BIOCHEMICAL STUDIES ON ANTIBIOTIC RESISTANCE AMONG STAPHYLOCOCCI FROM EYES AND OTHER CLINICAL SOURCES IN HEALTH AND DISEASE

ABSTRACT
THESIS SUBMITTED FOR THE AWARD OF THE DEGREE OF

Doctor of Philosophy
In
Biochemistry

BY
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Biochemical Studies on Antibiotic Resistance among Staphylococci from Eyes and other Clinical Sources in Health and Disease

Abstract

The emergence of multidrug-resistant bacteria is a phenomenon of concern to the clinician and the pharmaceutical industry, as it is the major cause of failure in the treatment of infectious diseases. Staphylococci have a record of developing resistance quickly and successfully to antibiotics. This defensive response is a consequence of the acquisition and transfer of antibiotic resistance plasmids and the possession of intrinsic resistance mechanisms. The acquired defense systems by staphylococci may have originated from antibiotic-producing organisms, where they may have been developed and then passed on to other genera. There are reports of emergence and high occurrence of staphylococcus strains resistant to methicillin, especially MRSA, from various parts of the World. These organisms are mostly resistant to multiple antibiotics; therefore the treatment of infections due to these organisms and their eradication is very difficult.

The present study was undertaken with the aim of determining epidemiology of clinical and carrier staphylococci and biochemical studies of their acquisition and dissemination of resistance.

The prevalence of methicillin-resistant Staphylococci was determined in 750 subjects infected/colonized with staphylococci providing 850 isolates. Of 850 strains 575 were isolated from clinical specimens, 100 from nasal cultures of hospitalized patients, 125 from nasal and 50 strains were isolated from ocular
swabs of hospital workers. It was shown that 35.1% (180/513) of *Staphylococcus aureus* and 22.5% (76/337) of coagulase-negative staphylococcal isolates were resistant to methicillin. Highest percentage of MRSA (35.5%) was found in pus specimens (n=151) while as urine samples (n=153) contributed 25.0% of methicillin-resistant coagulase-negative staphylococci. 29.7% of methicillin resistant *Staphylococcus aureus* isolates were found in nasal swabs (n=175) while as 10.0% and 16.5% of methicillin-resistant coagulase-negative staphylococci were observed in nasal swabs (n=50) and ocular cultures (n=50) respectively. The results indicated that major reservoir of methicillin resistant staphylococci in hospitals are colonized/infected inpatients and colonized hospital workers, with carriers at risk for developing endogenous infection or transmitting infection to health care workers and patients. The results were confirmed by restriction mapping and SDS-PAGE protein profiles of clinical and carrier methicillin-resistant staphylococcus aureus isolates. The multiple drug resistance of all MRSA (n=180) and MRCNS (n=76) isolates was detected. In case of both methicillin-resistant as well as methicillin-sensitive staphylococcal isolates zero resistance was found to fusidic acid and vancomycin while as highest resistance was found to penicillin G followed by ampicillin.

Selective methicillin-resistant staphylococcal isolates were subjected to plasmid isolation and curing treatments to study their mode of resistance. The plasmids were transformed into antibiotic sensitive *Escherichia coli* Strain DH5-α. In one of the experiments plasmid pJMR10 from *Staphylococcus aureus* coding for ampicillin (A'), gentamicin (G') and amikacin (Ak') resistance was transformed into *Escherichia Coli*. Transformation efficiency was about $2 \times 10^3$
transformants/μg of plasmid DNA. The minimal inhibitory concentrations (MICs) for A and G were lower in *E. coli* than in *S. aureus*. However, the MIC for AK was higher in *E. coli* transformants than in *S. aureus*. In one of Conjugation Studies transfer of erythromycin resistance was observed between clinical and carrier strains of *Staphylococcus aureus*. Furthermore Enzyme mediated resistance particularly through β-lactamase and chloramphenicol acetyl transferase was studied in selected strains. The substrate profile of β-lactamase so extracted was studied against different classes of pencillins and cephalosporins. The results indicated that Methicillin remains the most stable pencillin to the staphylococcal β-lactamase.

The anti-MRSA activities of a synthesized compound from an Inorganic Lab of A.M.U; was determined by *in vitro* testing. The results indicated that the Nickle-metalo, complex of 2-mercapto benzimidazole has good activity against MRSA (MIC > 64 μg/ml) and demands to be analyzed for clinical applications in future. The widespread occurrence and dissemination of β-lactamase and PBP mediated resistance leading to multiple antibiotics ineffective, thus increasing the cost of health care, needs to be tackled logistically by wise and judicious use of existing antibiotics and by developing ideal and cost effective antibiotics having least chances of acquiring resistance. Furthermore the hospital acquired MRSA infections through colonization of patients and hospital workers demands appropriate and timely measure to counteract this health problem.
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2003
This is to certify that the thesis entitled "BIOCHEMICAL STUDIES ON ANTIBIOTIC RESISTANCE AMONG STAPHYLOCOCCI FROM EYES AND OTHER CLINICAL SOURCES IN HEALTH AND DISEASE" herewith submitted by Mr. Javid Ahmad Dar in fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry of the Aligarh Muslim University, is an authentic record of research work carried out under our supervision and guidance. The work embodied in this thesis has not been presented in part or full to any other university or institute for the award of any degree.

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Dedicated
To my
Loving And Esteemed
Grand Parents,
Parents And Teachers
Research is to see what everybody else has seen and thinking what nobody has thought.

Albert Szent-Gyorgy

To get profit without risk, experience without danger, and reward without hard work, is as impossible as it is to live like without being born.

A. P. Gouthey
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Biochemical Studies on Antibiotic Resistance among Staphylococci from Eyes and other Clinical Sources in Health and Disease

Abstract

The emergence of multidrug-resistant bacteria is a phenomenon of concern to the clinician and the pharmaceutical industry, as it is the major cause of failure in the treatment of infectious diseases. Staphylococci have a record of developing resistance quickly and successfully to antibiotics. This defensive response is a consequence of the acquisition and transfer of antibiotic resistance plasmids and the possession of intrinsic resistance mechanisms. The acquired defense systems by staphylococci may have originated from antibiotic-producing organisms, where they may have been developed and then passed on to other genera. There are reports of emergence and high occurrence of staphylococcus strains resistant to methicillin, especially MRSA, from various parts of the World. These organisms are mostly resistant to multiple antibiotics; therefore the treatment of infections due to these organisms and their eradication is very difficult.

The present study was undertaken with the aim of determining epidemiology of clinical and carrier staphylococci and biochemical studies of their acquisition and dissemination of resistance.

The prevalence of methicillin-resistant Staphylococci was determined in 750 subjects infected/colonized with staphylococci providing 850 isolates. Of 850 strains 575 were isolated from clinical specimens, 100 from nasal cultures of hospitalized patients, 125 from nasal and 50 strains were isolated from ocular swabs of hospital workers. It was shown that 35.1% (180/513) of Staphylococcus aureus and 22.5% (76/337) of coagulase-negative staphylococcal isolates were resistant to methicillin. Highest percentage of MRSA (35.5%) was found in pus specimens (n=151) while as urine samples (n=153) contributed 25.0% of methicillin-resistant coagulase-negative staphylococci. 29.7% of methicillin resistant Staphylococcus aureus isolates were found in nasal swabs (n=175) while as 10.0% and 16.5% of methicillin-resistant coagulase-negative staphylococci were observed in nasal swabs (n=50) and ocular cultures (n=50) respectively. The results indicated that major reservoir of methicillin resistant staphylococci in hospitals are colonized/infected inpatients and colonized hospital workers, with carriers at risk for developing endogenous infection or transmitting infection to health care workers and patients. The results were confirmed by restriction mapping and SDS-PAGE protein profiles of clinical and carrier methicillin-resistant staphylococcus aureus isolates. The multiple drug resistance of all MRSA (n=180) and MRCNS (n=76) isolates was detected. In case of both methicillin-resistant as well as methicillin-sensitive staphylococcal isolates zero resistance was found to fusidic acid and vancomycin while as highest resistance was found to penicillin G followed by ampicillin.

Selective methicillin-resistant staphylococcal isolates were subjected to plasmid isolation and curing treatments to study their mode of resistance. The plasmids were transformed into antibiotic sensitive Escherichia coli Strain DH5-α. In one of the experiments plasmid pJMR10 from Staphylococcus aureus coding for ampicillin (A'), gentamicin (G') and amikacin (Ak') resistance was transformed into Escherichia Coli. Transformation efficiency was about 2 x 10^3 transformants/μg of plasmid DNA. The minimal inhibitory concentrations (MICs) for A and G were lower in E. coli than in S. aureus. However, the MIC for AK was higher in E. coli transformants than in S. aureus. In one of Conjugation Studies transfer of erythromycin resistance was observed between clinical and carrier strains of Staphylococcus aureus. Furthermore Enzyme mediated resistance particularly through β-lactamase and chloramphenicol acetyl transferase was studied in selected strains. The substrate profile of β-lactamase so extracted was studied against different classes of pencillins and cephalosporins. The results indicated that Methicillin remains the most stable pencillin to the staphylococcal β-lactamase.

The widespread occurrence and dissemination of β-lactamase and PBP mediated resistance leading to multiple antibiotics ineffective, thus increasing the cost of health care, needs to be tackled logistically by wise and judicious use of existing antibiotics and by developing ideal and cost effective antibiotics having least chances of acquiring resistance. Furthermore the hospital acquired MRSA infections through colonization of patients and hospital workers demands appropriate and timely measure to counteract this health problem.
CHAPTER-1
INTRODUCTION

Biochemical research in drug resistance demands its fundamental understanding at molecular grassroots level, and by unraveling the secrets of complexity of this emerging threat to human welfare, we can identify and devise logistic strategies for counteracting successfully the anti-chemotherapeutic mechanisms underlying microbial drug resistance.

The development of safe, effective antimicrobial drugs has revolutionized medicine in the past 60 years. Morbidity and mortality from microbial disease have been drastically reduced by modern chemotherapy. When antibiotics became widely available in the 1940s, they have been hailed as miracle drugs - magic bullets - able to eliminate bacteria without doing much harm to the cells of treated individuals. Yet with each passing decade, bacteria that defy not only single but multiple antibiotics have emerged, and therefore are extremely difficult to control. Today antibiotic resistance has either eliminated a number of previously valuable treatments, or made treatment more difficult or more costly because alternative agents must now be used.

When penicillin became widely available during the Second World War, it was a medical miracle, rapidly vanquishing the biggest wartime killer infected wounds. But just four years after drug companies began mass-producing penicillin in 1943, microbes began appearing that could resist it. The first bug to battle penicillin was Staphylococcus aureus. Thus, soon after the introduction of penicillin for clinical use a large-scale World Health Organization (WHO) survey showed that approximately 8% of strains were resistant; by 1956 the value was about 70%, consequently the emergence of multiply resistant S. aureus strains were responsible for many outbreaks of hospital infections during the 1950s.

The predominance of β-lactamase producing staphylococci in the 1950s led to an urgent search for β-lactamase stable penicillins that could be used for chemotherapy. The discovery and introduction of such compounds e.g.
methicillin, caused major decline in the incidence of multiply resistance
S. aureus strains during the 1960s. However, during the late 1970s and early
1980s strains of S. aureus resistant to multiple antibiotics including
methicillin and gentamicin emerged that were responsible for outbreaks of
hospital infections throughout the world. Such strains, referred to as
MRSA (methicillin-resistant S. aureus), not only cause difficulty in hospitals
but spread from them to nursing homes and to surrounding communities.
The pattern of resistance exhibited by MRSA imposes serious constraints on
the choice of antibiotics for therapy since the organisms involved are
resistant to virtually all the antibiotics in the clinician’s armamentarium, a
stockpile of more than 150 drugs. Vancomycin has become the antibiotic of
last resort, the only remaining drug effective against the most deadly of all
hospital-acquired infections, methicillin-resistant Staphylococcus aureus.
And yet vancomycin’s power - like that of the great, experienced warrior is
itself in jeopardy. In 1998 vancomycin-resistant S. aureus emerged in three
geographic locations that has increasingly worried physicians and hospital
workers.

Increasing reliance on vancomycin has led to the emergence of vancomycin-
resistant enterococci (VRE), bacteria that infect wounds, the urinary tract
and other sites. Until 1989 such resistance had not been reported in U.S.
hospitals but by 1993, more than 10% hospital acquired enterococci
infections reported to CDC were resistant. Streptococcus pneumoniae
causes thousands of cases of meningitis and pneumonia and 7 million cases
of ear infections in the United States each year. Currently, about 30% of S.
pneumoniae isolates are resistant to penicillin, the primary drug used to treat
this infection. Many penicillin-resistant strains are also resistant to other
antimicrobial drugs. In sexually transmitted disease clinics that monitor
outbreaks of drug resistant infections, doctors have found that more than
30% of gonorrhea isolates are resistant to penicillin or tetracycline, or both.
In recent years there has been a global resurgence of old foe - tuberculosis
as strains of multidrug resistant tuberculosis (MDR-TB) have emerged and
pose a particular threat to people infected with HIV, whereby the depressed
cell-mediated immunity of such individuals lead either to reactivation of a
previously latent *M. tuberculosis* infection or susceptibility to a newly acquired infection. Diarrheal diseases cause almost 3 million deaths a year—mostly in developing countries, where resistant strains of highly pathogenic bacteria such as *Shigella dysenteriae, Campylobacter, Vibrio cholerae, Escherichia coli* and *Salmonella* are emerging. A potentially dangerous “Superbug” known as *Salmonella typhimurium*, resistant to ampicillin, salfa, streptomycin, tetracycline and chloramphenicol, has caused illness in Europe, Canada and the United States. Infections caused by some multiply resistant strains of *Acinetobacter* and *Pseudomonas aeruginosa* are now virtually untreatable. Likewise resistance to antimalarial, antifungal and antiviral drugs is of increasing concern.\(^402,429\)

The extraordinary speed with which antibiotic resistance has spread amongst bacteria during the era of chemotherapy has been due, in large measure to the remarkable genetic flexibility of this group of organisms. Since the evolutionary history of living organisms is concerned with their adaptation to the environment, the adaptation of microorganisms to the toxic hazards of antimicrobial drugs is therefore probably inevitable.\(^38,73\)

At last six pathways\(^38,118,119,285,402\) may be involved in the appearance or spread of resistance in bacteria:

(i) Introduction of a few resistant organisms into a population where resistance previously was not present.

(ii) Acquisition of resistance by a few previously susceptible strains through genetic mutation.

(iii) Acquisition of resistance by a previously susceptible strain through transfer of genetic material.

(iv) Emergence of inducible resistance that is already present in a few strains in the population.

(v) Selection of a small, resistant subpopulation of organisms.

(vi) Dissemination of inherently resistant organisms locally within the setting.
Figure 1.1 A Scheme Showing the Route by which Antibiotic Resistance Genes are Acquired by Bacteria by Response to the Selection Pressure of Antibiotic Use. The resistance gene pool represents potential sources of DNA encoding antibiotic resistance determinants in the environment: this includes hospitals, farms, or other microenvironments where antibiotics are used to control bacterial development. After uptake of single or double stranded DNA by the bacterial host, the incorporation of resistance genes into stable replicons (DNA elements capable of autonomous replication) may take place by several different pathways, which have not yet been identified. The involvement of integrons, as shown here, has been demonstrated for a large class of transposable elements in the Entereobacteriaceae. The resulting resistance plasmids could exist in linear or circular form in bacterial hosts. The final step in the cycle - dissemination – is brought about by different gene transfer mechanisms. 118
Disease causing microbes thwart antibiotics by interfering with their mechanisms of action. For example, penicillin kills bacteria by attaching to their cell walls, then destroying a key part of the wall. The wall falls apart, and the bacterium dies. Resistant microbes however, either alter their cell walls so that penicillin can’t bind or produce enzymes that dismantle the antibiotics. In another scenario, erythromycin attacks ribosomes, structures within a cell that enable it to make proteins. Resistant bacteria have slightly altered ribosomes to which the drug cannot bind. The ribosomal route is also how bacteria become resistant to the antibiotics tetracycline, streptomycin and gentamicin.

Antibiotic resistance can be divided into seven basic groups depending on the mechanism involved:

- the presence of an enzyme that inactivates the antimicrobial agents;
- the presence of an alternative enzyme for the enzyme that is inhibited by the microbial agent;
- a mutation in the antimicrobial agent’s target which reduces binding of the antimicrobial agent;
- posttranscriptional or posttranslational modification of the antimicrobial agent’s target, which reduces binding of the antimicrobial agent;
- reduced uptake of the antimicrobial agent;
- active efflux of the antimicrobial agent; and
- overproduction of the target of the antimicrobial agent.

It is an established fact that acquired resistance to antimicrobial agents is more common in microorganisms isolated from hospital patients than in organisms causing community acquired infections. Hospitals and nursing homes provide an environment in which antibiotic resistant strains collect, concentrate and are maintained by a higher level of antibiotic usage than is normally found in the community. Antimicrobial agent-resistant bacteria may be introduced into hospitals and nursing homes by two different routes. They may emerge endogenously in patient’s flora during course of antimicrobial
Figure 1.2 Mechanisms of Antibiotic Resistance in Bacteria. Antibiotic-resistant bacteria owe their drug insensitivity to resistance genes. For example, such genes might code for "efflux" pumps that eject antibiotics from cells (a). Or the genes might give rise to enzymes that degrade the antibiotics (b) or that chemically alter and inactivate the drugs (c). Resistance genes can reside on the bacterial chromosome or, more typically, on small rings of DNA called plasmids. Some of the genes are inherited, some emerge through random mutations in bacterial DNA, and some are imported from other bacteria. (Reproduced from: Levy SB, 1998, The challenge of antibiotic resistance, Scientific American)

therapy, or they may enter with new residents who are already colonized or infected. Under antimicrobial chemotherapy the suppression of the normal flora - benign bacteria - innocent bystanders clearly creates a partial void that tends to be filled by resistant organisms from the environment or from other parts of the body. Such organisms behave as opportunists and may become pathogens. Hospital staffs and patients carry these so-called hospital strains and can cause epidemics of infections that were unknown before the antibiotic era.⁸⁴,¹⁹¹

Infections caused by antibiotic resistant staphylococci especially methicillin-resistant *Staphylococcus aureus* (MRSA) are a growing concern, particularly among patients in intensive care and surgical units, immunocompromised patients, and elderly patients in hospitals and nursing homes: Nasal carriage is a significant contributor to the epidemiology and pathogenesis of these healthcare associated infections.²⁶²,⁴¹⁵,⁵⁰⁰ Molecular biological studies in *S. epidermidis* endophthalmitis demonstrated that eyelid; conjunctiva or nose isolates were indistinguishable from intra-ocular isolates in up to 82% of patients with post-operative endophthalmitis, suggesting that the commonest source of infection is the patient's own flora.³⁷ The epidemiological pathways include endogenous spread, postoperative surgical wound infections, contaminated environmental surfaces, and patient-to-patient spread, frequently from patient to healthcare worker to patient. Moreover, there is now increasing evidence of the appearance of MRSA in the community.¹⁴⁵,¹⁹⁴,¹⁹⁷

Scientists and health professionals agree that decreasing the incidence of antimicrobial resistance will require improved systems for monitoring and controlling outbreaks of drug-resistant infections, developing new antibiotics, and a more judicious use of antimicrobial drugs.³⁶⁷,⁵⁰⁵ Understanding resistance offers the possibility of overcoming it, and so scientists also recognize the critical role that basic research plays in responding to this problem. These studies seek to define how bacterial pathogens acquire, maintain and transfer antibiotic-resistance genes. For example, studies of microbial physiology help scientists understand the biological processes that pathogens use to resist drug treatment. This knowledge can lead to the
development of novel strategies to overcome or reverse these processes. Investigations in molecular genetics and biochemistry with the aid of rapid improvements in gene sequencing technology are making it faster and easier to pin point the actual pathways and molecules involved in drug resistance. For examples, the studies of the molecular basis of drug resistance in micro-organisms have lead to the development of molecular tools to identify drug resistant strains, the identification of the genetic basis of resistance and resulting biochemical alterations in drug resistant species; the identification of methods to reverse resistance; and synthesis of drugs that are effective against drug-resistant strains of various pathogens.

1.1 OBJECTIVES OF THE PRESENT STUDY

In view of the significance and magnitude of the problem, the proposed research, ‘Biochemical Studies on Antibiotic Resistance among Staphylococci from Eyes and Other Clinical Sources in Health and Disease’ was undertaken with following aims and objectives:

1. Isolation of staphylococcal strains from eyes and other clinical sources in health and disease and their subsequent identification based on cultural, morphological and biochemical characteristics.

2. Determination of antibiotic resistance patterns of staphylococcal isolates from eyes and other clinical sources in health and disease against different classes of antibiotics.

3. Comparative study of antibiotic resistance profiles of methicillin-resistant and methicillin-sensitive staphylococcal isolates from eyes and other clinical sources in health and disease.

4. Comparative study of antibiotic resistance patterns of Staphylococcus epidermidis isolates from nose and clinically symptomatic eyes of patients having ocular infections, and normal eyes of hospital workers.

5. Comparative study of antibiotic resistance profiles of Staphylococcus aureus isolates from different clinical sources of patients having various infections, and nose of hospital workers.
6. Comparative study of antibiotic resistance profiles of *Staphylococcus aureus* isolates from wound infections and nose of patients, and nose of hospital workers in an Orthopaedic Surgical Ward.

7. Susceptibility testing and MIC determination of new antimicrobials against MRSA by agar diffusion, macrodilution and microdilution methods.

8. Isolation and characterization of plasmids from methicillin-resistant and methicillin-sensitive staphylococci.


10. Comparative study of plasmid profiles of methicillin-resistant and methicillin-sensitive *Staphylococcus epidermidis* isolates.

11. Transformation studies of staphylococcal isolates with known resistance markers.

12. Plasmid curing studies of staphylococcal isolates with known resistance markers.

13. Detection of conjugal transfer of antibiotic resistance among and between *Staphylococcus aureus* and *Staphylococcus epidermidis*.

14. Role of local environment of eye especially lower temperature on the transfer of antibiotic resistance in staphylococcal isolates.

15. DNA fingerprinting analysis of methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* isolates.

16. SDS-PAGE protein patterns of methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* isolates.

17. Study of β-lactamase and chloramphenicol acetyl transferase (CAT) from methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* isolated strains.
CHAPTER-2

REVIEW OF THE LITERATURE

Few developments in the history of medicine have had such a profound effect upon human life and society as the development of the power to control infections due to micro-organisms. World has been freed from the devastating disease that in earlier days decimated the population leaving a trail of misery and social disruption. No longer is surgery a desperate gamble with human life. The perils of childbirth are greatly lessened with the control of puerperal fever. The death of children and young adults from meningitis, tuberculosis and septicaemia, once commonplace is now a rarity. Research on antibiotics has contributed greatly to progress in chemotherapy of human, animal, and plant diseases, finding useful agents, developing modern techniques and processes of production, isolation, and identification of microbial products, and elucidating their structures, biosynthesis and mechanism of action. All these benefits are now accepted without question, but from the first resistance to the antibacterial agents announced itself as a potential problem. Sulphonamide resistance was evident through in vitro testing and animal experiments at an early stage. The discovery that microorganisms could produce an enzyme that destroyed penicillin was made almost as soon as the therapeutic potential of the drug itself was realized. All subsequent compounds have brought greater or less problems of resistance in their train and no systematic antimicrobial agent has yet been discovered that has proved to be beyond the ingenuity of micro-organisms to outwit. Bacteria are proved to be wily warriors, but even so, we have given them- and continue to give them – exactly what they need for their stunning success. By misusing and overusing antibiotics, we have encouraged super-races of bacteria to evolve. Moreover, advances in microbial biochemistry, physiology and genetics that have paralleled the development of antibacterial drugs have revealed an astonishing versatility, not only in the ways in which microbes can avoid or overcome the anti microbial assault but also in their capacity to share and disseminate that ability among their kind.
2.1 HISTORY OF ANTIMICROBIAL CHEMOTHERAPY

The history of antimicrobial chemotherapy can be divided into two major phases – ancient chemotherapy which takes us about 1600 A.D., and modern, which extends into the present. The origin of chemotherapy is lost in antiquity, but has some interesting points. All the ancient medicines, for example included eating earth, the favourite being red or yellow having a strong smell, or being greasy. These earths would be comparatively rich in moulds and may even have had a beneficial effect. Hippocrates, in 460 B.C., was the first to realize that disease was not caused by the anger of the Gods, by evil, or by magic, but rather by miasmas – impurities in the air, from this time the importance of magic and spells waned in their use until Aristotle, 150 years later, observed that phthisis, leprosy, scabies and, plague could be transmitted by contagion. This heralded the beginning of medicine as a subject, which could be studied in its own right. From then until the 16th Century little progress was made. Modern antimicrobial chemotherapy developed in three eras. The first, which lasted from about 1600 to 1900, involved the use of an extract of the bark of the Cinchona tree to treat malaria successfully and the subsequent development and use of other alkaloids. The first recorded treatment of malaria with an extraction of Cinchona bark is the wife of the Spanish Governor of Peru. Other alkaloids included extracts of the root of ipecacuanha for amoebic dysentery, a remedy widely used in Brazil and Asia and introduced into Europe early in the seventeenth century. The active constituent is the alkaloid ametine, identified in 1817 and shown to be effective against amoebic dysentery in 1891. The second era is that of synthetic compounds which began around 1900. The first success came from Germany, where Ehrlich’s work laid the foundation upon which all anti-microbial chemotherapy is built. Ehrlich began his scientific career in Koch’s laboratory (The founder of modern bacteriology). In 1906, in a speech at the opening of the institute he was to direct, he first described precisely this theory of substances, which would seek out and destroy microbes – the magic bullets – and he coined the word ‘Chemotherapy’ to describe his work. Later, in 1909, when Hata...
demonstrated in Ehrlich's institute that syphilis could be made to infect rabbits, work to find a cure was intensified. At that time 10 percent of the French population died of syphilis and 6 percent in Germany. The effectiveness of Salvarsan in treating syphilis was a milestone in chemotherapy. Because of some unpleasant side effects, a derivative—neosalvarsan—was introduced in 1912. Subsequently, more drugs were developed as an extension of Ehrlich's work, the most notable being suramin, introduced in 1920, developed from Trypan red and having useful activity against trypanosomiasis and mepacrine (quina crine, atebrine), first introduced in 1933, developed from methylene blue, as a an effective antimalarial agent of particular value in the Second World War. The interaction of chemical agents with micro-organism was recognized by another early scientist, Joseph Lister, who was the first scientist to study the inhibitory effect of chemicals on bacteria and to directly apply this knowledge to the practice of medicine Lister used phenol (1960s) to sterilize surgical instruments and in doing so greatly reduced the morbidity and mortality rates associated with surgery at that time.26 The next major step forward came in 1935 when Domagk in Germany began work on the effect of a number of dyes on mice infected with streptococci. One compound, prontosil, was effective and Domagk even had the courage to use it on his daughter, who was desperately ill from a streptoccal infection, before the drug had been tested in humans. The girl recovered dramatically. Prontosil was the first of the sulphonamides. The observation by the husband and wife team, the Trefouels, in the Pasteur Institute that prontosil was ineffective in vitro indicated that animals and humans converted the drug to an active component, which was the agent lethal to bacteria. Domagk quickly discovered that the drug hydrolysed to give sulphanilamide. In 1938 another sulphonamide, sulphapyridine, was produced by May and Baker Ltd., Dagenham, and became famous as M & B 693. This drug was spectacularly successful against pneumonia, meningitis, staphylococci and gonorrhoea. Notable achievements of synthetic antimicrobial agents since 1935 include isoniazid, p-amino salicylic acid and ethionamide for tuberculosis and nitroimidazole drugs for infections by anaerobes, amoebic dysentery, trichomoniasis and radiosensitisation of hypoxic tumours.
The third era of modern chemotherapy called the antibiotic era, although began in 1929 with Fleming's discovery of penicillin from Penicillum notatum, the real beginning was the recognition by Florey and his team of the chemotherapeutic potential and potency of the drug. The announcement in 1940 marks the beginning of the antibiotic revolution, which still continues today. Penicillin was used in the 1940s with enormous success against gonorrhoea, scarlet fever, pneumonia and meningitis, and began to replace sulphonamides in the treatment of puerperal fever and neosalvarsan for the treatment of syphilis. The systematic search, by an ingenious method, for an organism which could attack pyogenic cocci, conducted by Dubos in New York, led to the discovery of tyrothricin (garmicidin + tryrocidine), formed by Bacillus brevis, a substance which although too toxic for systemic use in man, had in fact a systemic curative effect in mice. This work exerted a strong influence in inducing Florey and his colleagues to embark on a study of naturally formed anti-bacterial substances, and penicillin was the second on their list. Florey gave a revealing account of these early developments in a lecture with the intriguing title “The Use of Microorganisms as Therapeutic Agents”: This was amplified in a later publication.

Two important events in the 1950s allowed the development of semisynthetic pencillin. First, the total synthesis of 6-aminopenicillanic acid (6APA) was achieved. Subsequently Rolinson and colleagues for Beecham Research Laboratories showed that many bacteria produce acylases capable of yielding 6APA from benzylpenicillin.

Hundreds of new antibacterial agents have been described in the world literature during the last 60 years. There have been many false dawns, but relatively few compounds have survived the stages of development and licensing to reach the pharmacist’s shelf. The dates of discovery and sources of the principal antibiotics are given chronologically in Table 2.1. Throughout this period there has been a tremendous progress in the field of antibiotic research particularly the development of analogues of existing drugs, stable to enzymatic inactivation, able to circumvent efflux mechanisms or able to bind to modified target sites. For example, linezolid representing a new class of antimicrobials, oxazolidinones, was approved in
the United States in early 2000, and was indicated for the treatment of patients with infections caused by vancomycin-resistant enterococci (VRE) and methicillin-resistant Staphylococcus aureus (MRSA). The regulatory approval provided cautions optimism in light of the growing resistance rates among Gram-positive organisms, which have necessitated the rapid development of newer agents such as linezolid, streptogramin combination (quinupristin/dalfopristin) and other novel antimicrobial agents (everninomicins, lipopeptides, glycyclyclines etc) to combat the resistance.

Table 2.1 Date of Discovery and Source of Natural Antibiotics

<table>
<thead>
<tr>
<th>Name</th>
<th>Date of Discovery</th>
<th>Microbe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>1929-1940</td>
<td><em>Pencillium notatum</em></td>
</tr>
<tr>
<td>Tyrothricin: Gramicidin Tryocidine</td>
<td>1939</td>
<td><em>Bacillus brevis</em></td>
</tr>
<tr>
<td>Griseofulvin</td>
<td>1939</td>
<td><em>Penicillium griseofulvum, Dierckx</em></td>
</tr>
<tr>
<td>Streptomycin</td>
<td>1944</td>
<td><em>Streptomycyes venezuelae</em></td>
</tr>
<tr>
<td>Bacitracin</td>
<td>1945</td>
<td><em>Bacillus licheniformis</em></td>
</tr>
<tr>
<td>Cholramphenicol</td>
<td>1947</td>
<td><em>Streptomycyes venezuelae</em></td>
</tr>
<tr>
<td>Polymyxin</td>
<td>1947</td>
<td><em>Bacillus polymyxa</em></td>
</tr>
<tr>
<td>Framycetin</td>
<td>1947-1953</td>
<td><em>Streptomycyesm lavendulae</em></td>
</tr>
<tr>
<td>Chlorotetracycline</td>
<td>1948</td>
<td><em>Streptomycyes aureofaciens</em></td>
</tr>
<tr>
<td>Cephalosporin C, N, and P</td>
<td>1948</td>
<td><em>Cephalosporium sp.</em></td>
</tr>
<tr>
<td>Neomycin</td>
<td>1949</td>
<td><em>Streptomycyes fradiae</em></td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>1950</td>
<td><em>Streptomycyes rimosus</em></td>
</tr>
<tr>
<td>Nystatin</td>
<td>1950</td>
<td><em>Streptomycyes noursei</em></td>
</tr>
<tr>
<td>Erythromycin</td>
<td>1952</td>
<td><em>Streptomycyes erythreus</em></td>
</tr>
<tr>
<td>Oleandomycin</td>
<td>1954</td>
<td><em>Streptomycyes antibioticus</em></td>
</tr>
<tr>
<td>Spiramycin</td>
<td>1954</td>
<td><em>Streptomycyes ambofaciens</em></td>
</tr>
<tr>
<td>Antibiotic</td>
<td>Year</td>
<td>Isolated From</td>
</tr>
<tr>
<td>----------------------</td>
<td>------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>1955</td>
<td><em>Streptomyces spheroides</em></td>
</tr>
<tr>
<td>Cycloserine</td>
<td>1955</td>
<td><em>Streptomyces orchidaceus</em></td>
</tr>
<tr>
<td>Vancomycin</td>
<td>1956</td>
<td><em>Streptomyces orientalis</em></td>
</tr>
<tr>
<td>Rifamycin</td>
<td>1957</td>
<td><em>Streptomyces mediterranei</em></td>
</tr>
<tr>
<td>Kanamycin</td>
<td>1957</td>
<td><em>Streptomyces kanamyceticus</em></td>
</tr>
<tr>
<td>Nebramycins</td>
<td>1958</td>
<td><em>Streptomyces tenebraes</em></td>
</tr>
<tr>
<td>Paromomycin</td>
<td>1959</td>
<td><em>Streptomyces rimosus</em></td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>1960</td>
<td><em>Fusidium coccineum</em></td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>1961-1962</td>
<td><em>Streptomyces flavopersicus</em></td>
</tr>
<tr>
<td>Lincomycin</td>
<td>1962</td>
<td><em>Streptomyces lincolnesis</em></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>1963</td>
<td><em>Micromonospora purpurea</em></td>
</tr>
<tr>
<td>Josamycin</td>
<td>1964</td>
<td><em>Streptomyces narvonensis</em></td>
</tr>
<tr>
<td>Tobramycin</td>
<td>1968</td>
<td><em>Streptomyces tenebraeus</em></td>
</tr>
<tr>
<td>Ribostamycin</td>
<td>1970</td>
<td><em>Streptomyces ribosidificus</em></td>
</tr>
<tr>
<td>Butirosin</td>
<td>1970</td>
<td><em>Bacillus circulans</em></td>
</tr>
<tr>
<td>Sissomicin</td>
<td>1970</td>
<td><em>Micromonospora myosensis</em></td>
</tr>
<tr>
<td>Rosaramicin</td>
<td>1972</td>
<td><em>Micromonospora rosaria</em></td>
</tr>
</tbody>
</table>

2.2 ANTIBIOTICS

Antibacterial agents comprise a large group of substances that inhibit bacterial growth (bacteriostatic agents), cause bacterial death (bactericidal agents), or destroy spores (sporicidal agents). Some bactericidal agents are also sporicidal and vice versa, but bacteriostatic agents are ineffective against resting spores. Antibacterial agents include disinfectants, antiseptics, preservatives and antibiotics. By tradition, bactericidal agents, which are used on inanimate objects, are termed 'disinfectants'. Disinfectants include antibacterial agents that are usually too toxic, irritant or corrosive to be applied to body surfaces or tissues, but are suitable for disinfection of equipment or the inanimate environment. Antiseptics include
bacterial inhibitors that are sufficiently free from toxic effects to be applied to body surfaces or exposed tissues and are agents, which should assist and not impair natural defence systems of the body. Although antiseptics have greater selective activity against bacteria than disinfectants, they are not suitable for the treatment of infections by systemic administration. Preservatives are frequently added to pharmaceutical, cosmetic and food products to inhibit bacterial contamination and proliferation and hence prevent infectivity or spoilage. The term 'biocide' includes disinfectants, antiseptics, and preservatives. It does not include antibiotics, which, inspite of being biocides in the strictest sense, tend to be categorized separately. Antibiotics are low-molecular-weight microbial metabolites that at low concentrations inhibit the growth of other microorganisms. A low – molecular weight substance is a molecule with a defined chemical structure having a relative mass of atmost a few thousand. We do not include among the antibiotics those enzymes, such as lysozyme from man, essential oils and alkaloids from plants, complex proteins such as colicins, and such oddities as the substance in the faceces of blowfly larvae, that also have antibacterial properties. It is true that the word 'antibiosis' was coined by Vuillemin in 1889 to denote antagonism between living creatures in general, but the noun 'antibiotic' was first used by Waksman in 1942, which gives him right to re-define it, and definition confines it to substances produced by microorganisms antagonistic to the growth or life of others in high dilution. The specifier "at low concentration" or "high dilution" is added to the definition, as even normal cell components and metabolic products as organic acids, hydrogen peroxide and alcohol can cause damage at excessive concentrations. For example, glycine, one of the constituents of every protein, has a strong bacterial effect on some bacteria if it is present in the culture medium in high concentrations. With the term "low concentration" we generally mean values well below 1mg/ml.

2.2.1 CLASSIFICATION OF ANTIBIOTICS

Since antibiotics exhibit a wide variety of properties the simplest way to categorize them is according to their structure and mechanism of action. In
order to include both synthetic and microbiologically produced drugs, here the classification of general antimicrobial agents is described.

2.2.1.1 Classification Based on Structure

1. Beta-lactams

The β-lactam antibiotics are the most varied and widely used of all the groups of antimicrobials. Starting from the discovery of benzylpenicillin in 1928 and its first clinical use in 1940, numerous natural and synthetic compounds have been described based upon the active structure of penicillin – cyclic amide – the β-lactam ring. β-Lactams account for 50% of all systemic antimicrobial use. Their success reflects low toxicity and the availability of so many derivatives. Apart from mycobacteria, intracellular pathogens, and cellwall deficient-species, few bacteria resist all of the β-lactams now available.

The main classes of beta-lactams based upon the nature of the cycle or of heterocatom included in the cycle (Table 2.2) are:

**Table 2.2 Diversity of Beta-lactam Antibiotics; Main Ring Structures, Names and Representative Antibiotics**

<table>
<thead>
<tr>
<th>Structure</th>
<th>Group</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Penicillin" /></td>
<td>Penam</td>
<td>Penicillins</td>
</tr>
<tr>
<td><img src="image" alt="Clavam" /></td>
<td>Beta-lactamase inhibitors (clavulanic acid)</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="Carbapenem" /></td>
<td>(Thienamycin, imipenem)</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="Cephem" /></td>
<td>Cephalosporins</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="Oxacillins" /></td>
<td>(Lanoksonef)</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="Monobactam" /></td>
<td>(Aztreonam)</td>
<td></td>
</tr>
</tbody>
</table>
Group I:

Penicillins

(i) **Natural Penicillin** (with a five-membered ring containing sulfur atom): Pencillin V (Phenoxy methylenicillin); Pencillin G (Benzylpenicillin)

(ii) **Penicillinase-resistant Penicillin**: Flucloxacillin, Nafillin, Methicillin, Cloxacillin, Oxacillin

(iii) **Amino penicillin**: Ampicillin, Amoxycillin

(iv) **Carboxypenicillin**: Carbenicillin, Ticarcillin

(v) **Ureidopenicillin**: Piperacillin, Mezlocillin, Azlocillin

(vi) **Other Penicillins**: Temocillin, Mecillinam

Group II:

Cephalosporins – Six-membered unsaturated rings with sulphur atom (cephems)

ii) **First-generation**: Cephalexin, Cefazolin

iii) **Second-generation**: Cefuroxime, Cefoxitin

iv) **Third-generation**: Cefixime, Ceftriaxone, Ceftazidime, Cefotaxime

v) **Fourth-generation**: Cefepime

Group III:

**Manobactam** (cyclic amides in a four-membered ring): Aztreonam

Group IV:

**Carbapenems** (five-membered ring with a double bond): Imipenem, Meropenem

Group V:

**Beta-lactamase inhibitors**: (contain a five-membered ring with an oxygen heteroatom) Clavulanic acid (+ amoxicillin), Clavulanic acid (+ ticarcillin), Salbactam (+ ampicillin), Tazobactam (+ piperacillin)
2. Aminoglycosides
Streptomycin, Neomycin, Kanamycin, Tobramycin, Paromomycin, Gentamicin, Sisomicin, Amikacin, Netilmicin

Amikacin and Netilmicin are semisynthetic aminoglycosides, derived from Kanamycin and sisomicin respectively. The suffix ‘mycin’ and ‘micin’ indicates that the compound was isolated directly or indirectly from Streptomyces spp. or Micromonospora spp., respectively.

3. Tetracyclines
Chlortetracycline, Oxytetracycline, Tetracycline, Demeclocycline, Methacycline, Doxycycline, Minocycline

4. Quinolones
Norflloxin, Ciprofloxacin, Ofloxacin, Pefloxacin Sparfloxacin, Trovafloxacin, Levofloxacin, Nalidixic acid, Cinoxacin.

The last two are quinolones while the rest are fluoroquinolones

5. Glycopeptides
Vancomycin, Teicoplanin

6. Macrolids
Erythromycin, Roxithromycin, Azithromycin, Oleandomycin, Clarithromycin

7. Lincosamides
Lincomycin, Clindamycin

8. Streptogramins
Pristinamycin, Virginiamycin, Synercid, Quinupristin, Dalfopristin

9. Phenicols (nitrobenzene derivative)
Chloramphenicol, Thiamphenicol

10. Sulfonamides
Sulfadiazine, Sulfamethoxazole, Para-aminosalicylic acid (PAS), Sulfadoxine

11. Trimethoprim (diaminopyrimidines)
Trimethoprim, pyrimethamine

12. Ansamycins
Rifampin

13. Fosfomycins
Fasfomycin

14. Nitrofurantoin
Nitrofurantoin, Furazolidone

15. Polypeptide antibiotics
Polymyxin B, Colistin, Bacitracin, Tyrothricin

16. Nicotinic acid derivatives
Isoniazid, Pyrazinamide, Thionamide

2.2.1.2 Classification Based on Mechanism of Action

The establishment of an infection in humans and animals by a pathogenic bacterium usually involves the following steps: (a) Attachment to the epithelial surfaces of the respiratory, alimentary or urogenital tract; (b) penetration of the epithelial surfaces by the pathogen; (c) interference with or evasion of, host defence mechanism; (d) multiplication in host tissues; (e) damage of the host's tissues. Steps (a) and (b) do not occur if the bacterium is introduced into the host directly through the skin by trauma or vector bite. Antibiotics usually prevent step (d) either by killing the pathogens or by slowing their growth to the point where host defence mechanisms can clear the infection. Since antibacterial agents exhibit a very wide variety of properties, the simplest way to categorize them is according to their site of action in the bacterial cell: agents acting on bacterial cell wall; agents acting on bacterial protein synthesis; agents acting on nucleic acid synthesis; and agents acting on bacterial cell membrane (Table 2.3- Table 2.4 and Table 2.5).
Table 2.3 Classifications of Antibacterial Agents According to their Site of Action

<table>
<thead>
<tr>
<th>Cell wall synthesis</th>
<th>Protein synthesis</th>
<th>Nucleic acid synthesis</th>
<th>Cell membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillins</td>
<td>Aminoglycosides</td>
<td>Sulphonamides</td>
<td>Polymixins</td>
</tr>
<tr>
<td>Cephalosporins</td>
<td>Chloramphenicol</td>
<td>Diaminopyrimidines</td>
<td>Gramicidin</td>
</tr>
<tr>
<td>Other β-lactams</td>
<td>Tetracyclines</td>
<td>Quinolones</td>
<td>Tyrocidine</td>
</tr>
<tr>
<td>Glycopeptides</td>
<td>Macrolides</td>
<td>Rifamycins</td>
<td>Valinomycin</td>
</tr>
<tr>
<td>Bocitracin</td>
<td>Lincosamides</td>
<td>Nitroimidazoles</td>
<td>Monensin</td>
</tr>
<tr>
<td>Cycloserine</td>
<td>Fusidic acid</td>
<td>Nitrofurans</td>
<td></td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>Streptogramins</td>
<td>Novobiocin</td>
<td></td>
</tr>
<tr>
<td>Isoniazid</td>
<td>Mupirocin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Indirect action on nucleic acid synthesis

*Not used in human medicine

Table 2.4 Inhibitory Effects of Antibiotics and Chemotherapeutic Agents Interfering with the Cell Wall Biosynthesis

<table>
<thead>
<tr>
<th>Drug</th>
<th>Inhibitory effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fosfomycin</td>
<td>Inhibition of the enzyme pyruvate-uridine-diphosphate-N-acetylglucosamine transferase by S-alkylation of a cysteine residue.</td>
</tr>
<tr>
<td>Fludalanine</td>
<td>Inhibition of the enzymes alanine racemase.</td>
</tr>
<tr>
<td>Alafosfalin</td>
<td>After active transport by an L, L-stereospecific peptide permease into the bacterial cell, alafosfalin is cleaved by an L,L-peptidase to L-alanine and L-1-aminoethyl-phosphonic acid which inhibits the enzymes alanine racemase and L-alanyl-uridine-diphosphate –muramyl ligase.</td>
</tr>
<tr>
<td>d-Cycloserine</td>
<td>Inhibition of the enzymes alanine racemase and d-alanine synthetase.</td>
</tr>
<tr>
<td>Antibiotic</td>
<td>Mechanism of Action</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-----------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>Inhibition of the pyrophosphatase which catalyzes the cleaves of the C$_{55}$ - isoprenoid alcoholpyrophosphate to the corresponding phosphate.</td>
</tr>
<tr>
<td>Vancomycin and ristocetins</td>
<td>Inhibition of the cross-linking (transpeptidation reaction) of the peptidoglycan chains by formation of stable stoichiometric complexes with the acyl-D-alanyl-D-alanine terminus of the peptidoglycan chains.</td>
</tr>
<tr>
<td>Penicillins and cephalosporins</td>
<td>Inhibition of several enzymes, the penicillin-binding proteins 1A, 1Bs, 2, 3, 4, 5 and 6 located in the cytoplasmic membrane, which are involved in the terminal stages of peptidoglycan synthesis. Morphological effects on the bacterial cell (E. coli): at low antibiotic concentrations: inhibition of cell division and formation of long filamentous cells; at higher concentrations: inhibition of cell elongation, cell lysis and release of the cell content as a spheroplast, which bursts unless osmotic protection is provided.</td>
</tr>
<tr>
<td>Thienamycin</td>
<td>Binding to the penicillin-binding proteins 1A, 1Bs, 2, 3, 4, 5, and 6 with highest affinity for penicillin-binding protein 2 and lowest affinity for protein 3. Morphological effect (on E. coli): at low concentrations: formation of large osmotically stable round cells; at higher concentration; cell lysis and release of the cell contents as a spheroplast.</td>
</tr>
<tr>
<td>Mecillinam</td>
<td>Higher specific binding to penicillin-binding protein 2. Morphological effect (on E. coli): formation of large osmotically stable round cells which continue to grow and eventually undergo lysis.</td>
</tr>
<tr>
<td>Point of attack</td>
<td>Inhibition</td>
</tr>
<tr>
<td>-----------------</td>
<td>------------</td>
</tr>
<tr>
<td>Purine and pyrimidine biosynthesis</td>
<td>DNA</td>
</tr>
</tbody>
</table>
Table 2.6 Inhibitory Effects of Antibiotics Affecting the Function of the Cytoplasmic Membrane

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Inhibitory Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrocidins, gramicidins, polymyxins</td>
<td>These surface-active polypeptide antibiotics increase the permeability of the cytoplasmic membrane and thus cause a massive exit of amino acids, purine and pyrimidine derivatives from the bacterial cell. This loss of important metabolites brings the biosynthetic reactions to standstill and leads to the death of the cell.</td>
</tr>
<tr>
<td>Natamycin, nystatin, amphotericin B, hachimycin, candidin</td>
<td>These polyene antibiotics cause impairment of the permeability of the cytoplasmic membrane of the fungal cell by forming complexes with certain membrane steroids such as ergosterol</td>
</tr>
</tbody>
</table>

2.2.2 ANTIBIOTICS AND MICROBIAL GROWTH

When bacteria are inoculated into a growth medium there is an initial lag phase before the onset of growth (Fig. 2.1). This is followed by a phase of exponential growth, the logarithmic phase. As available nutrients decline and toxic products accumulate, growth slows and then halts, i.e. the stationary phase.105

Antibiotics have been divided in two groups: bactericidal antibiotics (e.g. Pencillin), which kill or cause the lysis (dissolution) of invading bacteria, and bacteriostatic antibiotics (e.g. chloramphenicol), which merely inhibit bacterial growth and replication. Bacteriostatic agents rely on the host defences finally to defeat the infection and if antibiotic therapy is suspended microbial growth may resume. This division, however, is not clear cut, as bacteriostatic agents may become bactericidal if the concentration is raised and vice versa.
2.3 ANTIBIOTIC RESISTANCE

Antimicrobial action leads to the interaction of the drug with the microorganisms, the result of which may be inhibition or death of the organisms or instead may be the emergence of resistance that negates the effect of the antibiotic. The use of antimicrobial agents and the emergence of antibiotic resistance are intimately related. It has been shown on several occasions that use and misuse of various antibiotics has been associated with the emergence of resistant bacteria. However, the definition of resistance becomes clear when we take both microbiological as well as clinical aspects into consideration.
2.3.1 MICROBIOLOGICAL ANTIBIOTIC RESISTANCE

Microbiologically resistant isolate or species is one that processes one or more identifiable resistance mechanisms. Fleming described the phenomenon of resistance very clearly, if qualitatively, in initial tests of the antibacterial activity of penicillin: streptococci and staphylococci were sensitive, while Gram-negative bacilli such as *Escherichia coli*, were resistant. This is an example of inherent or intrinsic resistance, which defines the spectrum of an antibiotic. The spectrum may be narrow for agents such as benzylpenicillin or vancomycin (active against most Gram-positive organisms), colistin (active against most Gram-negative) or metronidazole (active only against anaerobes); or broad, for a wide variety of agents such as Tetracycline or chloramphenicol, which are active against most species of bacteria. When quantitative measurements of antimicrobial susceptibility are made, some species are clearly less sensitive than others (Figure 2.2).

Some strains of species that are normally inherently sensitive to an antibiotic may later acquire resistance to the drug. This phenomenon commonly arises when populations of bacteria have grown in the presence of the antibiotic. In some cases it is easily observable *in vitro*. For example, a culture of a strain of *Staphylococcus aureus* will contain individual organisms that are resistant to fusidic acid or rifampicin that grow out in the presence of appropriate concentrations of the antibiotic; the same phenomenon may occur in individual patients treated with these agents for staphylococcal infection. On the other hand, it may occur among clinical bacterial isolates on a demographic scale when the antibiotics are used extensively to treat large numbers of patients: this occurs to a greater or lesser extent with virtually all antibiotics in clinical use. The range of ‘sensitive’ and ‘resistant’ isolates of a given species may overlap (Figure 2.3). In such cases most sensitive isolates can be distinguished from most resistant ones but for the range of overlap it will be necessary to demonstrate that some isolates have a resistance mechanism not present in others.
Figure 2.2 Example of Distribution of the MICs of Benzylpenicillin for Isolates of a Sensitive Organism (Streptococcus pyogenes) and a Resistant Organism (Escherichia coli)

Figure 2.3 Distribution of the MIC of Gentamicin for Sensitive and Resistant Strains of Pseudomonas aeruginosa

2.3.2. CLINICAL ANTIBIOTIC RESISTANCE

Clinically a bacterial strain derived from a species that is susceptible to an antibiotic is said to be resistant when it is not inhibited to the minimal concentration of the antibiotic that inhibits the growth of the typical strains of that species. This strain may of course be inhibited by a higher
concentration of the same antibiotic. Therefore, it is obvious that the concept of resistance applies to a given bacterial strain, a given antibiotic and a given concentration. In fact, a bacterial strain is considered resistant to a given antibiotic if it can grow in the presence of a concentration equal to or greater than that which the antibiotic can reach in serum or tissues. Thus, we can say that a sensitive isolate is one that is likely to respond to therapy with the agent tested and a resistant isolate is one that will not. Between the two are isolates of intermediate or indeterminate susceptibility, over which range a positive clinical response is increasingly unlikely (Fig. 2.4). In practice the dividing lines imposed by breakpoints may not be so clear, since populations may again coalesce.

![Figure 2.4 Distribution of MICs among Bacterial Isolates that are Sensitive, Intermediate or Resistant to an Antibiotic](image)

2.3.3 SOME DEFINITIONS RELATED TO ANTIBIOTIC RESISTANCE

**Multiple-resistance**

When a microorganism is resistant to two or more antibiotics through unrelated mechanisms of resistance, one speaks of multiple resistances.

**Cross-resistance**

There are available in the market, and even more so among the cell physiologist's tools, many antibiotics that inhibit bacterial growth by the same
mechanism of action. Usually a bacterial strain that is resistant to an antibiotic is also resistant to other antibiotics of the same family. This phenomenon, called cross-resistance or co-resistance, is of considerable practical importance.

One-step and Multistep-resistance

It is possible to isolate resistant mutants from a population of bacteria susceptible to a given antibiotic by seeding a large number of cells on a solid culture medium containing a concentration of antibiotic several times higher than the MIC. Frequently, the bacteria in these colonies are resistant not only to the concentration of antibiotic used for the selection but also to higher concentrations. This is called one-step resistance, and occurs in the case of certain antibiotics for which the resistance is an all or none phenomenon, i.e., the cells are either susceptible to low concentrations or are not inhibited even by high concentrations. A typical example is resistance to streptomycin. In the case of other antibiotic, e.g., penicillin, it is not possible to isolate resistance cells by the method described above. Resistance of this type can be selected from liquid culture media by a method inappropriately called training. According to this method, the bacterial strain is grown in concentrations of antibiotic slightly below the MIC to select the less susceptible cells. These then give a population that includes cells capable of growing in the presence of slightly higher concentrations of antibiotic. By repeated passages of this type and by selecting from large populations in liquid culture, one can obtain strains that are notably more resistant. This gradual acquisition of resistance, found for pencillin and several other antibiotics, has been termed multistep resistance.

Resistance and Insusceptibility

For the purpose of clarity, it is preferable to reserve the word resistant for those bacterial strains that derive from a susceptible species and have lost their susceptibility to a given antibiotic through mutation or other alteration of their genetic heritage. Bacterial species that are “constitutionally” not inhibited by an antibiotic because, for example, they lack the structure on which the antibiotic acts, are called insensitive or insusceptible. The
expression intrinsic resistance, sometimes used instead of insusceptibility, should be avoided as it may create confusion.

Hypersensitive

There is a range of susceptibilities within a species, and some isolates of either sensitive or resistant species may prove to be more sensitive than normal: such strains are more familiar in the experimental than the diagnostic laboratory and are usually referred to as hypersensitive.

Antibiotic Susceptibility

Antibiotic susceptibility (sensitive strain) is usually defined by inhibition of an organism at concentration of antibiotic likely to be achieved in the blood after therapeutic doses. Ideally, the antibiotic concentration at the site of infection should exceed the minimum inhibitory concentration by at least two-to-fourfold in patients with serious infection. In practice, tissue concentrations are not precisely known and serum concentrations are used in the assessment of susceptibility.

Minimum Inhibitory Concentration (MIC)

The MIC is defined as the lowest concentration of an antibiotic that produces no visible growth after overnight (16-20 h) incubation of a standard inoculum of the organism with a series of dilutions of the drug in broth or agar. Many antibiotics are subject to inoculum density effects that may profoundly affect the MIC. An inoculum of $10^5$ colony-forming units/ml is commonly used in broth titrations, but the therapeutically correct inoculum has not been defined.

Minimum Bactericidal Concentration (MBC)

The MBC is usually defined as the minimum concentration of drug that reduces the colony count of viable organisms to less than 99.9% of its initial value after overnight incubation in broth: i.e. a 1000-fold reduction in the original inoculum. The MBC indicates the extent, but not the rate of killing, which may be more relevant to successful therapy in appropriate circumstance. Some antibiotics, notably β-lactam agents (in their action on
Gram-positive cocci) and quinolones, exhibit an optimal dosage effect so that less killing is observed as the drug concentration is raised.

**Tolerance**

Organisms are said to be 'antibiotic tolerant' when the MBC exceeds the MIC by 16-fold or more. The term is usually used to describe staphylococci or streptococci that are less susceptible than usual to the bactericidal action of β-lactam antibiotics. The clinical significance of tolerance is usually uncertain, but it may have implications for the treatment of certain forms of infective endocarditis.

**Postantibiotic Effect**

This effect refers to the continued inhibition of an organism for a period of time (sometimes several hours) after the concentration of an antibiotic has ostensibly fallen to below the MIC. Demonstration of the phenomenon *in vitro* is prone to considerable variation depending on the conditions of the test, and the therapeutic significance is unclear.

**Breakpoint**

The breakpoint concentration of an antibiotic is the concentration used to judge whether an organism is considered 'sensitive' or 'resistant' in antimicrobial susceptibility tests. Sometimes two breakpoints are used to establish categories of susceptible, resistant and reduced (intermediate) susceptibility. The later category implies that successful therapy may be possible if the drug is concentrated at the site of infection (e.g. excreted in high concentration in urine in urinary tract infection) or that high dosage might be appropriate. Breakpoint susceptibility testing refers to a method in which the susceptibility of organism to an antibiotic is tested only at the agreed breakpoint or breakpoints for that antibiotic.

**Frequency of Mutants and Frequency of Mutation**

Frequency of mutants refers to the fraction of resistant cells present in a sensitive population. Frequency of mutation is the fraction of resistant mutants that originate in a population at each generation (each doubling).
The frequency of mutants differs numerically from the frequency of mutation for many reasons:

1. The number of mutants present in a population derives both from those originating each generation and from the offsprings of the mutants formed in the preceding generations; consequently, the frequency of mutants tends to increase generation by generation.

2. Retromutation may occur generating susceptible cells from resistant ones, thus reducing the number of mutants.

3. The rate of growth of the resistant mutants may be different from that of the susceptible population; if, so often is the case, the former is lower, the frequency of resistant mutants tends to decrease with further generations.

2.3.4 HISTORY OF ANTIBIOTIC RESISTANCE

Paul Ehrlich gave the first account of microbial drug resistance in 1907, when he encountered the problem soon after the development of arsenical chemotherapy against trypanosomiasis. As the sulphonomides and antibiotics were brought into medical and veterinary practice resistance against these agents began to emerge. The first antibiotic resistance mechanism was identified a little more than 60 years ago, in 1940, when Abraham and Chain described the presence of a penicillinase, an enzyme that inactivates penicillin, in *Escherichia coli*. Just 4 years later Kirby reported the presence of a similar type of enzyme in *Staphylococcus aureus*. Therefore, even before penicillin came into widespread use, resistance to this drug already was recognized in both Gram-positive and Gram-negative organisms. Thus, soon after the introduction of penicillin for clinical use, a large-scale World Health Organization survey showed that approximately 8% of strains were resistant; by 1956 the value was about 70%. In the 1970s reports of organisms resistant not only to the penicillin-type drugs, but multiple other classes of antimicrobial agents, were common, as nosocomial pathogens which were responsible for many outbreaks of hospital infections, acquired a number of new resistance genes, such as those to aminoglycosides, chloramphenicol, and tetracycline. The predominance of
β-lactamase-producing staphylococci in the 1950s led to an urgent search for β-lactamase-stable penicillins that could be used for chemotherapy. The discovery and introduction of such compounds, e.g. methicillin, caused a major decline in the incidence of multiply resistant S. aureus strains during 1960s. Although Jevons first reported methicillin resistance in S. aureus in 1961, but during the late 1970s and early 1980s strains of S. aureus resistant to multiple antibiotics including methicillin and gentamicin emerged that were responsible for outbreaks of hospital infections throughout the world. Such strains, referred to, as MRSA not only cause difficulty in hospitals but spread from them to nursing homes and to surrounding communities. Vancomycin, which is somewhat toxic antibiotic requiring intravenous administration, is often the only drug effective against such staphylococci. However, there are several recent reports of vancomycin intermediate S. aureus (VISA) infections from different parts of the world. Moreover in the past few years a handful of organisms resistant to all known antimicrobial agents have emerged. Infections caused by some strains of enterococci, Acinetobacter, and Pseudomonas aeruginosa, are now virtually untreatable. Fortunately, these organisms remain rare; however, the conditions that favor the development and spread of such organisms are abundant. Therefore, we can expect resistant organisms to continue to emerge.

2.3.3 REASONS FOR THE EMERGENCE OF ANTIBIOTIC RESISTANCE

Pathways by Which Resistance Appears

At least six pathways (Table 2.7) may be involved in the appearance or spread of resistance in bacteria.

Table 2.7 Pathways of Resistance Introduction or Spread

<table>
<thead>
<tr>
<th>Pathway:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Introduction of a few resistant organisms into a population where resistance previously was not present</td>
</tr>
<tr>
<td>2. Acquisition of resistant by a few previously susceptible strains through genetic mutation</td>
</tr>
<tr>
<td>3. Acquisition of resistance by a previously susceptible strains through transfer of genetic material</td>
</tr>
</tbody>
</table>
4. Emergence of inducible resistance that is already present in a few strains in the population

5. Selection of a small, resistant subpopulation of organisms

6. Dissemination of inherently resistant organisms locally within the setting

This assumes that laboratory and surveillance artifacts, which can produce a false impression of resistance, have been eliminated. Both genetic determinants and epidemiological elements of spread are involved. Each of the six pathways will be considered in turn.

**Introduction of Resistant Organisms Into a Previously Susceptible Population**

Resistant strains often find new locales. For example, new strains may be introduced in the hospital setting by way of a patient from the outside, a health-care worker from outside or another institution, or a contaminated commercial product. This is illustrated by an episode in which strains of *Burkholderia cepacia*, an organism resistant to many antimicrobials, were introduced into a hospital in association with a contaminated iodophor solution; only patients treated with the implicated solution were affected. Introduction of resistant strains into hospitals from nursing homes and extended care facilities, as well as transfer in the opposite direction from hospital to nursing home, has become common for certain pathogens. After resistant organisms are introduced into a new health-care setting, they often are difficult to eradicate. For example, very few hospitals have been able to eradicate methicillin-resistant *Staphylococcus aureus* (MRSA) strains once they have been introduced, although some recent efforts were successful. Transfer from medical settings to the community also is common. For example, in some parts of the United States, MRSA strains recently became prominent in community-acquired infections, whereas previously they were prominent only in health-care institutions.

**Genetic Mutation**

Changes in only a few base pairs, causing substitution of one or a few amino acids in a crucial target (enzyme, cell structure, or cell wall) can affect chromosomal structural or control genes leading to new resistant strains.
The changed defense often is able to inactivate whole groups of antimicrobials that were thought capable of controlling the organism for years to come. Hospitalized patients treated with antimicrobials prone to select altered resistance traits can quickly become colonized or infected with these resistant strains.

**Transfer of Genetic Material**

Resistance can be acquired by a previously susceptible strain from another species or genus. Many of the antibacterial resistance genes are on plasmids that can, and do transfer themselves to another genus or species of bacteria. Although the ability of microorganisms to exchange genetic information has been known for several decades, the extent of this DNA exchange network is far greater than researchers previously imagined. Exchange of genetic information even between gram negative and gram-positive organisms has been reported, although the frequency of such events is hard to determine. Of particular interest is the pathway of exchange between staphylococci and enterococci. Already, the genes encoding β-lactamase production and high-level gentamicin resistance have moved from staphylococci into enterococci. This has resulted in resistance to penicillin, ampicillin, and the aminoglycosides, which has in many cases eliminated the synergistic activity of penicillins and aminoglycosides against these organisms. Of greatest concern, however, is that the transfer of vancomycin resistance genes from enterococci to staphylococci, and in particular S. aureus, will occur *in vivo*, as has already happened in the laboratory. This could make some methicillin resistant S. aureus strains, which are usually multiply resistant, virtually impossible to treat.

Many resistance genes encode proteins that either inactivate antimicrobial agents or block their site of action. One example of this is the TEM-type β-lactamase, which mediates resistance to penicillin, ampicillin, and first generation cephalosporins by hydrolyzing the β-lactam nucleus of the drug. The TEM β-lactamase (the name of which was derived from the initials of the little girl in a London hospital from whom the first ampicillin-resistant *E. coli* was isolated) is present in more than 70% of ampicillin-resistant *E. coli* as
well as in other enteric gram-negative rods. The enzyme is normally not capable of mediating resistance to drugs such as ceftazidime, cefotaxime, and ceftriaxone, the drugs usually held in reserve for the most difficult to treat infections. However, a change in just 3, 2 or even a single amino acid in the protein sequence of the TEM β-lactamase, at or around the active site of the enzyme, modifies the enzyme such that other antimicrobial agents, such as the cephalosporins, can now be hydrolyzed.\textsuperscript{134,334,485,490}

**Emergence of Strains With Inducible Resistance**

Chromosomal determinants for resistance to a given drug may not be expressed until the organism comes in contact with it or similar compounds. When permissive conditions appear (e.g., new antibiotics in use, introduction of new conjugative plasmids), the resistance can be manifested rapidly. The trigger for emergence may be the antimicrobial agent to which resistance is directed. In some case, exposure to another antimicrobial results in induction or derepression of a determinant (enzyme, etc.) that stimulates resistance as well to the studies drug.\textsuperscript{389,452}

**Selection of Resistant Stains**

Exposure to a stimulus that inhibits or kills the susceptible majority of a population allows a resistant subset of strains to grow at the expense of susceptible organisms. A minority of strains present in a given population may be resistant to the antimicrobial under study. The selecting factor usually is the antibiotic to which the subpopulation is resistant, but on occasion, a related agent can have a great impact as well. Non-drug factors such as those stimulating activity of reactions like acetylation or glucuronylation also can provide a selective advantage to organisms.\textsuperscript{314}

**Dissemination of Resistant Strains**

Organisms can spread from patient to patient, from one patient to another via a health care worker (e.g., on the hands of ward personnel), in contaminated commercial products (e.g., antiseptics), on other inanimate objects, or by widespread transfer of genetic material from the initial organism to others. This sixth pathway of transfer can be viewed as a
“common denominator” for both abrupt and less-abrupt increase in resistance.\textsuperscript{118,119,193}

**Investigating the Relative Importance of Different Pathways of Resistance**

Several new microbiologic techniques now are being used in conjunction with epidemiologic analysis to sort out the factors that play major roles in problems of multi-resistance. These tools can answer several questions: 1) Are the cases clustered by place? 2) Is there a common procedure being performed? 3) Are affected patients being treated with a common drug (the one for which resistance has developed, a drug of the same class as the one for which resistance has developed, or a drug that is an effective inducer? 4) Is the same type of resistance present in other organisms in the populations? 5) Are there single or multiple strains/ clones/ cassettes involved? These points and others must be analyzed to identify the pathways present and to identify selection factors (antimicrobial use etc.) that permit the resistant organisms to thrive. For examples, use of a ribosomal RNA probe allowed analysis of isolates of MRSA that were resistant as well to ciprofloxacin; the primary means by which resistant strains arose was found to be by transfer within the hospital setting (pathway 6), whereas mutational development of resistance in many strains at once (pathway 2), which was suspected initially, was not a major factor.\textsuperscript{452}

**Why Does Resistance Develop?**

Most current outbreaks of resistance in hospital or community settings, and virtually all endemic occurrences of resistance, involve a number of these pathways, each to a greater or lesser degree. For example all isolates of multiresistant *Serratia marcescens* in a hospital outbreak were related to transmission on the hands of health-care workers who handled contaminated collection basins while emptying the urine catheter collection bags. Here, both introduction of a new organism (pathway 1) and transfer within the hospital setting (pathway 6) explained the episode. In addition, there may be several concurrent manifestations of any one of these factors present in a given setting. Sorting out the multiple and concurrent means that lead to appearance and spread of resistance is an important but difficult
problem. Unfortunately, even reports of abrupt increase in resistance seldom have revealed which of the six pathways of resistance mechanism detailed above were operative. Therefore, the magnitude of each means of appearance or spread in today’s settings still is not clear. Despite these methodologic problems, three factors emerge as particularly important in the evolution of resistant microorganisms. First, simple mutations in common resistance genes have extended their spectrum of activity. Second, the exchange of genetic material (i.e. DNA) among microorganisms has moved some resistance genes from their original hosts into new organisms, causing them to become resistant to additional antimicrobial agents. Finally, the use of large quantities of antibiotics not just within the hospital environment but in community, farm, and aquaculture settings, has aided in the selection of new resistant organisms.275,328,336,452

Selective Pressure Caused by Use of Antimicrobial Agents

Selective pressure refers to the environmental conditions that allow organisms with novel mutations or newly acquired characteristics to survive and proliferate. Mutations that increase an organism’s resistance to antimicrobial agents occur naturally in bacteria. These mutations often involve changes in the cell wall that limit access of a drug to its site of action, or are alterations of the target site for an antibiotic. Whether the strain that harbors a mutation survives and replicates depends in large degree on the selective pressure placed on it (such as sub-inhibitory concentrations of antibiotics in the hospital environment). This is also true for an organism that acquires new DNA through genetic exchange. In an environment where large quantities of antimicrobial agents are present, or when inadequate doses of drugs are taken to eradicate infections, such as with Mycobacterium tuberculosis, the strains acquiring mutations or new DNA that lead to resistance will proliferate, whereas those susceptible to the antimicrobial agents will be killed. One organism that has developed significant levels of resistance likely in response to antibiotic pressure is Streptococcus pneumoniae. This organism causes more than 7 million cases of otitis media, 500,000 cases of pneumonia, and 3,000 cases of meningitis each year in the United States alone.349,452
Hospitals are usually the major breeding grounds and reservoir of resistant organisms. However, during the past decade, this began to change. The use of antimicrobial agents in animal feeds, in fish farming, and in children attending day care centers has provided the selective pressure for the development of new breeding grounds for resistant microorganisms. We are beginning to appreciate more fully the interrelatedness of bacterial pathogens causing disease in animals and humans. Thus, when quinolones were introduced into poultry to prevent infections in The Netherlands, strains of the diarrheal pathogen *Campylobacter jejuni* resistant to quinolones appeared not only in animals but in humans, too. Similar problems with increasing resistance to fluoroquinolones have been recognized in Spain and Finland. Therefore, our goal for prudent use of antimicrobial agents must extend beyond human medicine to include the veterinary, agriculture, and animal husbandry industries. 

2.4 MECHANISMS OF ANTIBIOTIC RESISTANCE

Two broad categories of antibiotic resistance are recognized: intrinsic (or intrinsic insusceptibility) and acquired. These categories also apply to biocides. The term 'intrinsic' is used to imply that inherent features of the cell are responsible for preventing antibiotic action and to distinguish this situation from acquired resistance, which occurs when resistant strains emerge from previously sensitive bacterial populations, usually after exposure to the agent concerned. Intrinsic resistance is usually expressed by chromosomal genes or by acquisition of plasmids and transposons. In the clinical setting, acquired antibiotic resistance results essentially from the selective pressure exerted on bacteria during the administration of antibiotics for chemotherapy. At the grass roots level mechanisms of antibiotic resistance can be learned on the basis of genetic and biochemical studies.

2.4.1 GENETIC MECHANISMS OF ANTIBIOTIC RESISTANCE

Antibiotic resistance can be determined by genes that reside in the host cell chromosome, on plasmids or on transposons. The genetic basis of these mechanisms is outlined below.
2.4.1.1 Resistance Determined by Chromosomal Genes

It is well established that resistance to certain antibiotics can be acquired by chromosomal mutations, i.e. changes in the base sequence of DNA. These mutations can be divided into two broad categories: microlesions, in which a single base pair has been altered, and macrolesions, in which more extensive changes have occurred. Both categories of mutations can be further subdivided. Microlesions comprise transition, transversion or frameshift mutations. Transition mutations involve base pair changes in which one purine is substituted for another purine, and consequently one pyrimidine for another pyrimidine. Transversions involve substitution of a purine for a pyrimidine and vice versa, whereas addition or loss of one or two base pairs constitutes a frameshift mutation. Macrolesions involve deletions, duplications, inversions and translocations (insertions).^285,293

2.4.1.2 Resistance Determined by Plasmids

Plasmids, i.e. extrachromosomal genetic elements that replicate independently of the chromosome, frequently carry genes that confer antibiotic resistance. The evolution and existence of plasmid-determined resistance mechanisms can be attributed to a number of factors. (a) In the absence of selection pressure by an antibiotic, the majority of cells in a particular need not maintain plasmids, thereby reducing the biochemical stress on the cell that would otherwise be required for replication and expression of plasmid DNA. (b) Genes encoded by plasmids are mobile because plasmids can be transferred both within and between certain species. Thus plasmids can be acquired from other bacteria in addition to inheritance from mother cells. In naturally occurring systems, plasmid transfer occurs by either conjugation or transduction. Conjugation is a process requiring cell-to-cell contact whereby DNA is transferred from a donor bacterium to a recipient. Transduction involves the transfer of genes by bacterial viruses (bacteriophages) and plays an important role in the natural transmission of antibiotic resistance plasmids amongst strains of S. aureus and of Strep. Pyogenes.^121,201
2.4.1.3 Resistance Determined by Transposons

Transposons are mobile DNA sequences capable of transferring (transposing) themselves from one DNA molecule (the donor) to another (the recipient). Unlike plasmids, transposons are not able to replicate independently and must be maintained within a functional replicon (e.g. plasmid or chromosome).

Transposons contain at their ends short regions that in any particular transposon are almost identical. These regions, or repeats, as they are termed, can lie in the same direction with respect to each other (direct repeats) or, more commonly, in the opposite direction (inverted repeats). The terminal repeats are believed to serve as recognition sequences for transposition enzymes (transposases) in their role of fusing the ends of the transposon with the recipient DNA. The central, or core, regions of transposons lying between the repeated ends frequently carry antibiotic resistance genes (Table 2.8), in addition to genes necessary for the process of transposition itself.\(^{402,389,335}\)

Table 2.8 Properties of Some Antibiotic Resistance Transposons

<table>
<thead>
<tr>
<th>Transposon</th>
<th>Size (kilobase pairs)</th>
<th>Resistance encoded</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tn1</td>
<td>5.0</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>Tn2</td>
<td>5.0</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>Tn3</td>
<td>5.0</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>Tn5</td>
<td>5.1</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>Tn7</td>
<td>14.0</td>
<td>Streptomycin, trimethoprim</td>
</tr>
<tr>
<td>Tn9</td>
<td>2.7</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>Tn10</td>
<td>9.3</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>Tn21</td>
<td>19.0</td>
<td>Streptomycin, sulphonamides</td>
</tr>
<tr>
<td>Tn1721</td>
<td>11.2</td>
<td>Tetracycline</td>
</tr>
</tbody>
</table>
**Found in staphylococci**

<table>
<thead>
<tr>
<th>Transposon</th>
<th>MLS group^a</th>
<th>Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tn551</td>
<td>5.3</td>
<td>MLS group^a</td>
</tr>
<tr>
<td>Tn552</td>
<td>6.1</td>
<td>Penicillin</td>
</tr>
<tr>
<td>Tn554b</td>
<td>6.7</td>
<td>MLS group, spectinomycin</td>
</tr>
<tr>
<td>Tn4001</td>
<td>4.7</td>
<td>Gentamicin, trimethoprim, kanamycin</td>
</tr>
<tr>
<td>Tn4002</td>
<td>6.7</td>
<td>Penicillin</td>
</tr>
<tr>
<td>Tn4003</td>
<td>3.6</td>
<td>Trimethoprim</td>
</tr>
<tr>
<td>Tn4201</td>
<td>6.6</td>
<td>Penicillin</td>
</tr>
</tbody>
</table>

**Found in enterococci**

<table>
<thead>
<tr>
<th>Transposon</th>
<th>MLS group^a</th>
<th>Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tn1546</td>
<td>10.8</td>
<td>Glycopeptides</td>
</tr>
</tbody>
</table>

^aMLS group: macrolids, lincosamides and streptogramins

^bTn554 is unusual among transposons in that it does not possess inverted or direct repeat sequences at its termini.

The existence of transposons that confer antibiotic resistance is one factor accounting for the emergence of multiply resistant (multiresistant) bacteria. Since transposons do not require extensive DNA homology to insert into the recipient molecule, it is possible for bacteria to acquire several different resistance genes by a series of transposon insertions in resident plasmids.167,333,335,378,398,458,462

### 2.4.2 BIOCHEMICAL MECHANISMS OF ANTIBIOTIC RESISTANCE

A variety of biochemical mechanisms for antibiotic resistance have been described, the most important of which are summarized in Table 2.9. However, it should be noted at the outset that resistance to a particular antibiotic could result from the combined expression of several of the listed mechanisms. For instance, the efficacy of a β-lactam antibiotic against a Gram-negative organism will be determined by the diffusion rate of the antibiotic across the outer membrane, its susceptibility to any periplasmic β-lactamase that may be present, and finally its affinity for target penicillin-binding proteins (PBP) located in the cytoplasmic membrane.
Consequently, β-lactam resistance may evolve by affecting one or more of these parameters.\textsuperscript{389,402}

<table>
<thead>
<tr>
<th>Mechanisms</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Alteration (antibiotic inactivation)</td>
<td>Resistance to aminoglycosides, β-lactams, fosfomycin and chloramphenicol</td>
</tr>
<tr>
<td>(2) Target site in the cell in insensitive to the inhibitor but still able to perform its normal physiological function</td>
<td>Resistance to aminoglycosides, β-lactams, glycopeptides, macrolides, quinolones, rifampicin, trimethoprim, tetracycline and mupirocin</td>
</tr>
<tr>
<td>(3) Decreased antibiotic accumulation:</td>
<td>Resistance to many antibiotics</td>
</tr>
<tr>
<td>(a) Impaired uptake</td>
<td>Resistance to tetracycline, macrolides, chloramphenicol and quinolones</td>
</tr>
<tr>
<td>(b) Enchanced efflux</td>
<td></td>
</tr>
<tr>
<td>(4) Bypass of antibitic-sentisive step by duplication of the target site, the second version being insusceptible to drug action.</td>
<td>Resistance to methicillin, sulphonamides, trimethoprim and mupirocin</td>
</tr>
<tr>
<td>(5) Overproduction of target so that higher antibiotic concentrations are needed to inhibit bacterial growth.</td>
<td>Resistance to trimethoprim</td>
</tr>
<tr>
<td>(6) Absence of an enzyme-metabolic pathway</td>
<td>Resistance to isoniazed in mycobacteria</td>
</tr>
</tbody>
</table>

Biochemical mechanism of antibiotic resistance is grouped into four classes, i.e. as resistance to inhibitors of nucleic acid, protein, and peptidoglycan synthesis and of membrane integrity.

2.4.2.1 Resistance to Inhibitors of Nucleic Acid Synthesis

2.4.2.1.1 Compounds That Interrupt Nucleotide Metabolism

Sulphonamides

Both chromosomal and plasmid-mediated resistance to sulphonamides has been described. Chromosomal resistance can result from two mechanisms.
The first involves hyperproduction of p-aminobenzoic acid (PABA) that overcomes the metabolic block imposed by the inhibition of dihydropteroate synthetase (DHPS). Resistance of this type was observed in certain organisms (e.g. S. aureus) soon after the introduction of sulphonamides for therapeutic purposes. Plasmid-mediated resistance to sulphonamides involves a bypass of the drug sensitive step by duplication of the corresponding target enzyme, the second (plasmid-encoded) version conferring resistance. Two different types (I and II) of plasmid-encoded sulphonamide-resistant DHPS enzymes have been found in Gram-negative bacteria. These enzymes bind sulphonamides about 10000-fold less efficiently than the chromosomally encoded (sensitive) enzyme, but resistance is achieved without sacrificing the efficiency with which natural substrate is bound.\(^{110,115}\)

2, 4-Diaminopyrimidines

Chromosomal mutations have been reported in E. coli that cause overproduction of dihydrofolate reductase (DHFR). Plasmid- and transposon-mediated resistance to trimethoprim have been found in a variety of Gram-positive and Gram-negative bacteria. Resistance involves a bypass of the antibiotic-sensitive step by duplication of the chromosomally encoded target DHFR enzyme, the plasmid- or transposon-encoded version conferring resistance. Ten different trimethoprim-resistant bacterial DHFR enzymes have been identified. These enzymes contain altered active sites, which render them resistant to trimethoprim.\(^{294,397}\)

2.4.2.2 Resistance to Compounds That Inhibit Enzymic Processes in Nucleic Acid Synthesis

2.4.2.2.1 Inhibitors of RNA Polymerase

Rifampicin inhibits RNA synthesis in bacteria by binding to the \(\beta\) subunit of DNA-dependent RNA polymerase. In E. coli acquired resistance to rifampicin results from amino acid substitutions, or small insertions and deletions, within three short highly conserved regions of the \(\beta\) subunit of RNA polymerase. The majority of mutations occur within region II between amino acid residues 507 and 534. Similar alterations within the corresponding
region of the RNA polymerase from strains of *M. tuberculosis* and *M. leprae* are associated with acquired resistance to rifampicin in these organisms.\textsuperscript{438}

### 2.4.2.2 Inhibitors of DNA Gyrase

The quinolone antibiotics inhibit bacterial DNA gyrase activity. Acquired resistance to the quinolones in several organisms (e.g. *E. coli*, *S. aureus*, *Campylobacter jejuni*, *M. tuberculosis*, and *M. smegmatis*) results from point mutations located within a part of *gyrA* (encoding the A subunit of DNA gyrase) termed the quinolone-resistance-determining region (QRDR). Two other types of chromosomal mutations that confer quinolone resistance have been reported in *E. coli*. *norB* mutants show low level resistance to some quinolones and other unrelated antibiotics as a consequence of decreased OmpF porin content and *norC* mutants are less susceptible to certain quinolones (e.g. norfloxacin and ciprofloxacin), but hypersusceptible to others. *norC* mutants are altered in both OmpF porin content and lipopolysaccharide structure. Efflux-based resistant to quinolones has recently been described in clinical isolates of *S. aureus* and *Proteus vulgaris*. The gene mediating resistance in *S. aureus*, *norA*, encodes a membrane protein that displays homology with other transport proteins coupled to the electrochemical proton gradient. A possible pathway for the selection of organisms expressing efflux-based resistance to quinolones has been suggested by recent studies. Several organisms express endogenous chromosomally encoded efflux systems that are to some extent able to remove quinolones from the cell. Selection pressure, exerted by the clinical use of quinolones, may have resulted in the emergence of resistant strains carrying acquired mutations within the endogenous transport system that improve affinity of the efflux system for quinolones.\textsuperscript{146,175,228,302,365,438,466,507}

### 2.4.2.2 Resistance to Inhibitors of Protein Synthesis

**Mupirocin**

Mupirocin inhibits isoleucyl tRNA synthetase, thereby leading to depletion of charged isoleucyl tRNA and cessation of protein synthesis. Because it is metabolized *in vivo*, mupirocin is restricted to topical use and it is within isolates of the skin pathogen *S. aureus* that acquired resistance to the
antibiotic has been reported. Resistant staphylococcal strains fall into two categories: those expressing high level resistance (i.e. upto 1000-fold more resistant than sensitive strains) and those expressing moderate level resistance (i.e. upto 60-fold more resistant than sensitive strains). High level resistance is associated with possession of two isoleucyl tRNA synthetases, the normal resident chromosomal enzyme (mupirocin sensitive) and a second mupirocin-resistant enzyme, which is usually plasmid encoded. Staphylococcal strains exhibiting moderate level resistance do not contain the gene encoding high-level resistance and possess only a single resistant isoleucyl tRNA synthetase which has presumably arisen by one or more point mutations in the resident chromosomal gene.\textsuperscript{210,327}

**Aminoglycoside-aminocyclitol Group**

Acquired resistance to these antibiotics can arise by mechanisms 1 (altered antibiotic), 2 (altered target site) and 3a (impaired uptake) described in Table 2.9. The most widespread mechanism of resistance involves modification of the antibiotics by bacterial enzymes that are either plasmid or transposon encoded. There are three classes of enzymes that modify aminoglycoside-aminocyclitol (AGAC) antibiotics: acetyltransferases (AACs), adenylyl-transferases (AADs) and phosphotransferases (APHs) AACs use acetyl CoA as a cofactor, whereas in the phosphorylation and nucleotidylation reactions ATP is the cofactor. AGAC resistance can also be expressed by mechanism 2 (Table 2.9). For instance, mutations in the \textit{rpsL} gene of \textit{E. coli} K-12 led to high level resistance to streptomycin by altering the nature of ribosomal protein S12 thereby preventing the antibiotic from binding to its target. Similar types of chromosomal mutations occur in other bacterial species, e.g. clinical isolates of \textit{S. aureus}.\textsuperscript{140,141,179,396}

**Chloramphenicol**

Acquired resistance to chloramphenicol is frequently due to inactivation of the drug. Inactivation is mediated by plasmid- or transposon-encoded chloramphenicol acetyltransferases (CATs) that convert the drug to 3-acetoxy chloramphenicol followed by conversion to 1, 3 diacetoxychloramphenicol, the final inactivation product. The acetoxy
derivatives are antibiotically inactive because they fail to bind to the ribosomal target. The type 1 enzyme has been most studied and the complete amino acid sequence of its subunits is known. A histidine residue at position 193 plays a critical role in the catalytic modifications of chloramphenicol and is likely to be located at the active site of the enzyme. Although the majority of plasmids conferring chloramphenicol resistance in *E. coli* encode CATs, at least one example of a plasmid-encoded non-enzymic resistance mechanism has been described. This type of resistance, encoded by the gene *cmlA* (contained in transposon Tn1696) involves active efflux of chloramphenicol mediated by the membrane-located CmlA protein. In gram-negative bacteria low-level resistance to chloramphenicol can arise by mutational loss of outer membrane porins through which the drug normally diffuses to reach the periplasm.\textsuperscript{225,423,424}

**Fusidic acid**

Fusidic acid is a steroid antibiotic that prevents bacterial protein synthesis by inhibiting functions associated with translocation factor protein (G factor or EFG). Gram-negative bacteria are intrinsically resistant to fusidic acid by virtue of poor antibiotic penetration across the outer membrane. Acquired resistance also occurs in Gram-positive bacteria that do not express intrinsic resistance to the antibiotic. Acquired resistance results from either chromosomal mutations or possession of plasmids. Chromosomal mutants invariably have a modified G factor, which has decreased affinity for the drug. In *S. aureus*, plasmid-mediated resistance to fusidic acid may involve decreased uptake of antibiotic across the cytoplasmic membrane, but the molecular basic of resistance is unknown. Surprisingly, the gene encoding resistance to fusidic acid also encodes a type I CAT. Firm evidence has been obtained that the CAT itself confers fusidic acid resistance and that a separate *fus* product not translated from an initiation codon internal to the *cat* message. However, fusidic acid is not inactivated or modified by enzyme, which appears to have a binding domain that sequesters a variety of aromatic compounds that are not necessarily closely related structurally.\textsuperscript{95}
Macrolides, Lincosamides and Streptogramins (MLS Group)

MLS antibiotics arrest protein synthesis by binding to the 50S ribosomal subunit. Resistance mechanisms specific to individual members of the MLS antibiotics occur but, in addition, resistance to all these antibiotics can be conferred by a single mechanism involving mono- or di-methylation of an adenine residue in 23S rRNA. Three distinct RNA methylase genes (ermA, emrB, emrC) have been reported in staphylococci, all of which confer complete cross-resistance to MLS antibiotics. Methylation prevents the drugs from binding to the 50S ribosomal subunit probably as a consequence of a conformational change in the 23S rRNA. Genes specifying MLS resistance by rRNA methylation are carried by plasmids and transposons. Recently a new resistance phenotype (MS) was observed in clinical isolates of S. aureus, in which cells are resistant to macrolides (M) and streptogramins (S) but remains sensitive to lincosamides.\[137,362,372,454,494\]

Resistance To Tetracyclines

Eight genetic determinants (tetA through tetF, tetK, and tetL) have been described in bacteria that encode tetracycline efflux proteins. Efflux is dependent on the proton-motive force and involves an electrically neutral proton-tetracycline antiport system with exchange of a monocationic magnesium-tetracycline chelate complex for a proton. It is assumed that a substrate (tetracycline) binding site fluctuating between high and low affinity states and a proton transfer site whose protonation-deprotonation affects the affinity of the binding site are present in the efflux proteins. Recently two types of ribosomal protection factors have been described, TetM and TetO, both of which are plasmid or transposon encoded. The tetM resistance system occurs naturally in certain streptococci, gonococci and Ureaplasma urealyticum, whereas tetO has been described in Campylobacter jejuni. The TetM and TetO proteins probably act in a catalytic manner to modify the tetracycline-binding site on the 30S ribosomal subunit. This might involve methylation of either ribosomal RNA or ribosomal protein, thereby rendering ribosomes insensitive to tetracycline. Chromosomal mutations in E. coli leading to loss of the outer membrane porin ompF, through which the
antibiotic normally diffuses, confer low level resistance to tetracycline (and other unrelated compounds).\textsuperscript{75,97,108,284,508}

2.2.4.2 Resistance to Antibiotics That Inhibit Peptidoglycan Synthesis

D-cycloserine

D-cycloserine is transported across the bacterial cytoplasmic membrane by the D-alanine transport system. In \textit{E. coli} this transport system is encoded by the chromosomal gene \textit{cycA}, which is involved in accumulation of D-alanine, D-glycine and D-serine as well as D-cycloserine. Mutation in \textit{cycA} confers resistance to cycloserine as well as defects in the transport of the other molecules. In streptococci, transport mutants analogous to those in \textit{E. coli} have been isolated as well as resistance mutants, which produce more of the target enzymes inhibited by D-cycloserine. Acquired resistance to cycloserine in \textit{M. tuberculosis} has been attributed to point mutation in the synthetase, but the mutants have been characterized in detail.\textsuperscript{389}

Fosfomycin

Fosfomycin acts at the level of phosphoenol-pyruvate: UDP-\textit{N}-acetylg glucosamine enoylpyruvil transferase, an enzyme involved in the biosynthesis of peptidoglycan. Resistance to the antibiotic has been reported in several types of Gram-negative bacteria and occurs principally by general mechanism 1 (drug alteration) and 3a (impaired uptake) (Table 2.9). Mechanisms of type 3a (impaired uptake) involve chromosomal mutation in the hexose-6-phosphate or L-glycerophosphate uptake systems by which fosfomycin is transported into susceptible bacteria.\textsuperscript{402}

Bacitracin

\textit{Bacitracin forms a complex with the membrane-bound pyrophosphate form} of the lipid carrier molecule involved in transferring disaccharide-pentapeptide unit to the nascent peptidoglycan chain. Gram-negative bacteria are intrinsically resistant to bacitracin so that discussion of acquired resistance to this antibiotic is only relevant for Gram-positive organism. Bacitracin-resistant mutants of \textit{S. aureus} have been isolates in the laboratory, but the mutations appear to revert at high frequency because resistant is readily lost on removal of the selective pressure. Clinical isolates
of Gram-positive bacteria resistance to bacitracin have been reported which may reflect the unstable nature of resistance mentioned above.\textsuperscript{389,402}

**Glycopeptide antibiotics**

Glycopeptide antibiotics interfere with glycan unit insertion by combining directly with peptidoglycan, in particular to the acyl-D-alanyl-D-alanine terminus of the peptidoglycan precursors. Acquired resistance to glycopeptides occurs in the enterococci, the genes conferring resistance residing within transport Tn\textsubscript{1546}. Conjugal transfer of various plasmids that have acquired Tn\textsubscript{1546} contributes to the spread of glycopeptide resistance genes in enterocci. Tn\textsubscript{1546} encodes two key proteins (VanA and VanH) that are responsible for changing the pathway of peptidoglycan synthesis to produce a modified peptidoglycan structure no longer able to bind glycopeptides.\textsuperscript{319,322,441}

**B-Lactam antibiotics**

Acquired resistance to B-lactam antibiotics can arise by three different mechanisms: (1) in Gram-negative organisms through reduction in permeability of the outer membrane to the antibiotic, (2) by synthesis of beta-lactamases, and (3) by modification of one or more PBPs. These mechanisms, either separately or jointly, account for acquired resistance to \(\beta\)-lactams. It has been known for many years that change in the Gram-negative outer membrane may affect the influx rate of \(\beta\)-lactams and thereby affect the resistance level. In particular, mutational loss of porins (e.g. OmpF and OmpC), through which many \(\beta\)-lactams normally diffuse, provides low levels of \(\beta\)-lactams resistance. \(\beta\)-Lactamases hydrolyse the cyclic amid bond in molecules thereby rendering the drugs unable to bind to bacterial PBPs. In the case of Gram-negative bacteria the enzymes are synthesized within the cell and are then secreted into the periplasmic region. Resistance results from the strategic location of \(\beta\)-lactamases between the antibiotic penetration barrier (the outer membrane) and the antibiotic targets (PBPs) located in the cytoplasmic membrane whereby the \(\beta\)-lactamases can sequentially destroy antibiotic molecules as they enter the periplasm. Therefore, in Gram-negative bacteria resistance due to \(\beta\)-lactamases produced by Gram-positive
bacteria are mainly excreted from the cell so that resistance is a population phenomenon, i.e the excreted enzyme spread into the external environment and can reduce the level of β-lactam to a concentration that permits growth of both enzyme-producing and non-enzyme producing bacteria. 17 6,77,78

Since β-lactam antibiotics are analogous of the cell wall substrate, mutations in PBPs affecting binding of the inhibitor (and thus potentially conferring resistance) might be expected to be lethal, as binding of the natural peptidoglycan substrate would also be affected. *Streptococcus pneumoniae* resistant to penicillin has several PBPs of altered size and deceased affinity for penicillins. Molecular analysis of PBP2 genes from susceptible and resistant organisms revealed that the gene from susceptible strains was uniform in sequence, whereas those from resistant isolates had a mosaic structure, containing regions identical to those in susceptible strains and regions up to 23% divergent in sequence. The modified PBP2 genes thus appear to have arisen by block replacement of parts of the pneumococcal PBP2 gene with homologous regions from some other species, probably by DNA transformation. Resistance mechanism (mechanism 4, Table 2.9) concerns β-lactam-lactam resistance in clinical *S. aureus* isolates mediated by the mec determinant. Resistance result from the synthesis of antibiotics unique PBP (molecular weight 74000) designated PBP2' or PBP2a that has a low affinity for β-lactam antibiotics. PBP2 is the only functional PBP at concentrations of β-lactam sufficient to inhibit the normal complement of PBPs in sensitive cells. The mec determinant confers resistance to all β-lactam antibiotics, including β-lactamases resistant compounds such as methicillin. The origin of the mec determinant is unknown, but may have been transferred to a single strain of *S. aureus* and then disseminated worldwide by clonal descent. 17,23,194,208

2.4.2.4 Resistance to Antibiotics That Inhibit Membrane Integrity

Plasmid-determined resistance to polymyxin has been described, but chromosomal mutants of *Ps. aeruginosa* resistant to polymyxin have been isolated. Resistance is associated with an increase in the content of an outer membrane protein (H1), which apparently decreases the requirement for
divalent cations from the outer membrane, it is suggested that protein H1 replaces cations at sites in the outer membrane, which would otherwise be susceptible to the antibiotics. Chromosomal mutations conferring resistance to polymyxin also occur in Gram-negative enteric bacteria such as S. typhimurium and E. coli. In these organisms the molecular basis of resistance differs from that of Ps. aeruginosa. In E. coli and S. typhimurium resistance to polymyxin is accompanied by decrease in the net ionic charge of outer membrane lipopolysaccharide following substitution of phosphate groups in the molecule by aminoethanol and aminocarabinose. These charges are responsible for decreased binding of the outer membrane, resulting in decreased susceptibility to polymyxin.\textsuperscript{389,402}

### 2.5 STAPHYLOCOCCI

Staphylococci were first observed and cultured by Pasteur and Koch. But Ogston in 1881 and Rosenbach in 1884 made the first thorough studies on staphylococci. The genus Staphylococcus was given its name by Ogston in 1881 when he observed grape-like clusters of bacteria in pus from human abscesses. Three years later, Rosenbach was able to isolate and grow these microorganisms in pure culture. He gave these bacteria the specific epithet Staphylococcus aureus Rosenbach because of the yellow-to-orange-pigmented appearance of their colonies. Rosenbach showed that S. aureus was responsible for wound infections and furunculosis, and that Staphylococcus epidermidis was a normal colonizer of the skin. Ever since Rosenbach first described the growth of this 'golden' coccus, surgeons have feared staphylococcal wound infections after surgery. Staphylococci also caused life-threatening disease after trauma and fatal pneumonia during the influenza season, killing young people in the prime of their lives. Therefore, in the pre-antibiotic era, S. aureus was known as a major life-threatening pathogen.\textsuperscript{106,255,268}

Because of penicillin's important contribution to the fight against S. aureus disease, the period between 1946 and 1950 was referred to as 'a golden age' for the treatment of staphylococcal disease. However, the rapid spread of resistant staphylococci soon led to this return of a major pathogen.\textsuperscript{145}
TAXONOMY

The family of Micrococcaceae consists of four genera:

- *Planococcus*
- *Stomatococcus*
- *Micrococcus*, and
- *Staphylococcus*

Planococci are not found in humans, whereas *Stomatococcus* and *Micrococcus* can colonize humans, but rarely produce disease. Staphylococci are Gram-positive nonmobile cocci that characteristically divide in different patterns to form irregular grape-like clusters (Fig. 2.5). They usually produce catalase. They are facultative anaerobes, but grow better under aerobic than under anaerobic conditions. Traditionally, they have been divided into coagulase-positive and coagulase-negative staphylococci. The coagulase-negative staphylococci can be subdivided at present into 32 different species (Table 2.10). Of the large number of staphylococci only three are commonly associated with human disease.

- *S. aureus*,
- *S. epidermidis*, and
- *Staphylococcus saprophyticus*

Figure 2.5 *Staphylococcus aureus* Showing Bunched Grape-like Colonies
<table>
<thead>
<tr>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. epidermidis</em></td>
<td>Winslow and Winslow 1908, Evans 1916, Schleifer and Kloos 1975</td>
</tr>
<tr>
<td><em>S. capitis</em></td>
<td>Kloos and Schleifer 1975</td>
</tr>
<tr>
<td><em>S. caprae</em></td>
<td>Devriese et al. 1983</td>
</tr>
<tr>
<td><em>S. saccharolyticus</em></td>
<td>Fourbert and Douglas 1948, Kilpper-Balz and Schleifer 1981</td>
</tr>
<tr>
<td><em>S. hominis</em></td>
<td>Kloos and Schleifer 1975</td>
</tr>
<tr>
<td><em>S. haemolyticus</em></td>
<td>Schleifer and Kloos 1975</td>
</tr>
<tr>
<td><em>S. warneri</em></td>
<td>Kloos and Schleifer 1975</td>
</tr>
<tr>
<td><em>S. pasteuri</em></td>
<td>Chesneau et al 1993</td>
</tr>
<tr>
<td><em>S. lugdunensis</em></td>
<td>Freney et al. 1988</td>
</tr>
<tr>
<td><em>S. auricularis</em></td>
<td>Kloos and Schleifer 1983</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>Rosenbach 1884</td>
</tr>
<tr>
<td><em>S. saprophyticus</em></td>
<td>Shaw, Still and Cowan 1951, Schleifer and Kloos 1975</td>
</tr>
<tr>
<td><em>S. cohnii</em></td>
<td>Schleifer and Kloos 1975</td>
</tr>
<tr>
<td><em>S. xylosus</em></td>
<td>Schleifer and Kloos 1975</td>
</tr>
<tr>
<td><em>S. kloosii</em></td>
<td>Schleifer, Kilpper-Balz and Devriese 1984</td>
</tr>
<tr>
<td><em>S. equorum</em></td>
<td>Schleifer, Kilpper-Balz and Devriese 1984</td>
</tr>
<tr>
<td><em>S. arlettae</em></td>
<td>Schleifer, Kilpper-Balz and Devriese 1984</td>
</tr>
<tr>
<td><em>S. gallinarum</em></td>
<td>Devriese et al. 1983</td>
</tr>
<tr>
<td><em>S. simulans</em></td>
<td>Kloos and Schleifer 1975</td>
</tr>
<tr>
<td><em>S. carnosus</em></td>
<td>Schleifer and Fischer 1982</td>
</tr>
<tr>
<td><em>S. piscifermentans</em></td>
<td>Tanasupawat et al. 1992</td>
</tr>
<tr>
<td><em>S. felis</em></td>
<td>Igimi et al. 1989</td>
</tr>
<tr>
<td><em>S. intermedius</em></td>
<td>Hajek 1976</td>
</tr>
</tbody>
</table>
### 2.5.1 CLINICAL SIGNIFICANCE OF S. AUREUS

The major habitats of the most pathogenic species, *S. aureus*, are the anterior nares and perineum. Newborn infants usually acquire *S. aureus* first on the skin (umbicial area) and later in the nose. Soon after the neonatal period some individuals become permanent carriers, often with the same strain. Although some spread of *S. aureus* may occur within a family, generally the established flora of the nose prevents acquisition of new strains. However, colonization with other strains may occur when antibiotic treatment is given that leads to elimination of the susceptible carrier strain. Because this situation occurs in the hospital, patients may become colonized with resistant staphylococci. Carriage rate of *S. aureus* in the nares of people outside the hospital varies from 10 to 40%. Hospital patients and personnel have higher carriage rates. The carriage rates of patients may increase during their stay in hospital due to colonization and nosocomial transmission. The rates are especially high in patients undergoing hemodialysis and in diabetics, drug addicts and patients with a variety of dermatologic conditions. The carrier state is clinically relevant because carriers undergoing surgery have more infections than non-carriers. This has led to the application of mupirocin (a local antibiotic) to the anterior nares in some centers just before open-heart surgery to lower the incidence.
of postoperative wound infection.\textsuperscript{64} Dispersers among the carriers are important because dispersers not only transmit staphylococci by direct contact, but also by air-borne transmission. Heavy perineal carriers almost always disperse large amounts of staphylococci. Staphylococci may accumulate rapid on the clothes and bedding of dispersers and may disseminate when these fomites are disturbed. Dust particles containing staphylococci may be carried for considerable distances.\textsuperscript{25,174,217,459}

Since 1944, an increasing proportion of hospital-acquired \textit{S. aureus} strains have developed resistance to penicillin because of their ability to produce beta-lactamase. By 1950, approximately 80\% of hospital-acquired infections were caused by these penicillinase producers. In the mid 1950s, penicillin-resistant \textit{S. aureus} of a new phage type (the so-called phage type 80) was first detected in Australia and then spread rapidly, causing a major pandemic of hospital-acquired infection. Because of the ‘resistance advantages’ of the new \textit{S. aureus} strain, referred to as ‘hospital staph’, there were again outbreaks on hospital wards. In 1959, the British Ministry of Health published guidelines for the prevention of further spread of the multi resistant [penicillin-streptomycin-tetracycline-erythromycin-resistant (PSTE)] \textit{S. aureus} strains. A temporary respite in the war against resistant \textit{S. aureus} occurred in the early 1960s, when Rolinson and Stevens discovered beta-lactamase stable penicillin, (methicillin). In the following years the introduction of the semisynthetic penicillins (methicillin, Oxacillin, flucloxacillin) and the application of infection control measures probably contributed to the spontaneous decrease in the epidemic of \textit{S. aureus} strains. However, coinciding with the disappearance of PSTE-strains was the appearance of celbenin (methicillin) –resistant staphylococci (MRSA). Early MRSA epidemics were reported in the early 1960s.\textsuperscript{235} Later, two surveillance studies conducted by the Cross Infection Reference Laboratory in London showed that the frequency of methicillin resistance in clinical isolates from London hospitals and from hospitals in south East England increased until 1963, when it was followed by a stationary phase (below or around 1\%) until 1968. The rate of methicillin resistance rose sharply in the period 1968-1969 (upto 5\%). After another decline of major MRSA epidemics, the first serious
hospital outbreaks were reported in 1970. The early MRSA epidemics could have been controlled by aminoglycosides. In the late 1970s however, resistance to gentamicin emerged in *S. aureus* and led to a new wave of hospital outbreaks.\textsuperscript{406,421} Since then the MRSA strains have spread all over the world, not only in hospitals, but also in the community. In the USA, the percentage of MRSA among all hospitals in the National Nosocomial Infection Survey System increased from 2.4% in 1975 to 29% in 1991. The incidence varied according to bed size, with hospitals of less than 200 beds reporting 14.9% of *S. aureus* isolated as MRSA, and hospitals of 500 beds reporting a 38.3% frequency. Within European countries a systematic survey of 40 laboratories documented a wide variation in incidence ranging from 0.1% in Northern European countries to over 30% in Spain, France and Italy.

Patients at highest risk for MRSA infection are:

- Those in large tertiary-care hospitals, particularly the elderly and immunocompromised;
- Those in intensive care units;
- Burns patients;
- Those with surgical wounds; and
- Patients with intravenous lines.

Duration of hospitalization, previous antibiotic treatment and proximity to a patient colonized or infected with the organism also predispose to MRSA infection. In acute care and nursing facilities, colonized and infected patients are the major MRSA reservoir. Person-to-person transmission of MRSA usually occurs via the hands of health care workers.\textsuperscript{80,456}

2.5.2 CLINICAL SIGNIFICANCE OF COAGULASE NEGATIVE STAPHYLOCOCCI

As a group, the coagulase-negative *Staphylococcus* species (CNS) are among the most frequently isolated bacteria in the clinical microbiology laboratory. One of the major problems facing the laboratory is distinguishing clinically significant, pathogenic strains of CNS from contaminant strains. The vast majority of infections (or diseases) assumed to be caused by CNS are a significant consequence of hospitalization.\textsuperscript{256} Recent reports on surveillance data taken from the National Nosocomial Infections Surveillance System during the late 1980s and early 1990s have indicated that CNS are
among the five most commonly reported pathogens (in fifth place at 9 to 9.7%, compared with 10 to 11.2% for Staphylococcus aureus) in hospitals conducting hospital-wide surveillance. CNS were the most frequently reported pathogens in nosocomial blood-stream infections (27 to 27.9%, with S. aureus next at 16 to 16.5%). The ranking and infection rates of CNS were quite similar among hospitals conducting surveillance in intensive care units (ICUs) and in those conducting surveillance hospital-wide. In contrast to the situation in the 1970s, major shifts have occurred in the decade of the 1980s and in the early 1990s in the etiology of nosocomial infection. Most noticeably, the shifts have been towards the more antibiotic resistant pathogens, of which the CNS are a major group. Current antibiotic-prescribing practices, including preoperative antibiotic prophylaxis, have led to the selection of antibiotic-resistant organisms. The increasing importance of CNS also may be due in part to the growing appreciation of this group of organisms as opportunistic pathogens and to the increase in the use of transient or permanent media devices, such as intravascular catheters and prosthetic devices, in seriously ill and immunocompromised patients (i.e. intensive care patients, premature newborns, and cancer and transplant patients). CNS infections often can be life threatening in these patients. CNS are a major component of the normal flora of the cutaneous ecosystem, including the skin and mucous membranes. In the cutaneous ecosystem, CNS generally have a benign relationship with their host and function as commensal or saprophytic organisms. However, if the cutaneous organ system has been damaged by trauma, inoculation by needles, or direct implantation of foreign bodies, these organisms can gain entry to the host. Depending upon their ability to adhere to host or foreign body surfaces, breach or avoid the host immune system, multiply, and produce products that damage the host, they may develop the lifestyle of a pathogen. Historical Perspective

In 1958, Smith and coworkers noted the potential pathogenicity of CNS by collecting data from patients with septicemia. Several years later, Pulverer and Halswick reported on 128 cases of endocarditis believed to be caused
by CNS. Prior to the 1970s, clinicians and microbiologists generally regarded CNS as contaminants in clinical specimens and S. aureus as the only pathogenic Staphylococcus species. In his Theodor Billroth Memorial Lecture at the Fifth International Symposium on Staphylococci and staphylococcal Infections, Pulverer shared his frustration with the medical community when he said, “In 1965 we sent a paper entitled ‘Coagulase-negative staphylococci as pathogenic agents’ to one of the leading medical journals in Germany. We had great problems in convincing the editors that we had no joke in mind but wished to report seriously about a lethal case of CNS endocarditis. In 1964, we observed this rather malignant disease in a 57-year-old man who died several months later despite a long-lasting and high-dosage penicillin treatment.” Brandt and Swahn reported that more than 1% of all cases of endocarditis might be due to CNS. In 1965, Wilson and Sturat reported that CNS were found in pure culture in 53 of 1,200 (4.4%) cases of wound infections. In 1971, Pulverer and Pillich investigated the incidence of CNS pyogenic infections in Cologne, Germany, presenting data for the years 1960, 1969 and 1970. CNS were found in about 10% of all pyogenic lesions observed in hospital patients, and in about 50% of these cases, CNS were believed to be present in pure culture. In 1962, Pereira reported that a certain group of CNS (now known as S. saprophyticus) caused urinary tract infections (UTIs). A few years later, Gallagher and coworkers and Mabeck also presented evidence that CNS cause UTIs. In 1971, Holt reported the colonization of ventriculoatrial shunts by CNS. Colonization was usually followed by septicemia. Looking through the literature Pulverer collected data from 2,276 ventriculoatrial or peritoneal shunt operations and estimated that 8% of the patients acquired shunt infections, with 58% of the cases probably caused by CNS.256,371,387

In light of recent advances there has been a considerable progress in the classification of staphylococci and in the development of methods for identifying them at the genus, species, subspecies, and strain levels has been made. By the 1980s, the range of infections believed to be caused by CNS, and especially by S. epidermidis, was quite wide and included bacteremia native valve endocarditis (NVE) and prosthetic valve
endocarditis; osteomyelitis; pyoarthritis; peritonitis; during continuous ambulatory dialysis; mediastinitis; prostatitis; infections of permanent pacemakers; vascular grafts and intravascular catheters; cerebrospinal fluid shunts and prosthetic joints and a variety of orthopedic devices; and UTIs. The CNS species *S. saprophyticus* was often regarded as a more important opportunistic pathogen than *S. epidermidis* in human UTIs, especially in young, sexually active females. It was considered to be the second most common cause of acute cystitis or pyelonephritis in these patients. Several other CNS species have been implicated at low incidence in a variety of infections. *S. haemolyticus* is the second most frequently encountered CNS species in the clinical laboratory. This species has been implicated in NVE, septicemia, peritonitis; *S. warneri* is believed to be the cause of some cases of vertebral osteomyelitis, NVE, and UTIs. *S. hominis* has been associated with endocarditis, peritonitis, septicemia, and arthritis. Some of the earlier reports indicating an association of *S. hominis* with infections may have been in error due to the confusion of this species with phosphatase-negative strains of *S. epidermidis*. *S. simulans* has been associated with some cases of chronic osteomyelitis and pyarthrosis.

2.6 STAPHYLOCOCCI AND CLINICAL MANIFESTATIONS

Staphylococcal infections are characterized by intense suppurative inflammation of local tissues with a tendency for the infected area to become encapsulated leading to abscess formation.

2.6.1 CLINICAL MANIFESTATIONS OF STAPHYLOCOCCUS AUREUS

FURUNCLE

The most common staphylococcal infection is the furuncle or boil, which is a localized painful superficial skin infection that develops in a hair follicle or gland. Similar infections at the base of the eyelashes are the common styes. The most common sites for boils are the neck and the buttocks, often as a result of wearing tight clothes. The infected patient is often a carrier of the *Staphylococcus* that causes the infection, usually in the anterior nares. A total of 65% of furuncles, especially those on the head and neck, are caused by a strain indistinguishable from that in the patient's nose, and there are
indications that perineal carriage is associated with furuncle on the legs. No specific pathogenicity factors are responsibly for the pathology of furuncles. Although about 50% of strains involved in carbuncles and furuncles have been found to produce PVL, only 2% of all S. aureus are producers. The course of infection is usually benign. No specific treatment is needed and most often the infection resolves after spontaneous drainage of the pus or surgical incision.56

CHRONIC FURUNCULOSIS

Approximately 2-3% of the populations have chronic furunculosis, with repeated attacks of boils are often caused by the same phage type of S. aureus. The most appropriate management of patients with recurrent furunculosis includes a course of an oral antibiotic combined with chlorhexidine washes and the use of an antistaphylococcal intranasal ointment.

CARBUNCLE

Staphylococcal infections may spread from a furuncle to the deeper subcutaneous tissues resulting in the development of one or more abscesses known as carbuncles. These abscesses occur mostly on the back of the neck, but may involve other skin sites. They may result in bloodstream invasion.

BULLOUS IMPETIGO

Certain strains of S. aureus can cause bullous impetigo. This characterized by small bullae, which form and burst. When this occurs in infants it may be described as pemphigus neonatorum. Bullous impetigo is a highly contagious superficial skin infection characterized by large blisters containing many staphylococci in the superficial layers of the skin. Bullous impetigo is most often seen in the infants and children under conditions where direct spread can occur (e.g. sharing of contaminated towels). Impetigo mainly occurs on the face and limbs. Extensive atypical bullous impetigo is associated with HIV infection. The strains often produce exfoliative toxin and impetigo can therefore be considered as a localized
form of scalded skin syndrome. The bullae are the result of epidermolytic toxins, which are serine proteases.

BOTRYOMYCOSIS

Botryomycosis is a rare chronic skin infection that resembles mycetoma or actinomycosis. The lesions may be nodular or granulomatous and many have draining sinuses.

SECONDARY CUTANEOUS INFECTION

The most common secondary infection occurs in people with eczema, especially atopic dermatitis. Colonization of lesions in adults approaches 100%; children are colonized only slightly less often. Skin ulcers, especially those of the legs in the elderly, may also become heavily colonized by S. aureus. Similarly, hidradenitis suppurativa, a chronic suppurative disease of the skin that occurs in areas bearing apocrine glands, most often the axilla and groin, also yields S. aureus.

PARONYCHIA

Another common S. aureus infection is paronychia, which involves the soft tissue around the nails.

DEEP LESIONS

Staphylococcus aureus can cause a wide variety of deep tissue, often metastatic, infections. These infections include osteomyelitis, arthritis, endocarditis and cerebral, pulmonary and renal abscesses, and breast abscesses in the nursing mother. Staphylococcus aureus can also cause bacterial pneumonia, which is almost always secondary (e.g. after influenza or other viral infections). In many of these situations, diabetes mellitus, leukocyte defects or a general reduction of host defenses by alcoholism, malignancy, old age or corticosteroid or cytotoxic therapy are predisposing factors. Severe S. aureus infections, including endocarditic, are particularly common in intravenous drug abusers.

WOUND INFECTIONS

Staphylococcus aureus is a notorious cause of wound infection. Infections can be major complications following surgery. The source may be the
patient’s own carrier state, other carriers (e.g. physicians or nurses) or other infected patients. Whether a staphylococcal wound infection occurs following surgery depends upon a complex interaction between:

- Host factors, including the status of the immune system and the presence of disease such as diabetes mellitus;
- Surgical factors, such as the disruption of tissue perfusion that accompanies the surgical procedure and whether foreign bodies are used;
- Staphylococcal factors, including substances that mediate tissue adherence and invasion or that enable staphylococci to survive the host defenses and antibiotics in the tissues; and
- The use of antimicrobial prophylaxis.

Staphylococcal infections at the site of intravenous lines can result in bacteremia with metastatic infection.°°

STAPHYLOCOCCAL SCALDED SKIN SYNDROME

This refers to a group of primarily cutaneous diseases (e.g. generalized scalded skin syndrome, staphylococcal scarlet fever and bullous Impetigo) caused by the staphylococcal exfoliatin toxins, exfoliatoxin A or exfoliatoxin B. Toxic epidermal necrolysis or Lyell’s disease is also known as generalized scalded skin syndrome. It results from the production of exfoliatin toxin in a staphylococcal lesion, which can be quite minor. The toxin is absorbed into the bloodstream, and intraepidermal desquamation may occur at remote sites from which S. aureus cannot be isolated. The disease is most common in neonates and in children under 8 years of age, and epidemics may occur in nurseries. The face, axilla and groin are most often affected first, but the erythema, bullous formation and subsequent desquamation of epithelial sheets can spread to all parts of the body. Generalized scalded skin syndrome is the most severe manifestation of exfoliatin-producing staphylococcal infections.^^®
TOXIC SHOCK SYNDROME

Toxic shock syndrome is a serious disease associated with *S. aureus*. As mentioned above it has been most commonly seen in young women during or immediately after menstruation and is associated with the use of highly absorbent intravaginal tampons. In such cases, *S. aureus* grows in large numbers in and around the tampon and liberates TSST-1. The disease is characterized by the development of high fever, vomiting, diarrhea, sore throat and muscle pain. Within 48 hours it may progress to septic shock with evidence of renal and hepatic damage. A skin rash may develop, followed by exfoliation at a deeper level than in scalded skin syndrome. Blood cultures are usually negative.50

STAPHYLOCOCCAL FOOD POISONING

Staphylococcal food poisoning results from the production of staphylococcal enterotoxin in food before ingestion. It is intoxication, not an infection. Because of the heat resistance of the toxin, toxicity persists even if the food is heated to boiling. Ingestion of the food results in acute vomiting and diarrhea within 1-5 hours. There is prostration, but usually no fever. Recovery is rapid, except sometimes in the elderly and in those with other disease. The diagnosis is suspected on the basis of exposure history and of the typical symptoms (nausea, vomiting, abdominal cramping and diarrhea). Worldwide, 30% of cases of food-borne illness are caused by staphylococci.

Table 2.11 Examples of Infections with *Staphylococcus aureus*

<table>
<thead>
<tr>
<th>Primary infection</th>
<th>Superficial infection</th>
<th>Deep infection</th>
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<td>Folliculitis</td>
<td>Abscess</td>
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<td>Furunculosis</td>
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<td>Impetigo</td>
<td>Pneumonia</td>
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<td></td>
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<td>Osteomyelitis</td>
</tr>
</tbody>
</table>
Septicaemia
Secondary Infection
Eczema
Decubitus ulcers
Toxin mediated disease
Food poisoning
Toxic shock syndrome
Scalded skin syndrome

2.6.2 CLINICAL MANIFESTATIONS OF STAPHYLOCOCCUS EPIDERMIDIS
CATHETER-RELATED INFECTIONS

*Staphylococcus epidermidis* is the most frequently isolated coagulase negative staphylococci species in catheter-related infections, especially in children. These infections have been reported in association with central hyperalimentation catheters, peripheral intravenous catheters, subclavian catheters, Hickmann and Broviac central lines and Swan-Ganz catheters. The diagnosis of a catheter-related infection is often a physician's nightmare. Catheters may be colonized without local signs of infection (i.e. without gross evidence of purulence or erythema at the insertion site of the catheter). Furthermore, blood cultures may yield *S. epidermidis* without the patient showing major clinical symptoms. A diagnosis of catheter-related infections is based on a proper collection of at least three blood cultures taken from different sites of venepuncture within 24 hours. In addition, culture of the intravascular catheter segment should be performed according to current standards. In most clinical microbiology laboratories, the intravascular segment of the catheter is cultured according to the method of Maki and colleagues: catheter segments are cultured after being 'rolled' over a blood agar plate and after incubation for 18 hours at 98.6°F (37°C), the number of CFUs are counted. Cultures yielding more than 15CFU/blood agar plate are associated with catheter-related bacteremia.²⁵⁶
CEREBROSPINAL FLUID SHUNT INFECTIONS

Both S. epidermidis and S. aureus strains are the most frequently encountered isolates in CSF shunt infections. Infections usually occur within 2 weeks of implantation or manipulation of the shunt. Clinical manifestations are usually nonspecific.\textsuperscript{347}

PERITONITIS

About 40% of patients who have CAPD develop peritonitis during the first year (overall incidence ranging from 0.6-6.3 episodes/patient-year) Criteria for diagnosis include a combination of:

- abdominal pain
- cloudy fluid containing more than 100 white blood cells/mm\(^3\) (the majority of which are PMNLs), and
- a positive dialysate culture.

\textit{Staphylococcus epidermidis} is by far the most frequently isolated organism in patients with CAPD peritonitis

ENDOCARDITIS

Infections of native cardiac valves with coagulase-negative staphylococci are uncommon, accounting for only about 5% of all cases of infective endocarditis. However, \textit{S. epidermidis} and \textit{S. aureus} account for the majority of nosocomial-acquired infective endocarditis infections. These bacteria are the single most common cause of infections of prosthetic cardiac valves, and \textit{S. epidermidis} is the cause of approximately 40% of cases of prosthetic valve endocarditis. In more than 80% of patients, \textit{S. epidermidis} valve endocarditis is associated with valve dysfunction or persistent fever during therapy. The infection is most frequently located in the valve sewing-ring, resulting in complications such as dehiscence, dysrhythmia and obstruction of the valve orifice. Fever usually persists during therapy because the valve ring abscess is relatively protected from antibiotics. \textit{Staphylococcus epidermidis} prosthetic valve endocarditis shows an indolent clinical picture without the classic endocarditis findings such as peripheral emboli and multiple positive blood cultures. Valve dysfunction and fever are the most frequent symptoms. Most patients are infected at the time of cardiac surgery,
but the incubation period can vary, with a latency of 1-2 months before symptoms arise.\textsuperscript{143,243}

**INFECTIONS IN IMMUNOCOMPROMISED PATIENTS**

The coagulase-negative staphylococci have emerged as pathogens in immunocompromised hosts, notably neonatal ICU patients and oncology patients. Most coagulase-negative staphylococci infections in these patients are related to the use of foreign devices, notably central intravascular catheters.

**URINARY TRACT INFECTION**

Among the coagulase-negative staphylococci S. saprophyticus can cause spontaneous acute urinary tract infection in previously healthy people. With a few exceptions these are women aged 16-25 years. The disease appears soon after the woman becomes sexually active and the symptomatology closely resembles that of urinary tract infection caused by *Escherichia coli*.\textsuperscript{213,214,304,483}

**2.7 HEALTHY FLORA OF STAPHYLOCOCCI AND SOURCES OF INFECTIONS**

The majority of staphylococcal infections arise from endogenous sources. This is true not only of sporadic infections, minor wound sepsis and post influenzal pneumonia in the community, but also of a considerable proportion of surgical wound infections and neonatal sepsis. Sometimes, however, staphylococci from another carrier or a contaminated object will reach, and cause infection in, a patient. Air-borne transmission may also occur. Carriage of *S. aureus* can be considered in the light of self-infection or as a source for cross-infection.\textsuperscript{25,188,262,430}

**2.7.1 CARRIAGE**

The carriage of *S. aureus* was first described by Halman (1937)\textsuperscript{188} and has since been studied intensively (William 1963).\textsuperscript{500} At any time about 35% of normal adults carry *S. aureus* in the anterior nares. Populations of primitive peoples are reported with lower carrier rates (7-25%) usually associated with high carrier rates of Enterobacteriaceae. However, successive monthly
random samples of about 240 individuals from a normal European population yielded variable nasal carrier rates, making it difficult to assess the significance of different rates usually measured in a small population. About 65% of anterior nasal carriers also carry on the turbinates and posterior nasal space and seeding from this site may account for apparent intermittent carriage in the anterior mares. High carrier rates are recorded for infants: by the age of 2 weeks rates of 60-70% of those born at home and 80-100% of those born in hospital. The rate declines to 20% by the end of infancy and rises again to reach the adult level by about 5-8 years. In elderly persons it declines to 20-25%. Repeated sampling of the same population yields cumulative nasal carrier rates of 60-90%; 20-35% of persons are persistent carriers, 30-70% are intermittent carriers and 10-40% are never carriers (Williams 1963). Persistent carriers usually harbour the same strain for months or years, indicating a marked stability in the nasal flora. Those who acquired a 'hospital Staphylococcus' in the nose while an in-patient, were frequently readmitted carrying their original domestic strain.

Information on throat carriage of S. aureus is discrepant, with reported rates varying from 4 to 64% (Williams 1963). A random sample survey of a normal population showed winter carriage rates of about 15% but in spring these dropped to about 3%, suggesting that the discrepant rates may be seasonally related. Faecal carriage rates in infants generally reflect nasal carriage; in normal adults faecal carrier rates are about 20% at a single examination and only small numbers of cells are found. This probably also reflects nasal carriage. Patients with chronic skin disease, especially the eczemas, are almost universally heavily colonized by S. aureus. Patients with other skin diseases are less prone to colonization unless large amounts of steroid have been used in therapy.

2.7.2 AIR BORNE DISSEMINATION

Few staphylococci are disseminated directly into the air from the respiratory tract; much larger numbers reach the environment from the body surfaces carried on air-borne desquamated skin scales. Some individuals disperse organisms very efficiently in this way; some, but not all, are heavy nasal carriers and may contribute to environmental contamination in hospital
wards. Profuse dispersal is more common in males than females and is related to dispersal from skin areas below the waist. Much dispersal results from friction of clothing removing scales from the skin surface but some dispersal takes place even from those who are naked. Nevertheless, only about 10% of air-borne staphylococci survive the first 24 h, about half dying during the initial desiccation process; the infectivity of these dried organisms is not known.\textsuperscript{415}

2.7.3 DISSEMINATION BY CONTACT

The undoubted reduction in wound sepsis rate which followed the introduction of ‘no touch’ dressing techniques and clear accounts of the role of hands in contaminating wounds indicate the importance of the this route of infection. Nasal carriers of S. \textit{aureus} frequently contaminate their fingers with their nasal strain and suppression of nasal carriage with antibiotic mupirocin has been shown to result in a reduction in hand carriage.\textsuperscript{56,80}

2.7.4 ACQUISITION AT CARRIER SITES

2.7.4.1 The Neonatal Period

Newborn babies are very susceptible to colonization with S. \textit{aureus}, which is the usual consequence of their first exposure to the organism; clinical sepsis seldom develops without prior colonization. Colonization of the nose, umbilical stump, or both rapidly approached 100% but if the stump was treated with hexachlorophane the nasal carrier rate was reduced. When the use of hexachlorophane ceased in the early 1970s carrier rates rose but the introduction of chorhexidine treatment reversed this trend. All are agreed that colonization predisposes to infection. Staphylococci may be spread in nurseries by the airborne or contact route. Rammelkamp and his colleagues compared the rate of colonization from an index source in the same room when they were attended to:

1. with unwashed hands
2. with effectively washed hands
3. with gloved hands and
4. without direct manual contact with the source of infection.
Briefly, contact with unwashed hands caused 5 times as many acquisitions as air-borne infection but if the hands were carefully washed or gloved the 2 routes of acquisition were about equal.\textsuperscript{282,393}

2.7.4.2 Later Acquistions

Patients admitted to hospital-acquired staphylococci in the nose roughly in proportion to the duration of stay in hospital and to the amount of air-borne staphylococci to which they are exposed,\textsuperscript{356} suggesting that the inhalation of air-borne particles bearing staphylococci is the most important route. However, patients admitted to dermatology wards also acquired \textit{S. aureus} on the chest and perineum, though at slightly lower rates than in nose. Patients with eczema acquired \textit{S. aureus} on the chest more often than did those with psoriasis, but nasal and perineal acquisition occurred at the same rate in each group of patients. Application of topical antibiotics was more likely to lead to nasal and chest acquisition than were systemic antibiotics but any antibiotic therapy was more likely to lead to acquisition than was absence of antibiotic therapy. This agrees generally with the many studies of nasal acquisition and the role of antibiotic therapy.\textsuperscript{190,328}

2.8 INFECTIONS OF THE EYE AND STAPHYLOCOCCI

Staphylococci are the most common, as well as the most thoroughly studied, cause of ocular infections. Staphylococci were once regarded simply as harmless conjunctival saprophytes. \textit{Staphylococcus} (usually \textit{S. epidermidis}) is present on the lid margins and very frequently in the corresponding conjunctival sac. Therefore, it is recommended that separate specimens be taken from the conjuctiva and lids. It is significant that experiments have established that ophthalmic Staphylococci produce powerful toxins (Allen). The development of tests for pathogenicity is also of major importance. With the advent of antibiotics, acute Staphylococcal infections (especially cellulites), which were previously dangerous can now be successfully treated. However, treatment of chronic Staphylococcal infections and prevention of their recurrence are still serious problems in ophthalmology. The importance of allergic reactions to staphylococcal infections is now frequently discussed; all staphylococci may be antigenic. Unfortunately,
allergy cannot be satisfactorily proven until the various types of sensitization are more fully understood.\textsuperscript{86,135,182,299,385,433}

2.8.1. BLEPHARITIS

Two clinical forms of blepharitis are seen: ulcerative form and squamous. Staphylococci play a primary role in the ulcerative form and a secondary role in the squamous type (usually seborrheic), where the \textit{Staphylococcus} acts as secondary invader. Either type may recur and persist for years. The conditions are usually associated with conjunctivitis and purulent inflammation of the hair follicles as well as of the adjacent meibomian glands (hordeolum externum and internum). Accumulation of bacteria under the crusts of the ulcer or inside the meibomian glands results in a process of long duration and, often, hypersensitivity phenomena. Tylosis ciliaris (thickening of the lid margins) and madarosis (loss of cilia) caused by destruction of the hair follicles are unpleasant complications of the process. These irreversible changes are dangerous to the cornea because of exposure and dysfunctions of the lacrimal film.\textsuperscript{416}

2.8.2 HORDEOLUM

Hordeolum internum is an acute inflammation of the meibomian glands, aggravated by Staphylococci. The process is stubbornly resistant to treatment (drugs do not readily penetrate the glands). Hoedeolum externum (acute inflammation of the Zeis gland) is usually a staphylococcal infection. The process is more accessible to treatment. If it is localized at the angulus externus, a tremendous edema may develop, as the lymphatic ways cross here. Hordeolosum, the persistent recurrence of several hordeola, may be seen in patients with chronic blepharitis or conjunctivitis or in debilitated persons. Staphylococci can also cause such dangerous diseases as orbital cellulitis and intraocular postoperative infection. Most cases of bacterial postoperative endophthalmitis are caused by \textit{Staphylococcus}. Frequently it is a hospital-acquired strain. The lid margins and nasal passages are potential sources of infection. For diagnosis, the aspiration of the anterior chamber and vitreous is necessary. Unfortunately, diagnosis is not always successful, particularly after treatment.\textsuperscript{416}
Staphylococci are the most common secondary invaders in many conditions: dacryocystitis, trachoma, pemphigus, keratomalacia, and some others. In such viral infections as measles, influenza, and adenoviral infection, superimposed staphylococcal infection may lead to serious complications. Most frequently, staphylococcal infections of the eye are associated with skin diseases.\textsuperscript{416}

2.8.3 CONJUNCTIVITIS

In adults, conjunctivitis is usually chronic and secondary to blepharitis, which is sometimes unnoticed, especially when mild. Chronic conjunctivitis is most troublesome, particularly in old people, since it is of long duration and recurrent. In these cases, factors besides staphylococci have to be considered: dryness of the conjunctiva, wearing of contact lenses, use of mascara, metabolic disturbances, and others. Acute conjunctivitis, seen usually in children, is less frequent than the chronic form and may develop independently of blepharitis. Acute conjunctivitis has a rather typical appearance: (1) the lower palpebral conjunctiva is predominantly affected, (2) the discharge may be scanty or heavy, but the underlying tissue is little involved, (3) the condition is initially acute and if not properly treated may easily become chronic, and (4) corneal complications are not infrequent. Acute staphylococcal conjunctivitis of the newborn differs from the condition as manifested in later life. It is a purulent but superficial type with very little tissue involvement, probably due to prevention by coagulase action. Nevertheless, it can occasionally be as acute as gonococcal conjunctivitis. Corneal involvement is uncommon, and there is no tendency to chronicity. The explanation for these peculiarities probably lies in some anatomic characteristics (absence of lymphatic tissue in the conjunctiva and rudimentary meibomian glands) peculiar to the neonate. Phyctenules are a frequent reaction to staphylococcal conjunctivitis, particularly in children. The lesions often ulcerate.\textsuperscript{66,158,290,313,375}

2.8.4 KERATITIS

Surprisingly, primary central staphylococcal hypopyon keratitis rarely results from corneal injury. The keratitis usually develops secondary to
staphylococcal conjunctivitis or blepharoconjunctivitis. The most frequent type of keratitis is marginal, characterized by a small, grayish infiltrate adjacent to but always separated from the limbus by a narrow clear zone. The small, multiple infiltrates may run along the entire limbus and the infiltrated area ulcerates readily. The process may be caused by staphylococcal toxin, and bacteria are usually not found in the scraping from the ulcer itself. The keratitis is mild and is generally cured together with the conjunctivitis. Epithelial punctate keratitis also may complicate staphylococcal infection. Tiny punctate epithelial erosions, most marked in the lower half of the cornea, can be seen only by staining with fluorescein. This condition is most often observed in staphylococcal conjunctivitis and is probably an allergic or mechanical phenomenon.\textsuperscript{266,310,503}

2.9 EMERGENCE OF RESISTANCE IN STAPHYLOCOCCI

Staphylococci have a record of developing resistance quickly and successfully to antibiotics. This defensive response is a consequence of the acquisition and transfer of antibiotic-resistance plasmids and the possession of intrinsic resistance mechanisms, some of which may have evolved prior to the formation of the genus \textit{Staphylococcus}. The acquired defense system by staphylococci may have originated from antibiotic producing organisms, where they may have been developed and then passed on to other genera. Presumably the genus \textit{Staphylococcus} has been one of the recipient genera as a consequence of coming in contact with antibiotic-producing bacteria and fungi in their natural habitats. Staphylococci are sometimes exposed to heavy metal ions present in their natural habitat or in the environment, e.g. by contact with certain metal-containing cosmetics, ointments, antiseptics and soils. They may also participate in genetic exchange with related soil bacteria, acting as reservoirs of heavy metal ion resistance genes.\textsuperscript{119}

Three different mechanisms of resistance to $\beta$-lactam antibiotics have been identified for staphylococci: the inactivation of penicillin (and cephalosporin) by penicillinase ($\beta$-lactamase) mediated hydrolysis of the $\beta$-lactam ring of the antibiotics; an intrinsic resistance resulting from a reduction in the affinity or the amount of the PBPs; and tolerance to the bactericidal effect of $\beta$-lactam
antibiotics due to an excess of autolysin inhibitor. Penicillin-resistant S. aureus strains began emerging shortly after the introduction of penicillin in medicine in the early 1940s. Today the percentage of penicillin-resistance strains has risen to 75-95%, with the highest percentage found among hospital (nosocomial) strains. Similar percentages have been observed for penicillin-resistant strains of the S. epidermidis species group. Most penicillin-resistance staphylococcal strains produce β-lactamase. β-Lactamase genes (bla) are most often found on class II plasmids that are distributed among many staphylococcal species. Resistance to methicillin and other β-lactamase-resistant penicillins was first observed in S. aureus soon after the introduction of methicillin into clinical use in Britain. The methicillin-resistant strains isolated in Britain came from hospitalized patients, were multiply antibiotic-resistant, belonged to phage III, and their resistance to methicillin was heterogeneous, affecting only a minority of the cell population. After the mid-1970s, large outbreaks of infection by methicillin-resistant S. aureus (MRSA) were recorded in many hospitals in Australia, USA, Britain, and Ireland. Many of these out breaks appear to have been caused by a single epidemic strain that was transferred between hospitals by the movement of patients. Genetic analyses and molecular typing methods have indicated that various isolates of MRSA may have had a clonal origin, and suggest that the methicillin-resistance determinant was acquired by S. aureus at some point in time before or around the time of the introduction of methicillin. During the 1980s, attention was drawn to the high incidence (20-85% of strains) of methicillin-resistant S. epidermidis (MRSE) in nosocomial infections. Methicillin resistance has now been detected in several other staphylococcal species, including S. haemolyticus, S. hominis, S. capitis, S. warneri, S. caprae, S. simulans, S. saprophyticus and S. sciuri.

Erythromycin-resistant staphylococci often have cross-resistance to macrolides (erythromycin, oleandomycin, spiramycin, clarithromycin, azithromycin), line cosamides (lincomycin, clindamycin) and streptogramin type B antibiotics (designated MLS-resistant). The different types of MLS antibiotics bind to the 50S ribosomal subunit at overlapping binding sites,
and binding interferes with transpeptidation and translocation reactions needed for peptide chain elongation. These sites are protected by the \(N^6,N^6\)-dimethylation of an adenine-2058 residue at the peptidyl transferase center of 23S rRNA, a reaction performed by an rRNA methylase. Three distinct rRNA methylase genes have been detected in staphylococci: \(ermA\), \(ermB\), and \(ermC\). Even though each of the \(erm\) gene classes can be distinguished by hybridization, the amino acid sequences of the encoded methylases are highly conserved, which suggests that they evolved from a common ancestor, possibly originating in the antibiotic-producing organism.

Tetracycline resistance is widespread among staphylococcal species and ranks along with \(\beta\)-lactam and MLS resistance as one of the most frequent types of antibiotic resistance found in natural populations of staphylococci. There are 3 major mechanisms responsible for aminoglycoside resistance in staphylococci. One mechanism involves changes in ribosomal proteins as a consequence of certain mutations in their structural genes, such that ribosomes can no longer bind streptomycin. A second mechanism involves the energization and permeability of the cell membrane. Some energy deficient, small-colony mutants of \(S.\) aureus have been shown to be resistant to aminoglycosides due to a diminished uptake of the antibiotics, presumably as a consequence of cell membrane impermeability. The third and most common mechanism of resistance involves modification of aminoglycosides by aminoglycoside-modifying enzymes so that the antibiotics are no longer capable of binding to ribosomes.

Trimethoprim resistance is either mediated by alterations in the expression of the intrinsic chromosomal \(dfr\) gene possibly resulting in overproduction of the native dihydrofolate reductase (DHFR) or a reduced affinity of the native DHFR for trimethoprim, or by the acquisition of a second chromosomal or plasmid \(dfr\) gene that encodes a trimethoprim-resistant DHFR capable of rescuing the reduction step leading to tetrahydrofolate in the presence of trimethoprim.

Glycopeptides are being used to treat severe infections caused by methicillin and other \(\beta\)-lactam-resistant staphylococci. Teicoplanin resistance
(MIC >16 μg ml\(^{-1}\)) and/or moderate vancomycin susceptibility (MIC 8-16 μg ml\(^{-1}\)) have been observed in \(S.\) \(h\)aemolyticus, especially in methicillin-resistant strains of this species. Strains of \(S.\) \(h\)aemolyticus and \(S.\) \(e\)pidemicis that are moderately susceptible to teicoplanin (MIC 16 μg ml\(^{-1}\)) are relatively common.\(^{36,173}\) Recently, infections with strains of MRSA that did not respond to vancomycin therapy have been reported. In May 1996, a vancomycin hetero-resistant strain (MIC, 18 μg / ml) was cultured from a chronically infected surgical wound in a 4-month old boy in Japan, who had been treated with the drug for 29 days. There have now been several reports of vancomycin intermediate-resistant \(S.\) \(a\)ureus (VISA) infections in the USA, France, UK, Germany, Italy, Greece, Korea, Thailand and Australia. VISA has been isolated from patients of all age groups who received prolonged courses of vancomycin.\(^{85,203,410,434,441}\) However it was not until June 2002 that the first clinical "VRSA" isolate was reported from a patient having chronic foot ulcer in Michigan, U.S.A.\(^{87}\)

Wide use of the fluoroquinolone ciprofloxacin has resulted in a steady increase in the incidence of fluoroquinolone-resistant (MIC ≥ 8 μg/ml\(^{-1}\)) staphylococci, especially among clinical isolates. The newer fluoroquinolones such as fleroxacin, levofloxacin and clinafloxacin are more active than ciprofloxacin against ciprofloxacin-susceptible staphylococci by \textit{in vitro} testing; however the problem still remains that fluoroquinolone-resistant mutants emerge following exposure to these antibiotics. Fluoroquinolone resistance has been identified in the species \(S.\) \(a\)ureus, \(S.\) \(e\)pidemicis, \(S.\) \(h\)aemolyticus, \(S.\) \(h\)ominisies and \(S.\) \(w\)ameri.\(^{15,266,296,409}\)

\section*{2.10 STAPHYLOCOCCAL PLASMIDS}

The genome of staphylococci consists of a chromosome to which may be added or subtracted various accessory elements, such as prophages, plasmids and transposons. The chromosome of \(S.\) \(a\)ureus strain NCTC 8325 is about 2800 kbp, and has been mapped by genetic and physical methods by Pattee\(^{373b}\). More than 100 loci have been mapped to date, including phenotypic markers and silent transposon insertions. The average size of the \(S.\) \(a\)ureus chromosome is 2741 ± 277 kbp.
Plasmids are facultative extrachromosomal genetic systems (elements) and, for the most part, they may be regarded as endosymbionts supplying the host cell with protective and various other adaptive functions. Plasmids are common in natural populations of most staphylococcal species.

The characteristics of many of the plasmids from *S. aureus* have been reviewed by Lyon and Skurray (1987) and Novick (1989). *S. aureus* plasmids have been classified into 3 general classes, I through III. Class I plasmids are of small size (1-5 kbp) and have a high copy number (10-55 copies per cell), and they usually encode a single antibiotic resistance or are cryptic. These plasmids are the most widespread throughout the genus *Staphylococcus*. Novick (1989) has subdivided class I plasmids into 4 subgroups or families on the basis of their nucleotide sequence and functional organization. Plasmids in this class are largely composed of cassettes or specific segments that are not transposons. They represent at least 10 different incompatibility groups and replicate by an asymmetric rolling-circle mechanism. Tetracycline-resistance plasmids (4-4.6 kbp) belonging to this family are highly conserved and are very common in a wide range of staphylococcal species. The erythromycin-resistance plasmids belonging to this family are usually 2-2.5 kbp in size and except for variation in the control region of the ermC gene, are also highly conserved.

Class II plasmids of *S. aureus* have been subdivided into 2 major families on this basis of incompatibility. These plasmids, commonly referred to as penicillin or β-lactamase plasmids, are relatively large (15-33 kbp), have a low copy number (4-6 per cell), and replicate using the θ replication mechanism. They carry various combinations of antibiotic and heavy metal resistance genes, many of which are located on transposons.

Class II plasmids have been identified in many other staphylococcal species but unlike class I plasmids, they are very uncommon in the species *S. hyicus, S. intermedius, S. simulans* and *S. lugdunensis*.

Most of the conjugative plasmids are class III plasmids that encode resistance to gentamicin and certain other aminoglycosides, such as tobramycin and kanamycin. Many also encode resistance to ethidium bromide and quaternary amines, whereas some also encode resistance to
penicillin and trimethoprim. These plasmids are relatively large (30-60 kbp) and have a conjugative transfer (tra) region that is often flanked by directly repeated 900 bp IS257 elements. They are also capable of mobilizing or cotransferring certain smaller coresident plasmids, which are not independently transferable.

2.10.1 PLASMID STABILITY AND THE EFFECT OF CURING AGENTS

The spontaneous loss of a particular trait or phenotypic characteristic in a strain may be the first indication that the character is plasmid-determined, though such loss does not necessarily mean that the genes responsible are plasmid-borne. Chromosomal mutations in other bacterial species occur at frequencies, which might be mistaken for plasmid loss. However, point mutations are often reversible whereas loss of a complete set of genes as occurs with loss of an entire plasmid is irreversible.

2.10.1.1 Plasmid Loss In Vitro

Plasmid loss does occur spontaneously in growing cultures at normal temperature but has also been found to occur in ageing broth cultures, or after storage on slope for several months. Plasmids determining penicillinase production and others determining tetracycline resistance, have been shown to be lost at higher frequencies during incubation at 42 °C-44 °C than at 37°C. Temperature-sensitivity is often a property of the plasmid itself rather than the host cell for introduction of the plasmid into a new host still leads to temperature-instability of the plasmid determinant. However, in some strains temperature sensitivity may be controlled by the host.

Growth at elevated temperature to accelerate plasmid loss is known as curing. There are a number of chemical curing agents, which have been shown to eliminate plasmids from various bacteria, such agents increase the rate of loss of plasmids without having any mutagenic effect on the cell. The usefulness of such agents lie chiefly in the identification of plasmid-determined properties. Various intercalative compounds, which interfere with DNA replication, have been found to efficiently eliminate resistance determinants from bacteria. Hahn and Ciak (1976) tested the ability of 18 DNA-complexing compounds to eliminate antibiotic resistance determinants.
from *Salmonella typhimurium*. The compound, which caused most efficient elimination of resistance determinants, was nitroacridine II, an intercalative agent. In *Staphylococcus aureus* acriflavine has been reported to eliminate erythromycin resistance and penicillin resistance. The precise mechanism of plasmid elimination by the acridine dyes is not yet clearly understood, but ethidium bromide, also an intercalative agent inhibits plasmid DNA synthesis by binding between the base pairs of DNA and inhibiting both DNA-polymerase and RNA-polymerase. An increased rate of loss of the penicillinase plasmid in *S. aureus* strain NCTC 8325 has also been achieved using rifampicin, a derivative of rifamycin, but attempts by other workers to cure the plasmid from the same strain were less effective and less reliable. An agent, which apparently cures by a completely different mechanism, is sodium dodecyl sulphate (SDS). This anionic surface-active agent eliminates R and F factors from *Escherichia* with high efficiency and also the penicillinase plasmids from *S. aureus*. The effect of SDS in *S. aureus* is obscure but in *E. coli* increased plasmid loss is thought to occur by selection of spontaneous variants rather than true curing. Other reported plasmid curing agents include novobiocin and guanidine hydrochloride. Because of the unreliability of the activity of curing agents in general, it is difficult to envisage any practical of therapeutic application *in vivo* of these chemical compounds. Attempts have been made to investigate the effects of some curing agents in mice infected with multi-resistant plasmid-carrying bacteria. However, the work indicated that no practical effectiveness could be expected from the curing drugs currently used.\(^{236,270,271}\)

### 2.10.1.2 Plasmid Loss *In Vivo*

Evidence for plasmid or antibiotic resistance loss *in vivo* is limited. Loss of resistance to five antibiotics from a strain of *S. aureus*, isolated repeatedly from a patient has been attributed by Lacey et al., (1973) to treatment with cloxacillin.\(^{273}\) Indeed, exposure of the strain to cloxacillin *in vitro* resulted in the loss of one or more of the antibiotic resistance markers. Loss of methicillin resistance, penicillinase production and neomycin resistance from a strain of *S. aureus* investigated over a three-month period in a hospital has also been observed. The prevalence of antibiotic resistant staphylococci
from the skin and nares of dermatology patients has been investigated by Noble (1977). He reported that in individuals colonized with antibiotic resistant \textit{S. aureus} more sensitive variants were found in the nose compared to the skin. This probably represented plasmid loss \textit{in vivo} and the particular distribution found may have been the result of either selection for resistance on the skin or for sensitive variants in the nose. The factors or mechanisms controlling plasmid loss or the selection of either resistant or sensitive variants \textit{in vivo} are not yet clearly understood. However, on skin the fatty acids, e.g. linoleic acid, may play an important role in determining the distribution of plasmid-bearing and plasmid-less cells.\textsuperscript{254,271,342}

\section*{2.11 Transfer of Antibiotic Resistance in Staphylococci}

Antibiotic resistant strains may occur as the result of (i) a pre-existing resistant strain (ii) a pre-existing mutant or (iii) as the result of transfer of resistance genes. These stains may then be selected to form a resistant population as a result of antibiotic use. In the last category, genes are often carried by plasmids, which can be transferred from one bacterial strain to another. A resistance gene can also be organized as part of a transposon which can transfer between bacterial replicons both chromosomal and plasmid to build up more complex groups of genes on single plasmids or to introduce them into the bacterial chromosome. The widespread presence of resistance plasmids for the same maker or a group of makers suggests that transfer occurs \textit{in vivo}; as it is unlikely that each has evolved separately on a large number of different occasions. However, an alternative explanation could be that the plasmid has been conserved to a high degree during the evolution of the bacterial host strain. In the staphylococci, transfer by transduction occurs readily \textit{in vitro}. Transfer can also be demonstrated by transformation and by a process requiring mixed culture in which phage can sometimes be shown to be directly involved. Protoplast fusion can also be used for the study of chromosomal recombinants and for the transfer of resistance plasmids.\textsuperscript{176,392,419}
2.11.1 TRANSDUCTION

Among the staphylococci, in vitro transduction techniques are well documented and have been invaluable in the elucidation of the genetics of antibiotic resistance in S. aureus.\textsuperscript{359,360} Transduction was first reported using a u. v induced lysogenic strain (PS 53 lysogenzed with phage 53) as donor and a phage sensitive culture (PS 53) as recipient. Transfer of streptomycin or novobiocin resistance at frequencies around $10^{-7}$ to $10^{-8}$ per phage particle indicated that co-transfer of genes by chance would be rare and co-transduction would indicate that genes were closely linked. Successful transduction has since been achieved for a wide range of chromosomal and plasmid markers at frequencies per phage particle ranging from about $10^{-4}$ to $10^{-10}$ using lysogenic recipient strains, temperate phage propagated by lytic growth and virulent phage. The fragments of DNA carried by transducing phage particles are largely homogenous. The amount of bacterial DNA, which can be transferred, depends on the amount, which can be incorporated into the phage particles. The size of the phage genome is about $28 \times 10^6$ daltons for transducing phage $\Phi$11 (previously P11) and $31 \times 10^6$ for the transducing and typing phage 80. This genome size probably represents about 1% of the total amount of DNA carried by the staphylococci cell. Plasmid isolated so far in staphylococci have a molecular weight range of about $1 \times 10^6$ to an upper limit of about $32 \times 10^6$ so that only at the larger end of scale will the transfer of the complete plasmid be restricted if it assumed that size is the only limiting factor. Many bacteria including staphylococci have restriction and modification systems, which degrade and modify DNA. This can be shown to affect the ability of a strain to act as recipient in in vitro experiments and could be important in the spread of resistance in vivo. Most examples of transduction in staphylococci have the characteristics of a generalized transduction system in which many different markers can be transferred. Novick (1967) isolated a high frequency transducing element in S. aureus as a result of recombination between the transducing phage P11 and a plasmid coding for penicillin and erythromycin resistance carried in the same donor strain.\textsuperscript{225,358,373}
2.11.2 TRANSFORMATION

Transformation involves the uptake and expression of DNA from a donor strain by a recipient strain and has been shown for both chromosomal and plasmid markers in *S. aureus*. The ability of a strain to be transformed is described as competence, although strictly the term should only apply to the uptake of DNA itself. Competence depends on a number of different factors, which have been well defined. The conditions under which transformation occurs are very similar to those required for the uptake of free phage DNA by transfection. The competence of a strain is dependent on the presence of a high concentration of divalent ions. Ba$^{2+}$, Mg$^{2+}$ and Ca$^{2+}$ ions all enhance competence in strain 8325 but Ca$^{2+}$ ions are the most effective at an optimum concentration of 0.1 mmol. If transformation is unsuccessful, this may also be due to nuclease production by the recipient strain or to the presence of host restriction. Experimentally, the former may be overcome by the isolation of nuclease-negative mutants in the recipient and the latter either by heating the recipient at 56°C for 2 min, which temporarily inactivates the restriction enzymes, or by the use of restriction-negative mutants. The method of preparation of the transforming DNA is also important.

Transformation provides an important technique for studying the genetic organization of the chromosome in *S. aureus* and has certain advantages over transduction in this respect. Larger fragments of DNA are involved and these are more heterogeneous than those generated in transduction. The uptake of DNA by transformation enables the study of fragments of plasmid DNA generated by restriction endonucleases or fragments, which have been cloned into a suitable plasmid carrier. Plasmids can also be studied in strains other than their normal host. These include naturally occurring plasmids and hybrid plasmids derived by the techniques of genetic engineering. In the former category, staphylococcal antibiotic resistance plasmids have been transferred into *Bacillus subtilis* to study the mechanisms of DNA uptake of these readily identifiable molecules and transfer of resistance plasmid from *S. epidermidis* and *B. subtilis* to *S. aureus* has also been demonstrated. In the later, penicillinase genes from a staphylococcal penicillinase plasmid
inserted into an *E. coli* vector plasmid, have been introduced into *E. coli* where gene expression occurs.\textsuperscript{432}.

**2.11.3 TRANSFER OF PLASMID GENES IN MIXED CULTURES**

The conventional transduction process described for staphylococci may not operate *in vivo* in the classical way due to the different environmental conditions. Transduction procedures usually employ high titres of free phage particles and high concentrations of cells, conditions that do not occur in the natural environment. A number of recent reports have shown that antibiotic resistance can be transferred from one strain to another in mixed broth culture. As staphylococci are often found in close proximity to one another under natural conditions, this form of transfer may well have contributed to the spread of antibiotic resistance *in vivo*.\textsuperscript{271}

**Transfer In Vitro**

Lacey and his colleagues have demonstrated the transfer of tetracycline, neomycin, penicillin, erythromycin and gentamicin resistances individually in mixed broth culture. The simultaneous transfer of three plasmid-mediating resistances to tetracycline, chloramphenicol and penicillin, in mixed broth culture has also been described. Apart from plasmid-mediated resistances, the transfer of chromosomal novobiocin, rifampicin and streptomycin resistances has also been shown to occur when donor and recipient culture are incubated together.\textsuperscript{271}

Engel et al. (1980) have achieved transfer of plasmid resistance from *Streptococcus pneumoniae* to *S. aureus* in mating experiments on filters. If phage were involved in this sort of transfer it would have to have an extremely broad host range. Further schaberg et al. (1982) have reported the conjugative transfer of plasmids between *Strep. Faecalis* and *S. aureus* in which phage appears to play no part. Conjugation in the streptococci is now well established so it would not seem surprising if other Gram-positive cocci also had the ability to transfer plasmids by a conjugation system. The transfer of antibiotic resistance plasmids between *S. epidermidis* and *S. aureus* also occurs in mixed broth culture. However, there is evidence against phage-mediated transfer in this report. The use of an encapsulated recipient strain and treatment of donor and recipient cells with γ-globulin,
which inhibit phage adsorption, did not inhibit plasmid transfer. Transfer of the gentamicin resistance plasmid occurred in the absence of calcium ions and in the presence of DNase. The frequency of transfer was markedly increased when donor and recipient cultures were incubated on filters, indicating a conjugative process involved in the transfer. Further the gentamicin resistance plasmids of two different species of coagulase-negative staphylococci could mobilize and facilitate the transfer of other resistance plasmids, analogous to the conjugative R-plasmids of the Gram-negative bacteria. It is therefore probable that conjugation occurs between *S. aureus* strains and also between coagulase-negative staphylococci and *S. aureus*. *In vivo* staphylococci accumulate in large numbers in pathological situations, e.g. boils, abscesses and on the skin of dermatology patients who are often subjected to antibiotic therapy. The close contact of cells in conditions such as these would favor conjugation as a means of transferring resistance.\[^{355,392}\]

### 2.11.3.2 Transfer *In Vivo*

Transfer of tetracycline resistance in mice has been demonstrated by the intravenous injection of the recipient strain of *S. aureus* followed by transducing phage. If transfer occurs naturally *in vivo* the site is more likely to be the skin or nasal epithelium rather than the internal tissues. The transfer of resistance to neomycin, penicillin, erythromycin, tetracycline and gentamicin has been attained on skin. Transfer of resistance to chloramphenicol and tetracycline has been shown to occur between strains of *S. aureus* in local lesions in mice.\[^{360}\] All these reports illustrate the potential of skin as the environment for the transfer of antibiotic resistance and probably other factors also. Naidoo and Noble (1981) found that the transfer of a plasmid mediating gentamicin resistance from *S. hominis* to clinical isolate of *S. aureus* and various *S. aureus* phage propagating strains occurred for some strains at higher frequency on skin than in broth culture.\[^{342}\] It would seem that for some strains skin facilitates transfer of plasmids. The few reports of plasmid transfer *in vivo* indicate that skin may be the environment for the transfer of resistance plasmids not only between *S. aureus* but also between the resident coagulase-negative staphylococci and *S. aureus*.\[^{229,360}\]
2.12 MOLECULAR BASIS OF METHICILLIN RESISTANCE IN STAPHYLOCOCCI

Methicillin-resistant strains of staphylococci were identified immediately upon the introduction of methicillin into clinical practice.\(^{39,235}\) Resistance was termed "intrinsic" because it was not due to destruction of the antibiotic by \(\beta\)-lactamase. The first outbreaks of infections caused by methicillin resistant staphylococci aureus occurred in European hospitals in the early 1960s.\(^{442}\) Since then, strains of methicillin-resistant \textit{Staphylococcus aureus} and methicillin resistant coagulase-negative staphylococci have spread worldwide\(^{251}\) and have become established outside of the hospital environment, particularly among patients in chronic care facilities and in parenteral drug abusers.\(^{325}\) There has been a steady increase in the prevalence of methicillin-resistant strains of \textit{S. aureus} isolated in hospitals in the United States over the years such that now approximately 25\% of nosocomial isolates of \textit{S. aureus} are methicillin resistant.

2.12.1 \textit{mec} DNA

Approximately 30 to 50 kb of additional chromosomal DNA, \textit{mec}, not found in susceptible strains of staphylococci is present in methicillin-resistant strains;\(^{207}\) \textit{mec} is always found near the pur-nov-his gene cluster on the \textit{S. aureus} chromosome (Figure 2.6). \textit{mec} contains \textit{mecA}, the structural gene for penicillin-binding protein 2a (PBP 2a); \textit{mecI} and \textit{mecR1}, regulatory elements controlling \textit{mecA} transcription; and 20 to 45 kb of \textit{mec}-associated DNA.\(^{186}\)
Figure 2.6 mecA Region of Different Staphylococcal Strains

IS; insertion sequence; mecA, methicillin-resistant gene; R1, regulatory element; Tn, transposon, vertical lines; position on restriction enzyme cleavage sites.

2.12.1.1 mecA

mecA encodes PBP 2a (also termed PBP 2'), an inducible 76-kDa PBP that determines methicillin resistance. There is no mecA homolog in susceptible strains. Both susceptible and resistant strains of S. aureus produce four major PBPs, PBPs 1, 2, 3, and 4, with approximate molecular masses of 85, 81, 75 and 45 kDa. PBPs are membrane bound DD-peptidases that have evolved from serine proteases, and their biochemical activity is mechanistically similar to that of the serine proteases. These enzymes catalyze the transpeptidation reaction that cross-links the peptidoglycan of the bacterial cell wall. β-Lactam antibiotics are substrate analogous that covalently bind to the PBP active site serine, inactivating the enzyme at concentrations that are approximately the same as the MICs. PBPs 1, 2, and 3, which have high affinity for most β-lactam antibiotics, are essential for cell growth and for the survival of susceptible strains, and binding of β-lactam by these PBPs is lethal. In methicillin-resistant cells, PBP 2a, with its low affinity for binding β-lactam antibiotics, can substitute for the essential functions of high-affinity PBPs at concentrations of antibiotic that are otherwise lethal. mecA is highly conserved among staphylococcal species. The mecA gene product, PBP 2a, is a high-molecular weight class B PBP. The same penicillin-binding motifs as those found in the penicillin-binding domains of high-affinity PBPs also are present in PBP 2a, but the precise structural basis for its low affinity is not understood. The mecA promoter region, the first 300 nucleotides of mecA, and its regulatory genes are similar in sequence to the analogous regions of the staphylococcal β-lactamases. The origins of mec are obscure. A mecA homolog with 88% amino acid similarity to mecA of methicillin-resistant staphylococci has been identified in Staphylococcus sciuri. Interestingly, the mecA homolog is ubiquitous in this species, but its phenotype is susceptible.
These and other data support the hypothesis that \textit{mecA} originated in a coagulase-negative \textit{Staphylococcus} species, possibly a close evolutionary relative of \textit{S. sciuri}. All methicillin-resistant strains of \textit{S. aureus} are clonal descendants from the few ancestral strains that acquired \textit{mecA}.\textsuperscript{21,207,267}

How \textit{mecA} was acquired by methicillin-resistant staphylococci is not known, but transposition is a plausible mechanism. The presence of one or more copies of the IS431 element (also known as IS257, a putative mobile element often associated with genes encoding a variety of resistance determinants) within \textit{mec}, inverted repeats at the ends of \textit{mec}, and identification of two open reading frames within \textit{mec} that may encode recombinases all suggest that \textit{mecA} and its associated DNA are mobile elements. The \(\beta\)-lactamase plasmid may provide a temporary insertion site for the \textit{mec}-containing transposon. There is one report, somewhat controversial because it has never been confirmed, of a \textit{mecA} containing transposon, Tn4291, residing in an insertion site on the \(\beta\)-lactamase plasmid p1524.\textsuperscript{15,167,207}

\textbf{2.12.1.2 \textit{mecl} and \textit{mecR1}}

\textit{mecl} and \textit{mecR1} are divergently transcribed regulatory genes located immediately upstream from the \textit{mecA} promoter. They are similar in molecular organization, structure function, and mechanism of regulation to staphylococcal \(\beta\)-lactamase regulatory elements, \textit{blal} and \textit{blaR1}. \textit{Blal} is a DNA-binding protein that represses \(\beta\)-lactamase gene transcription. \textit{BlaR1} is a signal-transducing PBP that in the presence of \(\beta\)-lactam antibiotics leads to \(\beta\)-lactamase gene transcription, although the precise mechanism by which this occurs is not known. \textit{mecl} and \textit{mecR1} perform analogous regulatory roles for \textit{mecA}. It seems likely that \textit{mecl} polymorphisms and \textit{mecA} promoter mutations found in most clinical isolates of methicillin-resistant \textit{S. aureus} reflect the selective pressure of \(\beta\)-lactam antibiotics for mutants lacking strong repressor activity, so that the amount of PBP 2a produced is sufficient to offer a survival advantage. PBP 2a production in strains with \textit{mecl} or \textit{mecA} promoter mutations is under the control of both \textit{mec} and \(\beta\)-lactamase regulatory genes.\textsuperscript{21,167,207,446}
2.12.1.3 mec-Associated DNA

meca, mecR1, and mecl are encoded by approximately 5 kb of DNA that is itself located within 25 to 50 kb of additional DNA that may contain up to 100 open reading frames.\textsuperscript{207,308} Transposons and insertion sequences are present, including Tn554 (which contains ermA, the gene encoding inducible erythromycin resistance), located 5' of meca, and one to four copies of IS431, at least one of which, IS431mec, is located 3' of meca. The distance between meca and IS431mec is highly variable due to deletion, rearrangement, and recombination events that may occur in this region. IS431 is an extremely common insertion sequence in the staphylococcal chromosome and plasmids and can be associated with a host of resistance determinants, including mercuric, cadmium, and tetracycline resistance. For example, the \textit{addD} gene, which encodes an enzyme for tobramycin resistance, is located on plasmid pUB110, which has integrated into mec associated DNA within IS431mec. The ability of IS431 elements through homologous recombination to trap and cluster resistance determinants with similar IS elements explains the multiple drug resistance phenotype that is characteristic of methicillin-resistant staphylococci.\textsuperscript{15,46}

2.12.2 HETEROGENEOUS RESISTANCE

A distinctive feature of methicillin resistance is its heterogeneous nature with the level of resistance varying according to the culture conditions and β-lactam antibiotic being used.\textsuperscript{195} The majority of cells in heterogeneous strains (typically 99.9% or more) are susceptible to low concentrations of β-lactam antibiotic, e.g., 1 to 5 μg/ml of methicillin, with only a small proportion of cells (e.g., 1 in 10^6) growing at methicillin concentrations of 50 μg/ml or greater. Most clinical isolates exhibit this heterogeneous pattern of resistance under routine growth conditions. Heterogeneous strains can, however, appear homogeneous (i.e., 1% or more of cells grow at 50 μg/ml of methicillin per ml) under certain culture conditions, such as growth in hypertonic culture medium supplemented with NaCl or sucrose or incubation at 30°C. Addition of EDTA (pH 5.2) or incubation at 37 to 43°C favours a heterogeneous pattern and may suppress resistance entirely. These
changes in expression of resistance with different culture conditions are transient and entirely phenotypic. Passage of a heterogeneous strain in the presence of β-lactam antibiotic alters the resistance phenotype by selecting for highly resistant mutant clones. These clones produce a homogeneous population of highly resistant cells that can grow at methicillin concentrations of 50 to 100 μg/ml. The trait tends to be unstable in these laboratory-selected clones. With repeated sub-culture in antibiotic-free medium, the proportion of highly resistant cells gradually diminishes and the original heterogeneous pattern reemerges. There are rare clinical isolates, with the COL strain of S. aureus being most extensively studied, and some laboratory-derived mutants that consistently are homogeneous despite repeated subculture.\textsuperscript{90,178,403}

2.12. 3 BORDERLINE RESISTANCE

Another type of methicillin resistance is that exhibited by so-called borderline (or low-level) methicillin-resistant strains. These strains are characterized by methicillin MICs at or just above the susceptibility breakpoint (e.g., oxacillin MICs of 4 to 8 μg/ml). Borderline strains may be divided into two categories on the basis of whether \textit{mecA} is present. Borderline strains that contain \textit{mecA} are extremely heterogeneous methicillin-resistant strains that produce PBP 2a. These strains have resistant subpopulations of cells, although it may be quite small, that can grow at high drug concentrations. Borderline strains that do not contain \textit{mecA} can be differentiated phenotypically from extremely heterogeneous \textit{mecA}-positive strains in that highly resistant clones do not occur. Borderline resistance in \textit{mecA}-negative strains has been hypothesized to result from modification of normal PBP genes or overproduction of staphylococcal β-lactamase.\textsuperscript{312} There is good evidence that modified PBPs can produce borderline resistance. Tomasz et al. reported alterations of penicillin binding by PBPs 1,2, and 4 in β-lactamase-negative, \textit{mecA}-negative borderline clinical isolates. Methicillinase activity distinct from plasmid-encoded staphylococcal β-lactamase also has been reported in borderline β-lactamase hyper-producers, suggesting that a novel β-lactamase, rather than overproduction per se, may be important. However, the putative
methicillinase gene has yet to be identified. Finally, PBP 2 sequence analysis of some β-lactamase-hyper-producing borderline strains has revealed mutations identical to some of those found in a β-lactamase-negative borderline strain that has modified PBPs.\(^8\) No clinical data suggest that the level of resistance expressed by \(meca\)-negative borderline strains leads to treatment failure. Data from animal studies show that the semisynthetic penicillinase-resistance penicillins are effective for infections caused by \(meca\)-negative borderline strains. Hence, other than the difficulty in differentiating \(meca\)-negative from \(meca\)-positive strains expressing borderline resistance, the phenomenon is probably of little, if any, clinical significance.\(^6\,\,^3\,\,^0\)

2.12.4 FACTORS AFFECTING RESISTANCE PHENOTYPE

Numerous genes that influence methicillin resistance phenotype have been identified. Staphylococcal β-lactamase plasmid can affect resistance in several ways. It may prevent spontaneous deletion of \(mec\) from the chromosome, which would produce a susceptible phenotype.\(^2\,\,^0\,\,^8\) Introduction of a β-lactamase plasmid into a homogeneously resistant recipient can lead to a heterogeneous pattern of resistance.\(^6\,\,^3\) β-Lactamase regulatory genes may affect the expression of resistance. Mutations altering the inducibility of β-lactamase have been associated with reduced (i.e., heterogeneous) expression of methicillin resistance. Inactivation of \(blaR1\) can produce heterogeneous resistance, presumably by the unopposed repressor activity of \(blal\). Similarly, strains containing functional \(mec\) regulatory genes produce little or no PBP 2a basally and are poorly inducible by many β-lactamase antibiotics, and induction is slow when it does occur, so that expression of resistance is relatively or completely suppressed. Mutations in the \(meca\) promoter region that affect repressor binding to its operator or otherwise alter gene transcription so that little or no PBP 2a is produced, or structural gene mutations that render the expressed protein nonfunctional may also result in a susceptible phenotype.
2.12.4.1 *fem* Factors

Transposon insertional mutagenesis producing susceptible mutants from methicillin-resistant strains has led to the identification of chromosomal genes, physically distinct from *mec*, that are necessary for full expression of resistance. These *fem* (for factor essential for methicillin resistance) or *aux* (auxiliary) factors are present in both susceptible and resistant strains.\(^{45,337}\) Six *fem* genes, *fema*, *femb*, *femc*, *femd*, *feme*, and *femf*, which map to numerous sites throughout the staphylococcal genome, have been characterized. Even more determinants affecting resistance are likely to be identified. Except for *feme*, which is of unknown function *fem* mutants have altered peptidoglycan composition.\(^{123}\) The *agr* (accessory gene regulator) locus and the *sar* (staphylococcal accessory regulator) locus are involved in controlling the expression of numerous staphylococcal virulence factors and exoproteins. Inactivation of either locus in a heterogeneous strain resulted in a slight decrease in the number of highly resistant cells, although the level of resistance expressed by these cells was unaffected. PBP 2a production was unaffected, but the amounts of PBP1 and PBP 3 were reduced, suggested that these PBPs could play a role in expression of resistance.\(^{124,184}\)

Although the *fem*, *aux*, and other factor appear not to explain heterogeneous or homogeneous resistance in wild-type strains (i.e. those that have been adequately characterized are present and functioning normally regardless of whether the phenotype is susceptible, heterogeneous, or homogeneous), a working model of resistance can be proposed based on an understanding of these factors. Heterogeneous strains may be deficient in a factor or lack a critical modification in a biochemical pathway, possibly for cell wall synthesis, that is important to the functions of PBP 2a, which, after all, is a "foreign" PBP. Homogeneous strains then arise from heterogeneous strains by β-lactam antibiotic selection pressure favouring mutants whose genetic background allows for a fully functional PBP 2a.\(^{206,446}\)

### 2.13 Vancomycin Resistance in Staphylococci

The glycopeptide antibiotic vancomycin was introduced clinically in 1958 for the treatment of Gram-positive bacteria. Data from the December 2000
Report of the National Nosocomial Infection Surveillance (NNIS) System indicated that about 75% of coagulase-negative staphylococci and 47% of S. aureus isolates from intensive care units were resistant to methicillin.\textsuperscript{116} Vancomycin remains the drug of choice for these infections. Vancomycin resistance among staphylococci was developed in laboratories even before the drug was in use clinically. However, this resistance was so difficult to induce that many felt it would be unlikely to occur in a clinical setting. That no vancomycin-resistant staphylococci were reported in the first 20 years the drug was used only strengthened this assumption. Unfortunately, this confidence was shattered by the first reports of vancomycin resistance in coagulase-negative staphylococci in 1979 and 1983. Though a cause for concern these reports did not generate a great deal of attention as coagulase-negative staphylococci are generally considered to be relatively avirulent organisms. A very different response greeted the first report of decreased susceptibility to vancomycin in S. aureus in 1997. Similar ones from other countries, including the United States, quickly followed this report. Given the well-known virulence of S. aureus, the isolation of these organisms generated enormous concern in the medical community and has prompted a flurry of activity aimed at limiting their emergence.\textsuperscript{87,173,434}

**Definition of Vancomycin Resistance**

NCCLS guidelines define staphylococci for which the MIC of vancomycin is \( \leq 4 \mu g/ml \) to be susceptible, while isolates for which the MIC is 8 to 16 \( \mu g/ml \) are intermediate and those for which the MIC is 8 to 16 \( \mu g/ml \) are intermediate and those for which the MIC is \( \geq 32 \mu g/ml \) are resistant. Japan, however, considers some isolates for which the MIC is 8 \( \mu g/ml \) to be resistant; as a result, some isolates reported as resistant in Japan have been reclassified as intermediate in the United States.\textsuperscript{451}

Confusion with respect of vancomycin resistance in staphylococci is also engendered by use of the term "heteroresistant staphylococci." This phenomenon, which is seen in both coagulase-negative staphylococci and S. aureus, refers to the variability of vancomycin susceptibilities among subpopulations of single isolate. A heteroresistant isolate contains two
populations of cells, a majority population that is susceptible to vancomycin and a minority population that is resistant. Heteroresistant is likely more common than pure resistant or diminished susceptibility, as evidenced by the fact that it was found in up to 20% of *S. aureus* isolates in one hospital in Japan.

**Laboratory Detection of Vancomycin Resistance**

Vancomycin resistance can be difficult to detect in the clinical microbiology laboratory. Disk diffusion sensitivity testing using the standard 30-µg vancomycin disk frequently misclassifies intermediately susceptible isolates as fully susceptible. In a recent study, 75% of microbiology laboratories from around the world misreported a glycopeptible-intermediate strain of *Staphylococcus epidermidis* as susceptible based on the results of disk diffusion testing. At this time, however, nonautomated MIC determinations by broth or agar dilution or by E test are the "gold standard" for determining vancomycin susceptibility. The Centers for Disease Control and Prevention (CDC) has published recommendations to guide vancomycin susceptibility testing of *S. aureus* isolates. These recommendations state that (i) primary testing of *S. aureus* requires at least 24 h of incubation, (ii) susceptibility determination with disk diffusion is not an acceptable method, and (iii) an MIC testing method should be used to confirm vancomycin susceptibility. Any *S. aureus* isolate for which the MIC is > 4 µg/ml should be sent to the CDC for confirmatory testing.87

**Infection Control Issues**

The epidemiologic importance of vancomycin resistance has brought a great deal of attention to infection control issues regarding the spread of vancomycin-resistant staphylococci. The infection control community is understandably nervous, given the rapid dissemination of vancomycin-resistant enterococci that has occurred in hospitals around the world. It has been established that *S. aureus* can be transmitted between patients by several routes, including environmental surfaces, hands of health care workers, and nasal shedding. It is also assumed that vancomycin-resistant staphylococci can be transmitted by the same mechanisms as their sensitive
counterparts. Indeed, there has been one reported outbreak of vancomycin-
intermediate S. aureus (VISA) in a French hospital among patients who had not received any glycopeptide antibiotics and whose primary risk for acquiring the organism was environmental exposure. Given the enormous public health concerns regarding the dissemination of these organisms, the Hospital Infections Control Practices Advisory Committee (HICPAC) of the CDC published infection control guidelines for all staphylococci for which the MIC of vancomycin is >8μg/ml in 1997.65,330

2.12.1 VANCOMYCIN RESISTANCE IN COAGULASE-NEGATIVE STAPHYLOCOCCI

Epidemiology of Vancomycin Resistance

Resistance to vancomycin among coagulase-negative staphylococci was first reported more than 20 years ago. However, the first report of a clinically significant isolate was in 1987. Since that time, there have been at least five other case reports of clinically relevant coagulase-negative staphylococci that had diminished susceptibility to vancomycin.287,410,411

Mechanisms of Vancomycin Resistance

The true mechanism of vancomycin resistance in these species, though currently unknown, is a topic of active investigation. Vancomycin acts by binding irreversibly to the terminal D- alanyl-D-alanine of cell wall disaccharide-pentapeptide precursors, thereby inhibiting production of the bacterial cell wall Enterococci have developed vancomycin resistance by replacing the terminal alanine with a lactate, which has a greatly reduced affinity for vancomycin. Studies of vancomycin resistant, coagulase-negative staphylococci have shown that these altered cell wall precursors are produced, but in amounts that are likely too small to account for the degree of resistance observed. Analysis of the cell wall peptidoglycans from highly resistant coagulase-negative staphylococci has demonstrated the presence of altered cross-links as compared to susceptible strains. It has been suggested that these altered cross-links may inhibit vancomycin binding to target peptides, but this hypothesis has yet to be proven. Vancomycin resistance in coagulase-negative staphylococci is likely multifactorial, but the exact mechanism await elucidation.441
2.12.2 VANCOMYCIN RESISTANCE IN S. AUREUS

Since first being reported in 1997, the threat of vancomycin resistance in S. aureus has been the topic of intense research and discussion. Although vancomycin resistance in S. aureus remains extremely rare and is less common than vancomycin resistance in enterococci or even coagulase-negative staphylococci, there is widespread concern that vancomycin-resistant S. aureus (VRSA) poses, by far, the greatest risk to patients, given the virulence of the organism. Though there have been only a few reports of S. aureus isolates with reduced susceptibility to vancomycin, the high prevalence of MRSA and vancomycin use, both thought to be risk factors for VRSA, make the widespread dissemination of these organisms an alarmingly realistic possibility. Furthermore, there is the equally alarming threat of the risk of transmission of these organisms between patients.

Epidemiology of Vancomycin Resistance in S. aureus

Until June 2002, there had been no verified clinical isolates of S. aureus that were truly resistant to vancomycin by the NCCLS standards. Instead, the organisms have had intermediate susceptibility, which has led to the term “vancomycin intermediate S. aureus” or “VISA”. The term “glycopeptide S. aureus” or “GISA” is synonymous, but because vancomycin is the only glycopeptide used in USA, most American physicians are more familiar with the acronym VISA. VISA isolates were first found in nature more than 15 years ago while investigators were screening isolates for vancomycin susceptibility. However, it was not until 1995 that the first clinical isolate was reported, which was from a French child who had been receiving vancomycin for an MRSA line infection. In 1996, a wound infection caused by VISA was reported in Japan in a child receiving vancomycin for an MRSA wound infection. The following year, the first VISA isolate was reported in the United States from Michigan. Since then, there have been at least seven confirmed cases of VISA from USA (Table 2.12). In June 2002, “VRSA” was isolated from a swab obtained from a catheter exit site from a Michigan (USA) resident aged 40 years with diabetes, peripheral vascular disease, chronic renal failure and foot ulcer. The Michigan Department of Community Health and CDC confirmed the identity of the strain. The MIC results of
Vancomycin, Teicoplanin and Oxacillin were ≥128 μg/ml, 32 μg/ml, and 16 μg/mL, respectively, by the broth microdilution method. The isolate contained the \textit{vanA} vancomycin resistance gene from enterococci and oxacillin--resistance gene \textit{meca}. The isolate was susceptible to chloramphenicol, linezolid, minocycline, quinupristin/dalfopristin, tetracycline, and trimethoprim/ sulfamethoxazole.\textsuperscript{66} The emergence of second clinical isolate of “VRSA” was reported in October 2002, from a patient admitted to a hospital in Pennsylvania and evaluated for a chronic foot ulcer and possible osteomyelitis. The isolate was again confirmed by CDC to be “VRSA” (Vancomycin MIC ≥ 32 μg /ml by broth microdilution testing).\textsuperscript{87b}

\textbf{Table 2.12 VISA Cases In The Untied States}\textsuperscript{441}

<table>
<thead>
<tr>
<th>State and year</th>
<th>Source</th>
<th>Underlying illness (es)</th>
<th>Vancomycin exposure (wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Michigan, 1997</td>
<td>Peritoneal fluid</td>
<td>Renal failure, MRSA peritonitis, cancer</td>
<td>18</td>
</tr>
<tr>
<td>New Jersey, 1997</td>
<td>Blood</td>
<td>Acute renal failure, MRSA bacteremia</td>
<td>18</td>
</tr>
<tr>
<td>New York, 1998</td>
<td>Blood</td>
<td>Renal failure, MRSA bacteremia</td>
<td>6</td>
</tr>
<tr>
<td>Illinois, 1999</td>
<td>Blood</td>
<td>Renal failure, MRSA endocarditis</td>
<td>3.5</td>
</tr>
<tr>
<td>Minnesota, 2000</td>
<td>Blood</td>
<td>Renal failure, MRSA osteomyelitis</td>
<td>18</td>
</tr>
<tr>
<td>Nevada, 2000</td>
<td>Abcess fluid</td>
<td>Complicated cholecystectomy with polymicrobial intrahepatic abscess (including MRSA)</td>
<td>10</td>
</tr>
<tr>
<td>Maryland, 2000</td>
<td>Blood</td>
<td>MRSA endocarditis, psoriasis, sleep apnea</td>
<td>14</td>
</tr>
</tbody>
</table>

\textbf{Mechanisms of Vancomycin Resistance}

The true mechanism of vancomycin resistance in \textit{S. aureus} is not known. It was initially feared that \textit{S. aureus} would acquire the \textit{van} genes that code for vancomycin resistance in \textit{Enterococcus} species; especially after this transfer was successfully accomplished in the laboratory. Further, vancomycin-resistant \textit{Enterococcus faecalis} emits a sex pheromone that promotes plasmid transfer, and it has been recently demonstrated that this same pheromone is produced by \textit{S. aureus}.\textsuperscript{355} Emission of this pheromone by \textit{S. aureus} organisms that are in proximity to vancomycin-resistant enterococci
that contain plasmids encoding \textit{van} genes could result in transfer of these resistance genes. However, thus far, neither the \textit{van} genes nor their altered peptidoglycan products have been recovered in vancomycin intermediate or resistant \textit{S. aureus} isolates. Instead, it appears that vancomycin resistance in \textit{S. aureus} is conferred by other alterations in the bacterial cell wall. 

Several years prior to the first clinical VISA isolates being reported, Daum et al. produced laboratory strains of VISA and VRSA that had much thicker cell walls than the sensitive parent strains. Subsequent investigators have demonstrated that cell wall synthesis and turnover are upregulated in VRSA isolates, leading to thicker and more disorganised cell walls. Further, it appears that resistant isolates have significantly less cross-linking in the peptidoglycan component of the cell wall. In order to exert an effect, vancomycin must reach the cytoplasmic membrane and bind with nascent cell wall precursors, thereby inhibiting their incorporation into the growing cell wall. It has been proposed that the thicker, disorganized cell walls can actually trap vancomycin at the periphery of the cell, thereby blocking its action. In fact, it has been shown that vancomycin can be recovered intact from the cell walls of VISA and VRSA isolates, indicating that the antibiotic is not being inactivated but merely sequestered by the bacteria. Furthermore, the altered cell walls appear to have a reduced affinity for vancomycin, as soluble targets are able to bind more antibiotic in the presence of vancomycin resistant isolates. Clearly, more research is needed to further elucidate the exact mechanism of vancomycin resistance, however, given what is currently known, it seems safe to assume that it will be multifactorial.

**Microbiology of VISA and VRSA**

The cell wall alterations in VRSA can cause difficulties with laboratory identifications. Morphologically, colonies of VISA and VRSA isolates often look smaller than their susceptible counterparts, which can lead some to confuse them with coagulase-negative staphylococci. Furthermore, vancomycin resistant strains may require more incubation time for coagulase detection. If the coagulase reactions are incubated for less than 4 h, the result may be falsely negative and the isolate may be misclassified as
coagulase-negative staphylococci. Thus, for any *Staphylococcus* with suspected vancomycin resistance, the coagulase test should be incubated for more than 4 h before being interpreted.\(^{441}\)

**Treatment of Infections Caused by VISA and VRSA**

Given the rarity of these infections, it is impossible to say what role the recently approved antibiotics quinupristin-dalfopristin and linezolid will play in their management. One study did show that both agents had good activity against three separate VISA strains; however, at least one of the clinical isolates was resistant to quinupristin-dalfopristin (Conway et al., *11th Annu Meet Soc Healthcare Epidemiol Am.*) Only linezolid has been used in reported clinical cases, being used once in conjunction with trimethoprim-sulfamethoxazole and doxycycline and once as a single agent (Conway et al., *14th Annu Meet Soc Healthcare Epidemiol Am.*) Again, though there was a microbiologic cure in both cases, only one of the patients survived. Though VISA isolates thus far have all been susceptible to linezolid, the recent report of linezolid resistance in an isolate of MRSA, combined with growing use of this agent, raises real concern over how long this uniform susceptibility will hold. Thus, there do appear to be at least a few treatment options for VISA and VRSA infections. However, until more experience exists, the best treatment strategy would appear to be to tailor therapy to the susceptibilities of the isolate in each case.\(^{487}\)

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### 2.14 CARRIER METHICILLIN-RESISTANT STEPHYLOCOCCUS AUREUS

#### 2.14.1 NASAL CARRIAGE

The prevalence of carriage of *S. aureus* in the anterior nares varies widely according to the population studied (Table 2.13) and is influenced by race, age, and exposure to antibiotics and the hospital environment. The particularly high rates for 5-7 year old normal children; nurses and hospitalized neonates are well known.\(^{500}\)
Table 2.13 Colonization and Sepsis in Reported Outbreaks of Infection with Multiply-resistant *S. aureus* (after Keane & Cafferkey, 1984)^35^

<table>
<thead>
<tr>
<th>Report</th>
<th>Number of Patients</th>
<th>% of Patients with Sepsis Colonized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saraglou, Cromer &amp; Bisno (1980)</td>
<td>7</td>
<td>100/0</td>
</tr>
<tr>
<td>Myers &amp; Linnemann (1982)</td>
<td>286</td>
<td>91/9</td>
</tr>
<tr>
<td>Saravolatz, Pohold &amp; Arking (1982)</td>
<td>193</td>
<td>85/14</td>
</tr>
<tr>
<td>Locksley et al. (1982)</td>
<td>35</td>
<td>80/20</td>
</tr>
<tr>
<td>Shanson &amp; McSwiggan (1980)</td>
<td>5</td>
<td>80/20</td>
</tr>
<tr>
<td>Peacock et al. (1980)</td>
<td>31</td>
<td>52/48</td>
</tr>
<tr>
<td>Craven et al. (1981)</td>
<td>174</td>
<td>47/53</td>
</tr>
<tr>
<td>Total</td>
<td>731</td>
<td>77/23</td>
</tr>
</tbody>
</table>

Repeated sampling of individuals reveals three categories: those who never carry, those who intermittently carry and those who are persistent nasal carriers of *S. aureus*. This suggests that both true non-carriers and persistent carriers of sensitive strains unlikely to acquire methicillin-resistant strains, possibly owing to host genetic factors^249^ or bacterial interference by the pre-existing nasal flora or bacterial interference by the pre-existing nasal flora. The throat may be a reservoir for the re-colonization of the anterior nares. Solberg (1965) found that 51% of nasal carriers also that throat isolates of *S. aureus* of which 69% were distinguishable from those in the nose.^25^ Adherence is doubtless a pre-requisite for colonization and there is evidence that *S. aureus* has a greater affinity in *vitro* for nasal epithelial cells derived from carriers than from non-carriers and greater affinity for epithelial cells from aczematous patients.^10^ Fibronectin, a family of high molecular weight glycoproteins is found in body fluids and on mucosal surfaces. The amount present varies with the cell cycle and with certain pathological conditions. *S. aureus* possesses a protein with two components of molecular weights 60,000 and 197,000 which bind specifically to fibronectin. This adherence in independent of protein A or the teichoic acid content of the staphylococcal cell wall and this may explain why they are so uncommonly
part of the nasal flora. Another family of glycoproteins, the lectins, is also present on human cell surfaces and as their function is to bind carbohydrates specifically they may bind to bacterial surface polysaccharides and thus promote adherence.  

The acquisition of MRSA has been associated with previous administration of two or more antibiotics, even when the site and timing of the infection was controlled by matched-pair analysis. Another case-matched study determined that patients acquiring MRSA infection received a mean of 5.1 antibiotics compared with 1.4 for those acquiring methicillin – sensitive S. aureus. Patients with MRSA infection received ampicillin and gentamicin significantly more often than controls and the duration of antimicrobial therapy was also longer (22 versus 9 days). The use of cephalosporins and aminoglycosides has also been significantly associated with acquisition of MRSA infection. 

2.14.2 CARRIAGE OF THE SKIN

The preferential colonization of the anterior nares, the perineum and axillae in normal subjects suggests site-specific factors and it is of interest that the lower part of the nasal vestibule is lined with skin which, like the skin of the perineum and axillae, contains vibrissae and sebaceous and apocrine glands. Although S. aureus may be deposited by transitory flora on other skin sites, especially the fingers and face, stable carriage at the sites is rare when compared with the anterior nares, the axillae and the perineum. Nevertheless in infants, S. aureus may colonise other sites including the umbilicus, groin and circumcision area. 

2.14.3 DISPERSAL OF S. AUREUS FROM CARRIAGE SITES

Using a definition of dispersal as the shedding of more than 10 cfu/cu ft of air, Ayliffe, Babb & Collins (1974) found that nasal carriers can disperse and that cultures of contact plates of their hair correlated better with dispersal than finger or face samples. Interestingly, they also showed in a small series that air counts were greater around subjects with nasal carriage alone than around those who were both nasal and perineal carriers. Any association of dispersal and nasal carriage has important implications for the management
of staff and patients who are nasal carriers of methicillin-resistant *S. aureus* during an outbreak. The patients with the heaviest load of *S. aureus* in their anterior nares are most likely to disperse.\textsuperscript{25,282,498}

### 2.14.4 CONTRIBUTION OF NASAL CARRIAGE TO INFECTION AND EPIDEMIC SPREAD

The contribution of patient's nasal *S. aureus* to auto-infection of surgical wounds has been known for many years. Table 2.13 summarizes several studies and shows that the incidence of surgical wound sepsis in nasal carriers ranged from 5.6 to 16.5\% and that between 47 and 89\% of these wounds were infected with phage types that were indistinguishable from those in the patient's nose. In contrast, post-operative infection rate for non-carriers was only 1.1 to 6.7\%. There are recent anecdotal accounts to suggest that wound infection with methicillin-resistant strains is preceded by nasal acquisition.\textsuperscript{191,394,415}

**Table 2.14 Prevalence of *S. aureus* on Skin\textsuperscript{85}**

<table>
<thead>
<tr>
<th>Site</th>
<th>Percentage of subjects carrying <em>S. aureus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Nose</td>
<td>34</td>
</tr>
<tr>
<td>Cheek</td>
<td>7</td>
</tr>
<tr>
<td>Forehead</td>
<td>5</td>
</tr>
<tr>
<td>Umbilicus</td>
<td>3</td>
</tr>
<tr>
<td>Groin</td>
<td>3</td>
</tr>
<tr>
<td>Forearm</td>
<td>2</td>
</tr>
<tr>
<td>Axilla</td>
<td>1</td>
</tr>
</tbody>
</table>

Overall it seems unlikely that methicillin-resistant staphylococci are less virulent than sensitive strains and a surprisingly small inoculum of *S. aureus* will cause wound infection. Foster & Hurt (1960) showed that when only 15 cocci from a culture of sensitive *S. aureus* were inoculated on to the slightly damaged forearm skin of healthy volunteers, $6.1 \times 10^6$ cfu were recoverable after 24 h and there was an associated erythema and seropurulent
exudates. These findings suggest that airborne transmission of methicillin-resistant *S. aureus* may readily result in wound infection, especially in surgical and burns units.\(^{80}\)

In summary, the earlier literature has predicted, often quantitatively, the significance of carriage during single-strain outbreaks. Nasal carriage presents a risk of autoinfection of surgical wounds and may be associated with significant dispersal, while nasal carriage among staff provides a source of organisms for nasal acquisition by hitherto non-colonized patients. There is evidence in literature that only single wards will decrease acquisition of hospital staphylococci by new patients. There seems little reason while methicillin-resistant strains should differ and recent experience of outbreaks with multiply resistant *S. aureus* is in line with results of these earlier studies on more sensitive strains.\(^{216,262}\)

### 2.14.5 ERADICATION OF CARRIAGE

Eradication of methicillin-resistant *S. aureus* have encouraged efforts to find an effective means of eliminating the carrier state. In a survey from the United States (Bryan *et al.*, 1980) 71% of hospitals sampled personnel during staphylococcal outbreaks and in two-thirds of this topical treatment was used in an attempt to eliminate carriage. Permanent eradication is difficult and in the many studies *S. aureus* recolonized at an unspecified time after treatment in 63 to 100% of subjects. Systemic or local antibiotic therapy, even with vancomycin, or with creams containing chlorhexihidine plus various antibiotics can reduce nasal carriage only temporarily.\(^{160}\) A novel agent, mupirocin (formely 'pseudomonic acid') shows great potential for the elimination of staphylococcal carriage. It has no potential as a systemic antibiotic as it is broken down to inactive compounds when administered intravenously. It is highly active against *S. aureus*, including methicillin-resistant strains, MICs of 0.015-0.06 mg/l and MBCs of around 16 mg/l.\(^{84}\) Using 2% mupirocin formulated in white soft paraffin containing anhydrous lanolin it was demonstrated in a controlled trial that all 32 healthy nasal carriers yielded negative cultures within two days of the start of treatment, and all subjects remained culture negative for at least two weeks after a 5-day course.\(^{84}\) Even 22 weeks after the end of the treatment only
50% of those sampled has resumed carriage, and phage typing indicated that in two-thirds of the subjects the anterior nares become re-colonized with a strain that was distinguishable from their pre-treatment isolate, that is, they recolonized rather than relapsed and so the use of this agent, in conjunction with single rooms or an isolation ward, may well contribute to the control of outbreaks caused by methicillin-resistant strains and avoid the necessity of using valuable systemic agents to treat the carrier state.25,190

2.15 BIOCHEMICAL BASIS OF ANTIBIOTIC RESISTANCE IN STAPHYLOCOCCI

It was initially assumed that resistance arose because of a change in the nature of a single gene. While this is certainly true of some bacteria, for example antibiotics change in the gene for folate synthesis confers sulphonamide resistance; it is not the sole reason. More commonly, resistant organisms have an additional gene (or genes) the product (s) of which confer resistance. For example, staphylococci resistant to penicillin have a gene specifically responsible for the synthesis of penicillinase, an enzyme absent from sensitive cells.306 Similarly Escherichia coli carrying R factor produces an intracellular enzyme that inactivates chloramphenicol by transferring the acetyl group to chloramphenicol from acetyl CoA.312 Bacteria may already possess these additional genes, but in other cases they may be acquired by mutations or transmission from other bacteria. The transmission of resistance is largely due to the acquisition of plasmids and/or transposons. Although transfer of resistance in the laboratory has been shown to occur via transformation, transduction, and conjugation, only conjugative transfer appear to be significant in vivo.288,293 The biochemical machinery conferring bacterial resistance to drugs of major importance in medicine is quite complex but provides a rational approach to the development of effective agents useful in treating resistant infections. Understandably, therefore, there is a considerable interest in the origins of the genes that encode drug-inactivating enzymes, drug efflux pumps and enzymes, that depress drug sensitivity by the covalent modification of drug targets.263 Antibiotic producing bacteria, such as streptomycetes, for example, protect themselves against the toxic effects of their own antibiotics,
with enzymes that specifically inactive compounds such as aminoglycosides, chloramphenicol and β-lactams. In addition, many streptomycetes express β-lactamases even though they do not produce β-lactamases, presumably as a protective measure against β-lactams synthesized by other organisms in the micro-environment. Pumped efflux of tetracycline and the enzymic modification of ribosomal RNA associated with resistance to erythromycin have both been identified in streptomycetes. A comparison of nucleic acid and protein sequence data supports the possibility that the genes for aminoglycoside-inactivating enzymes found in aminoglycoside resistant clinical isolates may have originated from streptomycetes. In general, however, evidence for a streptomyecete origin of drug resistance genes in pathogenic bacteria is limited. Among the mechanisms identified (Table 2.9), enzymatic inactivation of antibiotics is one of the most common biochemical processes that endangers resistance to a wide variety of antibiotic structural types in bacteria. Determinants for the enzymic inactivation of antibiotics in clinical isolates have arisen in most cases only by the inheritance of exogenous functions (genes). Once established in pathogen, genes encoding enzymes that catalyze covalent modifications of therapeutic agents can undergo mutations that remodel the active site of the enzyme, changing the spectrum of antibiotic substrates that may be modified. In his 1992 review, Neu showed the “phylogeny” of development of β-lactam antibiotics in response to the evolution of bacterial resistance to this class of antibiotics. Of the several known mechanisms of resistance to the β-lactam antibiotics (Table 2.9) the most elusive moving target is hydrolytic inactivation by β-lactamases. A single base change in the gene for a β-lactamase can change the substrate specificity of the enzyme that resulted in the development of extended – spectrum β-lactamases.

Staphylococci have an ability of developing resistance quickly and successfully to antibiotics. The acquired defence systems by staphylococci may have originated from antibiotic-producing organisms, where they may have been developed and then passed on to the other genera. The various biochemical mechanisms of antibiotic resistance for staphylococci are outlined in Table 2.15.
2.15.1 RESISTANCE TO $\beta$-LACTAM ANTIBIOTICS

Three different mechanisms of resistance to $\beta$-lactam antibiotics have been identified for staphylococci: the inactivation of penicillin (and cephalosporin) by penicillinase ($\beta$-lactamase) mediated hydrolysis of the $\beta$-lactam ring of antibiotic; and intrinsic resistance resulting from a reduction in the affinity or the amount of the PBPs; and tolerance due to an excess of autolysin inhibitor.$^{149,293}$ Most penicillin-resistant staphylococcal strains produce $\beta$-lactamase. $\beta$-lactamase genes ($bla$) are most often found on class II plasmids that are distributed among many staphylococcal species.$^{19,33,78,134}$

The products of three chromosomal genes, $bla_l$, $blaR1$, and $blaR2$, control the expression of the structural gene for $\beta$-lactamase, $blap$.$^{186,485}$

Figure 2.7 Interaction of Cephalosporins and Penicillins with $\beta$-lactamases. In Cephalosporins, opening of the $\beta$-lactam ring and expulsion of $R^1$ is followed by general disintegration of the molecule. In penicillins, opening of the $\beta$-lactam ring occurs.

Unlike Gram-negative bacteria, where $\beta$-lactamase is excreted in the periplasm, the beta-lactamase in Gram-positive bacteria including staphylococci is excreted into the environment. The number of beta-lactamases has steadily risen since the introduction of penicillin. Beta-
lactamases have been classified according to their functional aspects. This system is based on hydrolysis rates for a number of substrates and the level of inhibition by clavulanic acid, but simple point mutations may alter the classification. They have also been classified according to the nucleotide sequences that encode β-lactamases. Classes A, B, and D have serine at their active site, whereas class B has a zinc atom at the active site. Class A enzymes are encoded mostly on plasmids while class C enzymes are generally chromosomally encoded. Beta-lactamases range between 30 and 40 KDa in size. The β-lactamases of Staphylococcus aureus is an inducible enzyme. Enzyme production is very slow in the absence of penicillin or cephalosporin. The addition of minute quantities of antibiotic (as little as 0.0024 µg ml⁻¹ of medium) increases enzyme production enormously and the β-lactamase may account for more than 3% of the total protein synthesized by the bacterium. Resistance to methicillin and other β-lactamase resistant penicillins was first observed in S. aureus soon after the introduction of methicillin into clinical use in Britain. An altered PBP is involved in methicillin resistance in staphylococci. This PBP2a (PBP-2') is encoded by the mecA gene. mecA is encoded by a chromosomal insert called the mec determinant which probably originated outside staphylococci, perhaps via fusion between a β-lactamase gene and a PBP gene. The mec determinant is not readily self-transmissible but has spread within S. aureus and several coagulase-negative species, indicating that some horizontal transfer is possible.

2.15.2 RESISTANCE TO MACROLIDES, LINCOSAMIDES AND STREPTOGRAMINS

Erythromycin-resistant staphylococci often have cross-resistance of macrolids (roxithromycin, oleandomycin, spiramycin, clarithromycin, azithromycin), and not necessarily to all lincosamides (lincomycin, clindamycin) and streptogramin type antibiotics (designated MLS-resistant).

2.15.2.1 Target Modification

Modification of ribosomal target confers cross-resistance and remains the most frequent mechanisms of resistance to MLS antibiotics, although
enzymatic modification of the antibiotics and active efflux appears to be increasingly prevalent.\textsuperscript{281}

The different types of MLS antibiotics bind to the 50s ribosomal subunit at overlapping binding sites, and binding interferes with transpeptidation and translocation reactions needed for peptide chain elongation. These sites are protected by the N\textsuperscript{6}, N\textsuperscript{6} - dimethylation of an adenine – 2058 residue at the peptidyl transferase centre of 23S rRNA a reaction performed by an rRNA methylase.\textsuperscript{494} Three distinct rRNA methylase genes have been detected in staphylococci: \textit{ermA},\textsuperscript{362} \textit{ermB}\textsuperscript{280} and \textit{ermC}.\textsuperscript{212} Even though of the \textit{erm} gene classes can be distinguished by hybridization, the amino acid sequences of the encoded methylases are highly conserved, which suggests that they evolved from a common ancestor, possibly originating in the antibiotic producing organisms.\textsuperscript{362,463} The chromosomal \textit{ermA} gene is common among \textit{methicillin} – resistant (\textit{mecA}) strains of \textit{S. aureus} and \textit{S. epidermis}, but it is relatively uncommon in \textit{mecA}\textsuperscript{–} strains and usually accounts for only 6-11\% of those resistant to MLS antibiotics.\textsuperscript{454} \textit{ermB} is usually not found in staphylococcal species indigenous to primates; however, it appears to be relatively common in MLS – resistant strains of \textit{S. intermedius} from dogs, in which expression can be either inducible or constitutive, and MLS-resistant strains of \textit{S. hyicus} and \textit{S. xylosus} from pigs, in which expression in constitutive.\textsuperscript{137,233} The \textit{ermC} gene is most often located on class I plasmids of the pSN2 and pE194 families.\textsuperscript{132,259} These plasmids are present in very high frequencies (85-98\% of strains) in most staphylococcal populations following treatment of the host with tylosin, erythromycin or clindamycin.\textsuperscript{481,494} Expression of MLS resistance can be constitutive or inducible. The character of resistance is not related to the class of \textit{erm} determinant but, rather, depends on the sequence of the regulatory region, upstream from the structural gene for the methylase. Regulation by these regions occurs by a translational attenuation mechanism in which mRNA secondary structure influences the level of translation. In laboratory mutants and clinical isolates, single nucleotide changes, deletion or duplications in the regulatory region convert inducibly resistant strains to constitutively resistant ones that are cross-resistant to MLS antibiotics.\textsuperscript{212} In staphylococci,

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when expression is constitutive, the strains are resistant to all MLS antibiotics. Streptogramin A-type antibiotics escape resistance, and synergy with streptogramin B-type antibiotics is retained. When expression is inducible, the strains are resistant to 14- and 15-membered macrolides only. The 16-membered macrolids, the commercially available lincosamides and streptogramins remain active. This dissociated resistance is due to differences in the inducing abilities of MLS antibiotics, only 14- and 15-membered macrolides are effective inducers of methylase synthesis in staphylococci.

2.15.2.2 Antibiotic Inactivation

Unlike target modification, which causes resistance to structurally distinct antibiotics, enzymatic inactivation confers resistance only to structurally related drugs. Enzymes (Ere A and Ere B) that hydrolyze the lactone ring of the macrocyclic nucleus and phosphotransferases [type I (mphA) and type II] that inactive macrolides by introducing a phosphate on the 2' hydroxyl group of the amino sugar have been reported in members of the family Enterobacteriaceae and S. aureus. The gene linA mediates resistance to lincosamides. The product of linA has been partially purified and demonstrated to act as a lincosamide O-nucleotidyl transferase. Lactonases that are capable of cleaving the macrocyclic lactone ring structure of type B streptogramins have been identified in staphylococci (vgb gene). Two staphylococcal – related determinants vat and vatB, encoding an acetyltransferase that inactivates type A streptogramins, have been characterized. The vat and vgb genes are adjacent to each other on plasmid pIP630. This vat-vgb region is flanked by inverted copies of insertion sequence IS257, suggesting a role for this element in dissemination of these determinants.

2.15.2.3 Active Efflux

The presence of multicomponent macrolide efflux pump in staphylococci (msrA, msrB) has been documented. msr genes confer resistance only to 14 and 15 membered ring macrolides. The presence of a plasmid-mediated gene, vga, encoding for a putative ATP-binding protein, has been associated
with an active efflux of streptogramin A group compounds. This gene has been chiefly found in coagulase – negative staphylococci.\textsuperscript{137, 284, 362, 373}

2.15.3 RESISTANCE TO FLUOROQUINOLONES

Fluoroquinolone antibiotics exert their antibacterial effects by inhibition of certain bacterial topoisomerase enzymes, namely DNA gyrase (bacterial topoisomerase II) and topoisomerase IV. DNA gyrase is the only enzyme that can effect supercoiling of DNA.\textsuperscript{302} Inhibition of this activity by fluoroquinolones is associated with rapid killing of the bacterial cell.\textsuperscript{386} Topoisomerase IV is predominantly responsible for separation of daughter DNA strands during cell division. Three mechanisms for fluoroquinolone resistance have been postulated for staphylococci. One mechanism found in \textit{S. aureus} and \textit{S. epidermidis} involves mutations in the chromosomal gene \textit{gyrA}, encoding the DNA gyrase subunit A, so that its function is no longer inhibited by the antibiotic.\textsuperscript{175,228,439,440} A second mechanism found in \textit{S. aureus}, \textit{S. epedermids} and \textit{S. haemolyticus} involves mutations in the chromosomal gene \textit{norA} (or its regulatory region) that encodes a membrane efflux protein for hydrophilic fluoroquinolones and other unrelated antibiotics.\textsuperscript{351} The NorA protein has a hydrophathic amino acid profile consistent with a location in the cytoplasmic membrane and it exhibits a low level of fluoroquinolone efflux, with a preference for hydrophilic fluoroquinolones. NorA – mediated fluoroquinolone resistance is due to over expression of the wild-type gene \textit{norA}.\textsuperscript{239,365,507} A third mechanism found in \textit{S. aureus} involves mutations in the chromosomal gene \textit{grlA} that encodes the A subunit of DNA topoisomerase IV. Clinical isolates of \textit{S. aureus} that have mutations in both \textit{gyrA} and \textit{grlA} genes generally exhibit higher levels of ciprofloxacin resistance (MIC 16 – 128 \textmu g/ml\textsuperscript{1}). These data support the finding that in \textit{S. aureus}, \textit{grlA} mutations precede \textit{gyrA} mutations in developing resistance to ciprofloxacin. The \textit{S. saprophyticus} and \textit{S. sciuri} species groups inhibit an intermediate susceptibility (MIC 1 – 4 \textmu g/ml) to fluoroquinolones and this property may be related to their intrinsic resistance (MIC 16 – 64 \textmu g/ml) and nalidixic acid resistance (MIC 64-1024 \textmu g/ml), presumably due to modifications of the \textit{gyrB} gene and/or its regulation.\textsuperscript{215,296,409}
2.15.4 RESISTANCE TO TETRACYCLINES

Tetracyclines probably penetrate bacterial cells by passive diffusion. Tetracycline act by reducing the affinity of the A and P sites of the 30s ribosomal subunit for aminoacyl – transfer RNA, resulting in the inhibition of protein synthesis. Tetracycline resistance is widespread among Staphylococcal species. There are 2 mechanisms of tetracycline resistance recognized in staphylococci. The most common one involves an energy-dependent pumping (efflux) of tetracycline and doxycycline from the cell, so that levels of these antibiotics are reduced below that required to inhibit the ribosome. The efflux protein is most often encoded by the inducible gene \textit{tetK} which is located on class I plasmids of the pT181 family. The gram-positive \textit{tetK} and \textit{tetL} genes encoding tetracycline – efflux proteins are regulated by mRNA attenuation like gram-positive \textit{erm} genes encoding rRNA methylase and \textit{cat} genes encoding chloramphenicol acetyltransferase. Tetracycline specific exporters pump their substrate into the periplasm and not across the outer membrane, as found for the multidrug efflux pumps. The second mechanism, one that is controlled by the gene \textit{tetM}, involves ribosome protection such that protein synthesis is unaffected by the presence of tetracycline, or minocycline. However, to date the biochemical basis of tetracycline resistance mediated by TetM protein remains unclear. One possibility is that TetM stabilizes the ribosome-transfer RNA interaction in the presence of tetracycline.

2.15.5 RESISTANCE TO AMINOGLYCOSIDES

Aminoglycosides bind to the ribosomes and thus interfere, with protein synthesis. There are 3 mechanisms responsible for aminoglycoside resistance in staphylococci. One mechanism involves changes in ribosomal proteins as a consequence of certain mutations in their structural genes, such that ribosomes can no longer bind streptomycin. A second mechanism involves the energization and permeability of the cell membrane. Some energy-deficient, small –colony mutants of \textit{S. aureus} have been shown to be resistant to aminoglycosides due to a diminished uptake of the antibiotics, presumably as a consequence of cell membrane impermeability. The third and most common mechanism of resistance involves modification of
aminoglycosides by aminoglycoside-modifying enzymes so that the antibiotics are inactive to bind to ribosomes. Genes encoding these enzymes are either plasmid or transposon mediated. Evidence suggests that the enzymes have arisen from both antibiotic-producing organisms and by mutation of resident host genes. The modifying enzymes are present in the cytoplasm in quantities sufficient only to inactivate drug that enters during the slow uptake phase. Therefore resistant cells are characterized by growth in media containing unmodified antibiotic. There are classes of aminoglycoside-modifying enzymes: acetyltransferases (AACs); adenyltransferases/nucleotidyl-transferases (ANTs); and phospho-transferases (APHs). The AACs acetylate amino groups whereas ANTs and APHs adenylate and phosphorylate hydroxyl groups, respectively.4\(^{14}\)

Aminoglycoside resistance in staphylococci is well documented. The resistance of staphylococci to kanamycin has been to be caused by a kanamycin–neomycin phosphotransferase. Upto six genes have been identified.140,199 Often more then one resistance gene is present. One of the most remarkable aminoglycoside enzymes is the bifunctional AAC (6\(^{\prime}\)) APH (2\(^{\prime\prime}\)) enzyme, which is found on Tn4001 of S. aureus and in Enterococcus faecalis isolates. Nucleotide sequencing data suggest that the enzyme arose through the fusion of two genes, each encoding one of the partners. The epidemiology of Tn4001 is well studied and illustrates that substitutions, insertions and deletions play an important role in the adaptation of these elements to new hosts.209 Hybridization studies in Australia showed that Tn4001 could be present on the bacterial chromosome as well as on a number of structurally related plasmids,103 and this was linked to the rapid spread of gentamicin and tobramycin-resistant S. aureus in Australia. The same was also observed in North America but with shorter inverted repeats and insertion sequences flanking the resistance gene.297,396

2.15.6 RESISTANCE TO TRIMETHOPRIM AND SULPHONAMIDES

Trimethoprim and sulfonamides are synthetic agents that effect the biosynthesis of tetrahydrofolic acid, an essential derivative used in amino acid and nucleotide synthesis. Trimethoprim resistance is either mediated by alterations in the expression of the intrinsic chromosomal dfr gene (e.g. dfrB
of S. aureus or dfrC of S. epidermidis), possibly resulting in over production of the native dihydrofolate reductase (DHFR) or a reduced affinity of the native DHFR for trimethoprim, or by the acquisition of a second chromosomal or plasmid dfr gene (e.g. dfrA in S. aureus, S. epidermidis and S. hominis) that encodes a trimethoprim – resistant DHFR capable of rescuing the reduction step leading to Tetrahydrofolate in the presence of trimethoprim. The differences in resistance level of staphylococci correlate with differences in transcription caused by deletions adjacent to a copy of IS257 in Tn4003, which effects the promotor used by dfrA. The S. epidermidis trimethoprim–sensitive chromosomal DHFR gene dfrC, differs from dfrA by only four base pairs, strongly suggesting that dfrA originated from the S. epidermidis chromosomal gene. Site directed mutagenesis of dfrA and kinetic analysis of the purified DHFRs indicate that a single alteration (Phe-98 to Tyr) is responsible for the trimethoprim resistance of the type S1 DHFR.

Chromosomally encoded sulphonamide resistance has been described and resistance seems to be due to increased production of Periplasm-amino-benzoic acid. Furthermore alterations of dihydropteroate synthase (DHPS) could lead to low affinity for sulphonamides. Acquired sulphonamide resistance can result from the acquisition of plasmids that encode drug-resistant DHPS.

2.15.7 RESISTANCE TO CHLORAMPHENICOL

Chloramphenicol contains a nitrobenzene ring and is a derivative of dichloroacetic acid. Chloramphenicol inhibits protein synthesis by binding to the 50S subunit of 70S ribosome at a site that prevents the attachment of aminoacyl-transfer RNA (tRNA) to its binding site. Without this attachment, peptide bond formation cannot occur. Chloramphenicol attaches to the ribosome in proximity to the targets for erythromycin and clindamycin. The most common mechanism of resistance to chloramphenicol is the enzymatic inactivation of the compound. In resistant strains, chloramphenicol is acetylated by a cytoplasmic enzyme chloramphenicol acetyltransferase (CAT). CAT acetylates chloramphenicol at the C3-hydroxyl. Acetylated form of chloramphenicol cannot bind to the ribosome. Subsequent isomerization of the C3 – acetylated molecule results in the formation of substrate for a
second acetylation, resulting in 1,3-diacetyl-chloramphenicol. The ability of different CAT enzymes to catalyze this second acetylation varies, as do the conditions under which it occurs. In any case, the second acetylation is unlikely to be of substantial clinical significance, because the primary acetylation inactivates the molecule. There are at least a dozen different CAT enzymes. These enzymes may be plasmid encoded or chromosomally encoded. Nucleotide sequence data suggest some conservation of CAT, but their spectra of activity are essentially the same. An interesting feature of type I CAT (encoded by transposon Tn9) is its ability to bind (but not inactivate) fusidic acid, resulting in resistance to this unrelated antibiotic in addition to resistance to chloramphenicol. Nucleotide sequences, have defined seven distinct classes of cat genes. Unlike gram-negative bacteria expression of cat genes in Staphylococcus aureus is typically inducible, and expression appears to be regulated by translational attenuation in a similar manner to the erm genes conferring resistance to macrolides. The cat gene is preceded by a nine amino acid leader peptide, and the leader mRNA can form a stable stem-loop structure, which masks the ribosome binding site of the cat gene. Chloramphenicol appears to cause the ribosome to stall on the leader sequence, opening the stem-loop structure, thereby exposing the cat ribosome-binding site, allowing cat expression.
Figure 2.8 Modification of Chloramphenicol by Acetyltransferase

2.15.8 RESISTANCE TO GLYCOPEPTIDES

The glycopeptide antibiotics vancomycin and teicoplanin inhibit cell wall synthesis in Gram-positive bacteria by interacting with the terminal D-alanyl-D-alanine (D-Ala-D-Ala) group of the pentapeptide side chains of peptidoglycan precursors. This interaction prevents the transglycosylation and transpeptidation reactions required for polymerization of peptidoglycan. Since this mode of action is different from that of β-lactams, glycopeptides are being used to treat severe infections caused by methicillin and other β-lactam-resistant staphylococci. Although resistant to glycopeptides in enterococci is mediated through van genes but, resistant to glycopeptides in staphylococci is phenotypically diverse. The van genes have been introduced into a laboratory strain of S. aureus and were fully functional. Thus, the possibility that methicillin-resistant staphylococci will acquire resistance to vancomycin is very real. Teicoplanin-resistant derivatives of teicoplanin-susceptible S. aureus strains have also been obtained in vitro.
Studies of glycopeptide resistance in staphylococci have provided evidence that resistance may be related to alteration in the bacterial cell wall. Glycopeptide-resistant organisms have thicker, more irregular cell walls than glycopeptide-susceptible organisms. Although there is speculation about the role of PBPs that may compete with vancomycin for binding to cellular targets and changes in cell wall turnover (enhanced production of cell wall precursors), the exact mechanism for the heterogeneously reduced vancomycin susceptibility remains unclear.

2.15.9 RESISTANCE TO OTHER ANTIBIOTICS

Rifampin is a very potent bactericidal anti-staphylococcal agent with MICs of 0.05 µg/ml or less. It blocks protein synthesis by inhibiting RNA polymerase. High-level resistance occurs if rifampin is used alone due to point mutations in the \textit{rpoB} gene encoding B-subunit of the RNA polymerase, making the drug ineffective as monotherapy for clinical infections.

Mupirocin is pseudomonic acid, a natural product of \textit{Pseudomonas fluorescens}. Mupirocin inhibits and kills staphylococci by inhibiting isoleucyl-tRNA synthetase. Mupirocin contains an epoxide side chain structurally similar to isoleucine and competes with this amino acid for the binding site on isoleucyl-tRNA synthetase. As a result of this, no isoleucine is incorporated into the nascent peptide chain and protein synthesis is aborted. Resistance to mupirocin has been described in staphylococci. Low level resistance (MIC < 100 µg/ml) may be mediated by altered access to binding sites on isoleucyl-tRNA synthetase. This results due to point mutations in the gene of the target enzyme. High level resistance (MIC>1000 µg/ml), is due to the presence of a modified isoleucyl-tRNA synthetase gene, which has been located on a conjugative plasmid encoding gentamicin resistance, that renders mupirocin ineffective. Analysis of this gene product suggests it was acquired from another organisms. 210,327

Fusidic acid a protein synthesis inhibitor interferes with ribosome-associated elongation factor G, the 50S ribosome translocation protein. The chromosomal mutation \textit{(fusA)} lowers the affinity of the G factor for the antibiotic in \textit{S. aureus}. A plasmid-borne determinant \textit{(fasB)} also encodes for decreased permeability. 95
<table>
<thead>
<tr>
<th>Resistance Phenotype and Responsible Protein</th>
<th>MW (kDa)</th>
<th>Major Activity or Mechanisms</th>
<th>Gene</th>
<th>Gene Location</th>
<th>Species Distribution</th>
<th>References</th>
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<tbody>
<tr>
<td>Penicillin (β-lactams)</td>
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<tr>
<td>Penicillinase (A-D) (β-lactamase)</td>
<td>A: 28.8</td>
<td>Inactivation of penicillin and cepahalosporin by hydrolysis of the β-lactam ring A, B and C require induction by penicillin or its analogues</td>
<td>blaZ</td>
<td>Plasmids (mainly class II and III) &gt;chromosome bla1-blaR1-blaZ region is often on transposon (Tn552, Tn4002, Tn 4201)</td>
<td>S. aureus, S. epidermis</td>
<td>135,334,485,490</td>
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<td>β-Lactamase repressor Blal</td>
<td>14.9</td>
<td>Transcriptional repressor for the system</td>
<td>blal</td>
<td></td>
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<td>β-Lactamse entirepressor BlaR1</td>
<td>69.2</td>
<td>Receptor for β-lactams and the depression of blaZ</td>
<td>blaR1</td>
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<td>Methicillin (β-lactamase resistant penicillins)</td>
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<td>Penicillin-binding protein</td>
<td>74-78</td>
<td>mecA</td>
<td></td>
<td></td>
<td>Chromosome: S. aureus</td>
<td>123,195,308,432</td>
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<thead>
<tr>
<th>Resistance Phenotype and Responsible Protein</th>
<th>MW (kDa)</th>
<th>Major Activity or Mechanisms</th>
<th>Gene</th>
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<tr>
<td>PBP2a (PBP2')</td>
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<td>Methicillin-resistance repressor MecI</td>
<td>14.8</td>
<td>Transcriptional repressor for the system</td>
<td>mecl</td>
<td>Smal fragment G mecl-mecR1-mecA region may be on a transposon and sometimes on plasmids</td>
<td>S. epidermidis</td>
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<tr>
<td>Methicillin-resistance MecR1 protein (or antirepressor)</td>
<td>68.5</td>
<td>Derepression of mecA Blal and BlaR1 can substitute for MecI and MecR1</td>
<td>mecR1</td>
<td></td>
<td>S. haemolyticus</td>
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<tr>
<td>Aux (auxiliary) or Fem (factors essential for expression for methicillin resistance) (A-D)</td>
<td>A: 50.6</td>
<td>A and B: biosynthesis of pentaglycine interpeptide bridge of peptidoglycon C: amidation of α-carboxyl group of D-glutamic acid in peptide subunits of the peptidoglycan</td>
<td>femA, femB</td>
<td>Chromosome: Sma I fragment A</td>
<td>S. aureus</td>
<td>46,123,172, 366</td>
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<tr>
<td></td>
<td>B: 49.7</td>
<td>D: Controls rate of synthesis of unsubstituted disaccharide pentapeptide precursor</td>
<td>femC</td>
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<td>Resistance Phenotype and Responsible Protein</td>
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<tr>
<td>Macrolide, lincosamide and streptogramin B (MLS)</td>
<td>28.3-29</td>
<td>$N^6; N^6$-dimethylation of adenine -2058 residues at peptidyl transferase centre of 23S rRNA, resulting in a decreased affinity of 50S ribosomal subunit for MLS antibiotics</td>
<td>$ermA$</td>
<td>Chromosome: on transposon Tn554 which prefers a single $att$ site</td>
<td>$S. aureus$ $S. epidermis$</td>
<td>333,335,454</td>
</tr>
<tr>
<td>23S rRNA methylases (or methyl transferases)</td>
<td>28.3-29</td>
<td>Require induction by erythromycin or are made constitutively</td>
<td>$ermB$</td>
<td>Plasmids and chromosome: on transposon Tn551 which can transpose</td>
<td>$S. aureus$ $S. intermedius$ $S. hyicus$</td>
<td>137,246,357</td>
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<td>Resistance Phenotype and Responsible Protein</td>
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<td>Major Activity or Mechanisms</td>
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<td>Macrolilide and streptogramin B (MS)</td>
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<tr>
<td>ATP-dependent efflux protein MsrA (member of ATP-binding ABC cassette transporter superfamily)</td>
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Plays a role as ATP-dependent efflux pump to remove MS antibiotics and requires a transmembrane complex
Possibly inactive macrolides

Plasmids (mainly class I) > chromosome

*ermC*  
*S. aureus*  
*S. epidermis*  
Most species of staphylococci  
*Bacillus subtilis*

*S. xylosus*  
Several other genera

*S. hominis*, S. cohnii > S. epidermis  
Several other species

137  
212, 226, 276, 494
<table>
<thead>
<tr>
<th>Resistance Phenotype and Responsible Protein</th>
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<tbody>
<tr>
<td>Lincosamide</td>
<td></td>
<td>Inactivates lincosamides. Requires a nucleoside 5'-triphosphate as nucleotidyl donor and Mg$^{2+}$ as cofactor</td>
<td>lineA, lineA' (lin gene group)</td>
<td>Plasmids (class I)</td>
<td>S. haemolyticus, S. aureus, S. hominis, S. cohnii, Several species, other</td>
<td>67,280,281</td>
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<tr>
<td>3-Lincomycin, 4-clindamycin O-nucleotidyltransferase</td>
<td>19</td>
<td></td>
<td></td>
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<tr>
<td>Tetracycline</td>
<td></td>
<td>Plays role as energy-dependent efflux pump to remove tetracycline. Uses antiport mechanism of transport exchanging a proton for a metal-tetracycline complex. Requires induction by very low amounts of tetracycline. Regulation by translational attenuation.</td>
<td>tetK</td>
<td>Plasmids (class I) &gt; chromosome</td>
<td>S. aureus, S. haemolyticus, S. hiipidermis, Many Other Species Of Staphyloco cci Bacillus subtilis</td>
<td>494</td>
</tr>
<tr>
<td>Tetracycline efflux proteins (metal tetracycline/H$^+$ antiporters)</td>
<td>32-35</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Resistance Phenotype and Responsible Protein</td>
<td>MW (kDa)</td>
<td>Major Activity or Mechanisms</td>
<td>Gene</td>
<td>Gene Location</td>
<td>Species Distribution</td>
<td>References</td>
</tr>
<tr>
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</tr>
<tr>
<td>Tetracycline and minocycline</td>
<td>72.6</td>
<td>Permits productive binding of aminoacyl-tRNAs to ribosomes in the presence of tetracycline and minocycline</td>
<td>tetM</td>
<td>Chromosome</td>
<td>S. intermedius</td>
<td>75,348,495</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Requires tRNA modification (miaA) activity Homology with</td>
<td></td>
<td>Chromosome: Tn916 and Tn916-like transposons</td>
<td>S. aureus Other gram positive genera</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Plasmid</td>
<td>Gram-negative genera</td>
<td></td>
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<tr>
<td>Resistance Phenotype and Responsible Protein</td>
<td>MW (kDa)</td>
<td>Major Activity or Mechanisms</td>
<td>Gene</td>
<td>Gene Location</td>
<td>Species Distribution</td>
<td>References</td>
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<tr>
<td>Aminoglycosides</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Aminoglycoside modifying enzymes:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aminoglycoside 6' - acetyltransferase -</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aminoglycoside 2''-phosphotransferase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[AAC (6') + APH(2'')]</td>
<td>56</td>
<td>Bifunctional: 2 domains</td>
<td>aac(6')-aph(3')</td>
<td>Plasmids</td>
<td>S. aureus</td>
<td>396,462,464,468</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAC(6') acetylates amino</td>
<td></td>
<td>(mainly class III) and</td>
<td>S. epidermis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>groups of tobramycin netilmicin, amikacin, and gentamicin C at 6' position</td>
<td></td>
<td>chromosome on transposons (Tn4001 and Tn4001-like, Tn403 Tn3851)</td>
<td>Enterococcus</td>
<td>458</td>
</tr>
<tr>
<td></td>
<td></td>
<td>APH (2'') phosphorylates</td>
<td></td>
<td></td>
<td>S. aureus</td>
<td>103,140,179</td>
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<tr>
<td></td>
<td></td>
<td>2''.hydroxyl groups</td>
<td></td>
<td></td>
<td>Enterococcus</td>
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<tr>
<td>Aminoglycoside 3'-'phosphotransferase</td>
<td>31</td>
<td>Phosphorylates 3'-hydroxyl</td>
<td>aph (3') - IIIa (aphA)</td>
<td>Plasmids</td>
<td>S. aureus</td>
<td>414,140,179</td>
</tr>
<tr>
<td>[APH(3')-III]</td>
<td></td>
<td>groups of kanamycin, neomycin, paromomycin, amikacin and gentamicin B</td>
<td></td>
<td>and chromosome : Sma I fragment A</td>
<td>S. epidermis</td>
<td></td>
</tr>
<tr>
<td>Aminoglycoside-aminocyclito-4'-adenylvtrtransferase</td>
<td>34</td>
<td>Adenylylates tobramycin</td>
<td>ant (4')-la (aadD)</td>
<td>Plasmids</td>
<td>S. aureus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>isepepamicin, paromomycin.</td>
<td></td>
<td></td>
<td>S. epidermis</td>
<td></td>
</tr>
<tr>
<td>Resistance Phenotype and Responsible Protein</td>
<td>MW (kDa)</td>
<td>Major Activity or Mechanisms</td>
<td>Gene</td>
<td>Gene Location</td>
<td>Species Distribution</td>
<td>References</td>
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</tr>
<tr>
<td>adenyltransferase (aminoglycoside 4'-adenyltransferase) [ANT (4',4'')-I]</td>
<td></td>
<td>paromomycin, kanamycin, neomycin and gentamicin A at 4'-hydroxyl groups and also dibekacin at the 4''-hydroxyl group</td>
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<tr>
<td>Aminoglycoside 9-adenyltransferase (ANT (9) -I)</td>
<td>29</td>
<td>Adenylylates spectinomycin</td>
<td>ant(9)-Ia (spc)</td>
<td>Chromosome: on transposon Tn554</td>
<td>S. aureus</td>
<td>332</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>S. epidermidis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Frequently found in meCA strains several other species</td>
<td></td>
</tr>
<tr>
<td>Trimethoprim</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>S1 Dihydrofolate reductase (S1 DHFR)</td>
<td>18-19</td>
<td>Marked reduced affinity for trimethoprim High-level resistance: (MIC&gt;1000 mg ml⁻¹ Phe-Tyr change at position 98 (compared with native DHFR) is major determinant of resistance</td>
<td>drfA</td>
<td>Plasmid&gt; chromosome: on transposon-like Tn4003 in an operon with thyE and ORF</td>
<td>S. aureus</td>
<td>110,115,294,397</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>S. epidermidis</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S. hominis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Several other species</td>
<td></td>
</tr>
<tr>
<td>Resistance Phenotype and Responsible Protein</td>
<td>MW (kDa)</td>
<td>Major Activity or Mechanisms</td>
<td>Gene</td>
<td>Gene Location</td>
<td>Species Distribution</td>
<td>References</td>
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</tr>
<tr>
<td>Chloramphenicol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>22.5-23</td>
<td>Acetylates chloramphenicol via acetyl coenzyme A to yield mono- and diacylated derivatives unable to bind to the ribosome Requires induction by chloramphenicol Regulated by translational attenuation</td>
<td><em>cat</em></td>
<td>Plasmids (class I)</td>
<td><em>S. aureus</em></td>
<td>83,225,412, 413,423</td>
</tr>
<tr>
<td>acetyltransferase (CAT)</td>
<td>mono trimeric</td>
<td></td>
<td></td>
<td></td>
<td>Several other species</td>
<td></td>
</tr>
<tr>
<td>Fluoroquinolons</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA gyrase subunits</td>
<td>A: 99.2</td>
<td>Subunit A: Ser → Leu or Phe change at position 84 and Glu → Lys change at position 88 result in moderate to high fluoroquinolone resistance Subunit B: mutations may result in</td>
<td><em>gyra</em></td>
<td>Chromosome: Sma I fragment G Part of operon: <em>recF-gyrB-gyrA</em></td>
<td><em>S. aureus</em></td>
<td>178,228,302, 439,440</td>
</tr>
<tr>
<td>[DNA topoisomerase (ATP-hydrolysing)]</td>
<td>B: 72.5</td>
<td></td>
<td><em>gyrB (nov)</em></td>
<td></td>
<td><em>S. epidermis</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Many other species Bacillus subtilis</td>
<td></td>
</tr>
<tr>
<td>Resistance Phenotype and Responsible Protein</td>
<td>MW (kDa)</td>
<td>Major Activity or Mechanisms</td>
<td>Gene</td>
<td>Gene Location</td>
<td>Species Distribution</td>
<td>References</td>
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</tr>
<tr>
<td>Mutidrug efflux protein (or transporter) Nor A</td>
<td>42.2</td>
<td>Plays role as energy dependent efflux pump to remove hydrophilic fluoroquinolones and some other unrelated antibiotics</td>
<td>norA or its regulatory region flqB may affect level of expression of norA</td>
<td>Chromosome Smal fragment D Infrequently on plasmids</td>
<td>S. aureus, S. epidermidis, S. haemolyticus</td>
<td>239,351,365,507</td>
</tr>
<tr>
<td>DNA topoisomerase</td>
<td>A: 90</td>
<td>Subunit A: Ser→Phe or Tyr change at position 80</td>
<td>grlA (cfxB, ofxC of flq)</td>
<td>Chromosome: Smal fragment A</td>
<td>S. aureus</td>
<td>146,466</td>
</tr>
<tr>
<td>Resistance Phenotype and Responsible Protein</td>
<td>MW (kDa)</td>
<td>Major Activity or Mechanisms</td>
<td>Gene</td>
<td>Gene Location</td>
<td>Species Distribution</td>
<td>References</td>
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</tr>
<tr>
<td>IV subunit A</td>
<td>B: 74.4</td>
<td>results in either high or low fluoroquinolone resistance subunits A and B are closely related to subunits A and B-of DNA gyrase grlA mutations may be prerequisite for resistance by gyrA mutations</td>
<td>locus) grlB</td>
<td>Part of Opeon: grlB-grlA</td>
<td></td>
<td>128</td>
</tr>
<tr>
<td>DNA-dependent RNA polymerase β subunit</td>
<td>142-151</td>
<td>Mutations in rifampin binding area (area1) of β subunit may result in rifampicin, rifamycin, and streptovaricin resistance Specific amino acid changes have not been documented</td>
<td>RpoB (rif)</td>
<td>Chromosome: Smal fragment D Part of Operon: rpl-rpoB-rpoC</td>
<td>S. aureus</td>
<td>S. epidermidis</td>
</tr>
</tbody>
</table>
CHAPTER-3

MATERIALS AND METHODS

In order to carry out the proposed research work "Biochemical Studies on Antibiotic Resistance among Staphylococci from Eyes and Other Clinical Sources in Health and Disease", the organisms were isolated, characterized and identified by various biochemical tests, and subsequent experiments were performed by standard methods and protocols described in this chapter.

3.1 MATERIALS USED

The various materials including antibiotics and chemicals used while performing different experiments in the present study were shown in Table 3.1 and Table 3.2.

Table 3.1 List of Antimicrobial Agents with their Disk Contents Purchased from Hi-Media Laboratories, Pvt. Ltd. Mumbai, India

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>SYMBOL</th>
<th>Disk Content a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>Ak</td>
<td>30 μg</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>A</td>
<td>10 μg</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>Cz</td>
<td>30 μg</td>
</tr>
<tr>
<td>Cephalaxin</td>
<td>Cp</td>
<td>30 μg</td>
</tr>
<tr>
<td>Cephotaxime</td>
<td>Ce</td>
<td>30 μg</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>C</td>
<td>30 μg</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Cf</td>
<td>5 μg</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>Cd</td>
<td>2 μg</td>
</tr>
<tr>
<td>Co-trimoxazole (trimethoprim/ Sulphamethoxazole)</td>
<td>Co</td>
<td>1.25/23.75 μg</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>Fc</td>
<td>10 μg</td>
</tr>
<tr>
<td>Chemicals /Materials</td>
<td>Source</td>
<td></td>
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<tr>
<td>------------------------------</td>
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<td></td>
</tr>
<tr>
<td>Acetone</td>
<td>SRL, India</td>
<td></td>
</tr>
<tr>
<td>Acridine orange</td>
<td>Hi-Media, India</td>
<td></td>
</tr>
<tr>
<td>Acriflavine</td>
<td>Hi-Media, India</td>
<td></td>
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<tr>
<td>Acrylamide</td>
<td>Hi-Media, India</td>
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<tr>
<td>Agar agar</td>
<td>Hi-Media, India</td>
<td></td>
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<tr>
<td>Agarose</td>
<td>Hi-Media, India</td>
<td></td>
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<tr>
<td>Amikacin sulphate</td>
<td>Hi-Media, India</td>
<td></td>
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<tr>
<td>Ammonium chloride</td>
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</tr>
<tr>
<td>Ammonium oxalate</td>
<td>SRL, India</td>
<td></td>
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<tr>
<td>Ammonium persulphate</td>
<td>Hi-Media, India</td>
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<tr>
<td>Amoxycillin</td>
<td>Hi-Media, India</td>
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<tr>
<td>Ampicillin</td>
<td>Hi-Media, India</td>
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<tr>
<td>Bacitracin</td>
<td>Hi-Media, India</td>
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</table>

In accordance to Performance Standards for Antimicrobial Disk Susceptibility Tests, NCCLS.

Table 3.2 Chemicals/Materials Used in Experimental Work
<table>
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<th>Chemical Name</th>
<th>Supplier</th>
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<td>Benzyl penicillin</td>
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<tr>
<td>Bis-acrylamide</td>
<td>Hi-Media, India</td>
</tr>
<tr>
<td>Bromocresol purple</td>
<td>Hi-Media, India</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>Hi-Media, India</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>SRL, India</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>Hi-Media, India</td>
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<tr>
<td>Cephalexin</td>
<td>Hi-Media, India</td>
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<tr>
<td>Cephotaxime</td>
<td>Hi-Media, India</td>
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<tr>
<td>Chloramphenicol</td>
<td>Ranbaxy, India</td>
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<tr>
<td>Chloroform</td>
<td>SRL, India</td>
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<tr>
<td>Ciprofloxacin</td>
<td>Hi-Media, India</td>
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<tr>
<td>Coomassie brilliant blue R-250</td>
<td>Hi-Media, India</td>
</tr>
<tr>
<td>Cotton swab</td>
<td>Hi-Media, India</td>
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<tr>
<td>Crystal violet</td>
<td>Hi-Media, India</td>
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<tr>
<td>DNA</td>
<td>Hi-Media, India</td>
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<tr>
<td>DNase test agar</td>
<td>Hi-Media, India</td>
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<tr>
<td>EDTA</td>
<td>SRL, India</td>
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<tr>
<td>Erythromycin</td>
<td>Hi-Media, India</td>
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<td>Ethanol</td>
<td>E. Merck, India</td>
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<tr>
<td>Ethedium bromide</td>
<td>Sigma, USA</td>
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<tr>
<td>Glacial acetic acid</td>
<td>E. Merck, India</td>
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<td>Glycerol</td>
<td>SRL, India</td>
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<tr>
<td>Hydrochloric acid</td>
<td>SRL, India</td>
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<tr>
<td>Hydrogen peroxide</td>
<td>SRL, India</td>
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<tr>
<td>Iodine crystals</td>
<td>Hi-Media, India</td>
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<td>Chemical</td>
<td>Supplier</td>
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<tr>
<td>Isoamyl alcohol</td>
<td>SRL, India</td>
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<tr>
<td>KCl</td>
<td>SRL, India</td>
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<tr>
<td>KOH</td>
<td>Hi-Media, India</td>
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<td>Luria-Bertani broth</td>
<td>Hi-Media, India</td>
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<td>Lysostaphin</td>
<td>Sigma, USA</td>
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<td>Lysozyme</td>
<td>Sigma, USA</td>
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<tr>
<td>Mannitol salt agar</td>
<td>Hi-Media, India</td>
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<tr>
<td>2-mercaptoethanol</td>
<td>Sigma, USA</td>
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<td>Methicillin</td>
<td>Ranbaxy, India</td>
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<td>MgCl₂</td>
<td>SRL, India</td>
</tr>
<tr>
<td>MnCl₂</td>
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<tr>
<td>MOPS</td>
<td>Sigma, USA</td>
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<td>Mueller-Hinton agar</td>
<td>Hi-Media, India</td>
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<td>Mueller-Hinton broth</td>
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<td>Na₂HPO₄</td>
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<td>NaH₂PO₄.H₂O</td>
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<td>NaOH</td>
<td>SRL, India</td>
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<tr>
<td>Novobiocin</td>
<td>Hi-Media, India</td>
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<td>Nutrient agar</td>
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<td>Nutrient broth</td>
<td>Hi-Media, India</td>
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<td>Oxacillin salt agar</td>
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<tr>
<td>Oxacilline</td>
<td>Ranbaxy, India</td>
</tr>
<tr>
<td>Phenol</td>
<td>SRL, India</td>
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<tr>
<td>Phenol red</td>
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<td>Phenolphthalein phosphate agar</td>
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<tr>
<td>Potassium iodide</td>
<td>Hi-Media, India</td>
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</tbody>
</table>
3.2 SOURCE OF ORGANISMS

All the bacterial strains of Staphylococci used in this study were isolated from:

1. Eye (n=120) and nose (n=50) of the patients having various ocular infections/diseases, who attended the out patient department (OPD) or were admitted in the wards, and normal eyes (n=50) of the hospital workers of the Institute of Ophthalmology, J.N. Medical College, Aligarh Muslim University, Aligarh India.

2. Nose (n=50) of the patients having wound infections (n=56) and nose (n=50) of the hospital workers attending the patients having wound infections in an Orthopaedic Surgical Ward of J.N. Medical College, A.M.U., Aligarh.

3. Different clinical sources (n=399) of patients having various infections/diseases, who attended the out patient department (OPD) or were admitted in the wards and nose (n=75) of hospital workers of S.K. Institute of Medical Sciences Kashmir.
The details of isolated staphylococcal strains have been depicted in Table 3.3.

Table 3.3 Distribution of Staphylococcal Isolates (n=850) from Eyes and Other Clinical Sources in Health and Disease

<table>
<thead>
<tr>
<th>Clinical Source</th>
<th>No. of Staphylococcal Isolates n=850 (100%)</th>
<th>No. of Staphylococcus aureus Isolates n=513 (60.3%)</th>
<th>No. of Coagulase-negative Staphylococcal Isolates n=337 (39.7%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conjunctiva</td>
<td>76 (8.9)</td>
<td>21 (4.1)</td>
<td>55 (16.3)</td>
</tr>
<tr>
<td>n=76 (8.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corneal scrapings</td>
<td>29 (3.4)</td>
<td>6 (1.2)</td>
<td>23 (6.8)</td>
</tr>
<tr>
<td>n=29 (3.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitreous humour</td>
<td>15 (1.8)</td>
<td>3 (0.6)</td>
<td>12 (3.6)</td>
</tr>
<tr>
<td>n=15 (1.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pus^a</td>
<td>151 (17.8)</td>
<td>115 (22.4)</td>
<td>36 (10.7)</td>
</tr>
<tr>
<td>n=151 (17.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>153 (18.0)</td>
<td>76 (14.8)</td>
<td>77 (22.8)</td>
</tr>
<tr>
<td>n=153 (18.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>70 (8.2)</td>
<td>58 (11.3)</td>
<td>12 (3.6)</td>
</tr>
<tr>
<td>n=70 (8.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebrospinal fluid</td>
<td>14 (1.6)</td>
<td>4 (0.8)</td>
<td>10 (3.0)</td>
</tr>
<tr>
<td>n=14 (1.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Semen</td>
<td>18 (2.1)</td>
<td>18 (3.5)</td>
<td>-</td>
</tr>
<tr>
<td>n=18 (2.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catheter tip</td>
<td>18 (2.1)</td>
<td>16 (3.1)</td>
<td>2 (0.6)</td>
</tr>
<tr>
<td>n=18 (2.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other^b</td>
<td>31 (3.6)</td>
<td>21 (4.1)</td>
<td>10 (3.0)</td>
</tr>
<tr>
<td>n=31 (3.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nasal swabs^c</td>
<td>225 (26.5)</td>
<td>175 (34.1)</td>
<td>50 (14.8)</td>
</tr>
<tr>
<td>n=225 (26.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ocular swabs^d</td>
<td>50 (5.9)</td>
<td>-</td>
<td>50 (14.8)</td>
</tr>
<tr>
<td>n=50 (5.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a^ Pus includes pus from wound and pus from abscess.
^b^ Other includes: blood culture, blood, and catheter tip.
^c^ Nasal swabs includes: nasal swab.
^d^ Ocular swabs includes: conjunctival swab and corneal scrapings.
a Strains isolated from wound exudate, ulcer, abscess etc.

b Sputum or tracheal aspirate, ear fluid, drained fluid, tissue sample, vaginal swabs etc.

c Strains isolated from nose of patients having wound infections and hospital workers attending wound infections n=(50+50), nose of hospital workers (n=75) and nose of patients having eye infections (n=50) respectively in Orthopaedic Surgical Ward of J.N. Medical College, A.M.U. Aligarh; S.K. Institute of Medical Sciences Kashmir, and Microbiology Section, Institute of Ophthalmology, A.M.U., Aligarh.

d Strains isolated from normal eyes of hospital workers of Institute of Ophthalmology, A.M.U., Aligarh.

3.3. PROCEDURE FOR OBTAINING CULTURES

The cultures were obtained with moist cotton swabs or sterile loops under aseptic conditions as described by Fedukowiez and Stenson in 1983. All the glassware were thoroughly washed with tap water followed by distilled water and were sterilized in the hot air oven at 170±10 °C for at least one hour. The cotton swabs were sterilized by autoclaving at a temperature of 121°C and 151 b/² pressure for 15-20 minutes.

3.3.1 CORNEAL CULTURES

The conjunctival cultures are inadequate in case of corneal ulcer; therefore, direct sampling from the corneal ulcer bed is necessary both for smears and cultures. Topical anaesthesia, xylocaine (4%) was used before taking culture, depending upon the response of patients. The exudates and necrotic material were removed from the ulcer crater with a moist swab/loop, which was then cultured. Causative organism are often best recovered underneath this debris, therefore, additional specimens were obtained with a sterile swab or loop by taking care to avoid excess pressure especially with deep ulcers or severe thinning.

3.3.2 NASAL CULTURES

A single sterile cotton swab moistened in nutrient broth was swirled in the anterior portion of each naris of five clockwise and five counter clockwise rotations. The swab was immediately reintroduced into the culture tube, transported to lab and inoculated on blood agar plates. The plates were incubated at 37 °C for 48 h. S. aureus isolates were selected by
mannitol salt agar and identified by catalase and coagulase testing of colonies with typical morphology.

### 3.3.3 CULTURES FROM OTHER CLINICAL SOURCES

The isolation of staphylococci from various clinical specimens was performed as per routine operations in the concerned clinical laboratories. However, some recommended procedures for collecting and processing specimens as described in the American Society for Microbiology’s Manual of Clinical Microbiology, were followed.\(^{14,82,390}\) The cultures from clinical specimens were taken with the help of cotton swabs or inoculation loops and streaked on blood agar and loops and nutrient agar plates. The plates were incubated at 37 °C for 48 hour and then stored at room temperature for 4 days to enhance pigment production and colony morphology.

### 3.4 PREPARATIONS AND COMPOSITION OF MEDIA

There is a wide variety of media available for the growth of microorganisms in the laboratory. Most of these are available from the commercial sources with all the components premixed and requiring only addition of water and sterilization for use. When it is desirable to grow bacteria on a solid medium, a solidifying agent such as agar (polysaccharide derived from a marine alga) is added to the medium.

All the media used in the study were either prepared in the laboratory or supplied by Messers Hi-Media Pvt. Ltd. Mumbai.

### 3.4.1 NUTRIENT BROTH (Collee and Marr, 1989)

Nutrient broth was used for growing the bacterial strains prior to testing their antibiotic sensitivity and minimum inhibitory concentrations (MIC's) of some antibacterial agents.

**Composition:** (Ingredients/litre)

- Peptone 5 g, Sodium chloride 5 g,
- Beef extract 1.5 g, Yeast extract 1.5 g
- pH 7.4±0.2
Dissolved the ingredients in water, heated to obtain a homogenous solution and poured this solution into clean test tubes (3 ml into each tube) stopper with cotton plugs. The tubes were autoclaved for 15-20 minutes.

3.4.2 NUTRIENT AGAR (Finegold and Martin, 1982)

It is the simple or basal medium commonly used in microbiological laboratories, whenever, needed. It contains all basic nutritional requirements, sources of energy, carbon, nitrogen and mineral salts.

**Composition:** (Ingredients per litre)

- Peptone 5 g, Sodium chloride 5 g, Beef extract 1.5 g, Yeast extract 1.5 g, Agar 15 g, pH (approx.) 7.4±0.2

Dissolved the ingredients in distilled water in a conical flask, plugged firmly with cotton, wrapped in aluminium foil. Autoclaved it at 121 °C for 15 minutes and poured into sterile petriplates under aseptic conditions. Allowed to cool to form a homogenous layer about 4 mm in thickness. A few plates from each batch were incubated overnight at 37 °C to check their sterility.

3.4.3 BLOOD AGAR

Blood agar in addition to being an enriched medium is an indicator medium showing the hemolytic properties of bacteria.

**Composition:**

- Nutrient agar 100 ml
- Sheep a blood 5-10 ml

The method of preparation of this medium was essentially the same as that for nutrients agar. The sterile citrated sheep's blood (5-10%) was added to autoclaved nutrient agar from a screw capped bottle after the mixture had cooled down to about 50°C±20°C in a water bath and was poured aseptically into sterile petriplates.

3.4.4 MANNITOL SALT AGAR (Cappuccino and Sherman, 1983)

It is both a selective and an indicator medium. Most strains of *S. aureus* ferment mannitol.
Composition: (Ingredients per litre)

Beef extract 1 g, Proteose peptone 10 g,
Sodium Chloride 75 g, D-Mannitol 10 g, Phenol Red 0.025, Agar 15 g,
pH 7.4±0.2

Dissolved the ingredients in distilled water and adjusted the pH by adding diluted acid or base and autoclaved.

3.4.5 PHENOLPHTHALEIN PHOSPHATE AGAR

(Barber and Kuper, 1951)^40

This indicator medium assists the identification of S. aureus in mixed cultures.

Composition: (Ingredients/litre)

Nutrient agar 980 ml,
Sodium phenolphthalein diphosphate (0.6% soln.) 20 ml, pH 7.4

Dissolved sodium phenolphthalein diphosphate in water to 0.6% concentration, filtered and at once added 20 ml of this solution to melted nutrient agar just before pouring into sterile petriplates.

3.4.6 DNA AGAR

It used for identifying S. aureus strains.

Composition: (Ingredient/litre)

Tryptose 20 g, DNA 2 g, Sodium chloride 5 g,
Agar powder 12 g, pH 7.3

Dissolved the ingredients in distilled water and autoclaved for 15-20 minutes.

3.4.7 MUELLER-HINTON AGAR

Of the many media available, the NCCLS^344,345,346 considers Mueller Hinton agar to be the best for routine susceptibility testing of non-fastidious bacteria for the following reasons:

- It shows acceptable batch-to-batch reproducibility for susceptibility testing.
• It is low in sulfonamide, trimethoprim, and tetracycline inhibitors.
• It gives satisfactory growth of most non-fastidious pathogens.

**Composition:** (per litre)

- Casen acid hydrolysate 17.5 g,
- Beef heart infusion 2.0 g
- Starch, soluble 1.5 g
- Agar 17.0 g

*Final pH (at 25 °C) 7.3±0.2*

**Preparation of Mueller-Hinton Agar:**

Mueller-Hinton agar preparation includes the following steps:

1. Mueller-Hinton agar should be prepared from a commercially available dehydrated base according to the manufacture's instructions.

2. Immediately after autoclaving, allow it to cool in a 45 to 50°C water bath.

3. Pour the freshly prepared and cooled medium into glass or plastic, flat-bottomed petridishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 60 to 70 ml of medium for plates with diameters of 150 mm and 25 to 30 ml for plates with a diameter of 100 mm.

4. The agar medium should be allowed to cool to room temperature and, unless the plate is used the same day, stored in a refrigerator (2 to 8°C).

5. Plates should be used within seven days after preparation unless adequate precautions, such as wrapping in plastic bags, have been taken to minimize drying of the agar and unless they have been shown to perform correctly with the quality control organisms.

6. A representative sample of each batch of plates should be examined for sterility by incubating at 30 to 35 °C for 24 hours or longer.
The pH of each batch of Mueller-Hinton agar should be checked when the medium is prepared. The agar medium should have a pH between 7.2 and 7.4 at room temperature after gelling.

If, just before use, excess surface moisture is present, the plates should be placed in an incubator (35 °C) or a laminar flow hood at room temperature with lids ajar until excess surface moisture is lost by evaporation (usually 10 to 30 minutes). The surface should be moist, but no droplets of moisture should be apparent on the surface of the medium or on the petridish covers when the plates are inoculated.

### 3.4.8 Oxacillin Salt Agar

The oxacillin salt-agar was used for the detection of MRSA. Oxacillin salt-agar was prepared by taking Mueller Hinton agar and was supplemented with NaCl (4% w/v; 0.68 mol/L) and 6 mg oxacillin/L.

### 3.5 Identification and Characterization of Bacterial Isolates

After incubating the culture plates for 18-24 hours at 37 °C, the isolated colonies were obtained, which were later picked up aseptically preserved on nutrient agar slants at 4 °C. Each of the isolate was marked with a prefix -JS (e.g., JS-1, JS-2, JS-3—etc) and subculture on fresh nutrient agar slants, time to time.

All the isolated bacteria were further studied for their morphological, cultural and biochemical characteristics as per recommendations of Buchanan and Gibbons (1974) and Finegold and Martin (1982).

#### 3.5.1 Cultural Characters

The cultural characters included the appearance of colonies to naked eye, their size, outline, elevation, translucency, color, pigmentation and hemolytic characters.

#### 3.5.2 Morphology and Staining (Hucker’s modification)

Gram's staining was employed to determine whether a particular organism was Gram-positive or Gram-negative and its size, shape, arrangement of cells and grouping.
A. Staining Reagents

1. (a) **Stock crystal violet solution:**

   Crystal violet (85% dye) – 20 g
   
   Ethanol (95%) – 100 ml

(b) **Stock oxalate solution:**

   Ammonium oxalate – 1 g
   
   Distilled water – 100 ml

**Working solution:**

Diluted the stock crystal violet solution 1:10 with distilled water and mixed it with 4 volumes of stock oxalate solution and stored in a glass stoppered bottle.

2. **Gram’s Iodine solution:**

   Iodine crystals – 1 g
   
   Potassium iodine – 2 g

Dissolved these two in 5 ml of distilled water. Then following solutions were added.

   Distilled water – 240 ml
   
   Sodium bicarbonate solution (5% aqueous) – 60 ml

The contents were mixed well and stored in amber glass bottles.

3. **Decolourizer:**

   Ethanol (95%) – 200 ml
   
   Acetone – 200 ml

   Mixed, and stored in glass stoppered bottles.

4. **Counter stain:**

   Stock Safranine-D - 2.5 g
   
   Ethanol (95%) - 100 ml
Working Solution:

Diluted the stock safranine solution to 1:5 or 1:10 with distilled water and stored in glass stoppered bottle.

B. Staining Method (Finegold and Martin, 1982)

(a) A thin film of the material to be examined was prepared on a glass slide, dried and heat fixed.

(b) Flooded the slide with crystal violet stain and allowed to remain for 10 seconds.

(c) Poured off the stain by washing the slide with iodine solution.

(d) Rinsed off this mordant after 10 seconds with running water.

(e) The slide was then decolourized with alcohol - acetone solution for about 10-20 seconds.

(f) The slide was then counterstained with safranine for 10 seconds and washed off with water.

(g) Blotted the slide between clean sheets or filter paper and examined microscopically, under oil immersion.

3.5.3 BIOCHEMICAL REACTIONS

The biochemical reactions were studied as described elsewhere.\textsuperscript{268,337,369}

3.5.3.1 Mannitol Fermentation Test

Mannitol fermentative capability of the test strains of staphylococci was examined according to method described by Cappuccino and Sherman (1983).\textsuperscript{82}

Principle:

The mannitol salt agar containing high salt concentration (7.5% NaCl) is inhibitory to the growth of most bacteria other than \textit{Staphylococcus} species and also performs a differential function as it contains carbohydrate mannitol, which some staphylococci are capable of fermenting. The phenol red pH indicator is used for detecting acid produced by mannitol
fermentating staphylococci. The *Staphylococcus aureus* exhibit a yellow zone surrounding their growth.

**Method:**

Spreaded the inoculum under test thinly over the mannitol salt agar plate and incubated for 18-24 hours at 37 °C. Strains of *Staphylococcus aureus* fermented mannitol and exhibited yellow zones surrounding their growth in contrary to *Staphylococcus epidermidis* that do not ferment mannitol and hence did not produce change in colouration.

### 3.5.3.2 Catalase Test (Cappuccino and Sherman, 1983)

During aerobic respiration, microorganisms produce H$_2$O$_2$ and in some cases extremely toxic superoxides. Accumulation of these substances will result in the death of organisms unless they are enzymatically degraded. These substances are produced when aerobes, facultative anaerobes and microaerophiles use the aerobic respiratory pathways in which oxygen is the final electron acceptor during degradation of carbohydrates for energy production. The organisms capable of producing catalase or peroxidase rapidly degrade hydrogen peroxide as follows:

\[
\text{Catalase} \\
2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \\
\text{Peroxidase} \quad \text{Free oxygen}
\]

**Method:**

Transferred a small portion of overnight grown culture under study with the help of a clean glass rod into a tube containing 30% of (v/v) hydrogen peroxide and watched for the evolution of bubbles of free O$_2$, indicative of catalase activity.

### 3.5.3.3 Coagulase Test

The enzyme coagulase is produced by strains of *Staphylococcus aureus*, which coagulates human and rabbit plasma by converting the fibrinogen into fibrin. Coagulase plays an important role in the pathogenesis of staphylococcal infections. This test was performed for differentiating
coagulase positive from coagulase negative strains, as the demonstration of coagulase production is the best single test to identity pathogenic *Staphylococcus* species.  

**(a) Tube Test** (Gillespie, 1943)  

Citrated human or rabbit plasma  

Saline (0.85% NaCl)

Soluble or free coagulase can be demonstrated by adding a drop of young staphylococcal broth culture (overnight) into a tube containing 0.5 ml citrated human or rabbit plasma diluted 1 in 10 with isotonic (0.85% NaCl) saline. It turns the citrated plasma into a firm gel.

**Method:**

1. Took 0.5 ml of the diluted plasma in each of the three test tubes and inoculated 0.1 ml of 18-24 h. broth culture (strain under test) into the plasma of one tube.

2. A control test was setup with known coagulase positive culture in the second tube, whereas, the third tube was left unseeded as negative control to confirm that it did not coagulate spontaneously.

3. All the three tubes were incubated at 37 °C, and examined for coagulation at 1, 3 and 6 h and again if still negative after standing overnight at room temperature. Examination at each of the prescribed times was necessary as the coagulum may liquefy after it has been formed. Test was read positive in which plasma had been converted into a stiff gel and read the test negative in which plasma remained wholly liquid or free flowing.

**(b) Slide Test** (Williams and Harper, 1946)

**Method:**

1. Placed a drop of saline (0.85% NaCl) solution on a clean microscopic slide and with minimum of spreading, emulsified the material obtained from one or two colonies of culture under test in it.
Dipped the tip of inoculating wire into the undiluted plasma warmed to room temperature and added its traces to the bacterial suspension on slide. Flamed the inoculating wire and repeated the procedure for the control bacterial suspension.

Coagulase was demonstrated when clumping of staphylococci occurred within 20 seconds of adding a trace of undiluted plasma to a saline suspension of organism on glass slide after verifying that there is no spontaneous agglutination.

3.5.2.4 Phosphatase Plate Test (Barber and Kuper, 1951)

This test assists the identification of Staph. aureus in mixed cultures. All coagulase-positive S. aureus strains produce phosphatase and their colonies liberate phenolphthalein.

**Method:**
Inoculum under test was spreaded thinly over the phenolphthalein phosphate agar plate and incubated for 18-24 h at 37 °C. After incubation, placed 0.1 ml of ammonia solution (SG 0.88) in the lid of plate and placed the dish with culture over the ammonia for a minute or so. Colonies of S. aureus strains became bright pink due to liberation of phenolphthalein.

3.5.3.5 Deoxyribonuclease Plate Test (Collee and Marr, 1989)

The following method is modified from Jeffries (1961). This test determines the ability of coagulase +ve strains of Staph. aureus to produce nuclease that hydrolyses DNA.

**Method:**
Inoculated material from the test culture by spotting it onto a small area in the middle of one of the marked sections of the DNA-agar plate. Similarly spot inoculated other cultures to be tested on the other sections, including DNase positive control strain. Incubated the plate aerobically for 18-24 h at 37 °C. After incubation, flooded the plate with 1 mol/litre (3.6%) hydrochloric acid to precipitate unhydrolysed DNA and let the plate to stand for a few minutes until a white cloudiness was seen in the agar. Examined the plates under strong indirect light against a dark background. Those spot cultures
were read as DNase positive which were surrounded by a clear unclouded zones (3mm from the edge of colony) comparable with those around control culture and cultures with markedly smaller zones and cloudiness, were read as DNase negative strains.

3.5.3.6 Heamolysin production

The culture under test was inoculated on blood agar plates with the help of sterile inoculating loop and incubated for 18-24 h at 37 °C. Heamolysin producing organisms lysed red blood cells and thereby, exhibited clear zones around their colonies.

3.5.3.7 Novobiocin Sensitivity

The resistance to novobiocin distinguishes S. saprophyticus from other clinically important Staphylococcus species. In routine identification of staphylococci from urine, it is generally sufficient to test by including a 5μg novobiocin disc in the disc diffusion test for antibiotic sensitivities. S. epidermidis shows a large zone of inhibition of growth, e.g. over 15 mm in diameter around 7 mm disc, while S. saprophyticus shows a much smaller zone or grows right up to the disc.

3.5.3.8 β-lactamase test

β-lactamases are the main cause of bacterial resistance to penicillins and cephalosporins. β-lactamase detection was carried out by two simple tests described as:

3.5.3.8.1 Iodometric Test

Hydrolysis of penicillin yields penicilloic acid, which reduces iodine, decolorizing the starch-iodine complex. This reaction can be exploited to detect β-lactamase activity in tubes or on paper strips.

(A) Tube method: Benzylpenicillin, 6mg/ml in 0.1 M phosphate buffer pH 6.0, is distributed in 0.1 ml quantities in tubes or a microtitre tray. Bacterial growth from agar is suspended in these solutions until they are heavily turbid. The suspensions are held at room temperature for 30-60 min then 20 μl volumes of 1% (w/v) soluble starch in distilled water are added, followed
by 20 μl of 2% (w/v) iodine in 53% (w/v) in aqueous potassium iodide. β-lactamase activity is demonstrated by decolourization of the iodine within 5 min. Positive and negative controls are vital as extraneous protein reduces iodine and heavily inoculated tests may give false-positive results.

(B) Paper strip method: To prepare iodometric paper strips, 0.2 g of soluble starch is added to 100 ml of distilled water and dissolved by boiling. After cooling 1 g of benzylpenicillin is added. Filter papers (1x5 cm, Whatman No. 3) are soaked in this solution, and then air-dried for 2 h. The strips, which are stable for 1 year at -20 °C, are moistened with 2% (w/v) iodine in 53% aqueous potassium iodide before use. They are then smeared with colonies from an overnight culture plate. Decolorization within 5 min indicates β-lactamase activity. Positive and negative controls are mandatory.

3.5.3.8.2 Acidometric Test

Hydrolysis of the β-lactamase ring generated a carboxyl group, acidifying unbuffered systems. The resulting acidity can be tested in tubes or on filter papers.

(A) Tube method: For the tube method, 2 ml of 0.5% (w/v) aqueous phenol red solution is diluted with 16.6 ml distilled water and 1.2 g of benzylpenicillin is added. The pH is adjusted to 8.5 with 1M NaOH. The resulting solution, which should be violet in colour, can be stored at 20 °C. Before use, 100 μl portions are distributed into tubes or microtitre wells and inoculated with bacteria from culture plates to produce dense suspensions. A yellow colour within 5 min indicates β-lactamase activity. Positive and negative controls must be run in parallel.

(B) Paper Strip Method: For the acidometric paper method, filter paper (Whatman No.1) is cut into 5x1 cm strips and soaked in a freshly prepared solution containing 125 g/l benzylpenicillin, 0.1% (w/v) bromocresol purple and 1.25 mM NaOH. The strips are dried and can be stored at 4 °C for 6 months with a silica gel desiccant. Before use the strips must be moistened with non-acidic distilled water. Bacteria from agar cultures are smeared on the strip and development of a yellow colour within 5 min indicates β-lactamase activity. Controls must be run in parallel.
3.5.3.9 Chloramphenicol acetyltransferase Test

The most common mechanism of resistance to chloramphenicol is the enzymatic inactivation of the compound. In resistant strains the cytoplasmic enzyme, chloramphenicol acetyltransferase (CAT) acetylates chloramphenicol at the C3-hydroxyl, leading to its inability to bind to the ribosome. CAT was detected by a rapid method called modified Slack's method. A plate of Mueller-Hinton agar was flooded with a barely turbid broth culture (McFarland Opacity of 0.5) of chloramphenicol susceptible *E. coli* ATCC 25922. The plate was dried to remove the surface fluid and discs of Whatmann no.1 filter paper 8.5 mm were placed on the inoculated plate. With the help of an inoculating wire loop, growth was scraped from the surface of the *S. aureus* culture to be tested. This heavy inoculum of the organism was applied evenly over the surface of the 8.5 mm filter paper (6 mm size) discs wetted with chloramphenicol, were carefully placed centrally on the larger Whatmann filter disc, which has been inoculated with the test organism. CAT produced by test strain diffuses through the larger outer disc and inactivates the chloramphenicol and hence *E. coli* growth is not inhibited.

If chloramphenicol has diffused into the external medium in the active form it inhibits *E. coli*, thereby producing a zone of inhibition of *E. coli* and this is associated with the production CAT. Results are interpreted within 4 h and zone of inhibition less than 18 mm signifies CAT positive strain and a zone of inhibition more than 18 mm is interpreted as CAT negative.

### 3.6 ANTIBIOTIC SUSCEPTIBILITY TESTING

A variety of laboratory methods can be used to measure the in vitro susceptibility of bacteria to antimicrobial agents. However the method employed in the present study is the standardized method recommended by the NCCLS Subcommittee on Antimicrobial Susceptibility Testing which is based on the method originally described by Bauer et al. This is the most thoroughly described disk method for which interpretive standards have been developed and supported by laboratory and clinical data.
3.6.1 PROCEDURE FOR PERFORMING THE DISK DIFFUSION TEST

3.6.1.1 Storage of Antimicrobial Disks
Cartridges containing commercially prepared paper disks specifically for susceptibility testing are generally packaged to ensure appropriate anhydrous conditions. Disks were stored as follows:

- Refrigerated the containers at 8 °C or below or froze at −14 °C or below, in a non-frost free freezer until needed.
- The unopened disk containers were removed from the refrigerator or freezer 1 to 2 hours before use to equilibrate these to room temperature.
- Once a cartridge of disks has been removed from its sealed package, it was placed in tightly sealed, desiccated box.

3.6.1.2 Turbidity Standard for Inoculum Preparation
To standardize the inoculum density for a susceptibility test, a BaSO₄ turbidity standard, equivalent to a 0.5 McFarland standard was used. BaSO₄ 0.5 McFarland standard was prepared as follows:

(1) A 0.5 ml aliquot of 0.048 mol/L BaCl₂ (1.175% w/v BaCl₂.2H₂O) was added to 99.5 ml of 0.18 mol/l H₂SO₄ (1% v/v) with constant stirring.
(2) The correct density of the 0.5 McFarland standard was verified by determination of absorbance at 625 nm with a 1cm path length and was found to be 0.08 to 0.10.
(3) The turbidity suspension was transferred in 4 to 6 ml aliquots into screw-cap tubes of the same size or those used in growing or diluting the bacterial inoculum.
(4) The tubes were tightly sealed and stored in the dark at room temperature.
(5) The turbidity standard was vigorously agitated on a mechanical vertex mixer before each use and inspected for a uniformly turbid appearance.
3.6.1.3 Inoculum Preparation

(A) Growth Method: The growth method was performed as follows:

1. A least three to five well-isolated colonies of same morphological types were selected from an agar plate culture. The top of each colony was touched with a loop and the growth was transferred into a tube containing 5 ml of a suitable medium such as tryptic soy broth.

2. The broth culture was incubated at 35 °C until it achieved or exceeded the turbidity of the 0.5 McFarland standard (usually 2 to 6 hours). This results in a suspension containing approximately 1 to 2×10^8 CFU/ml.

3. The turbidity of the actively growing broth culture was adjusted with sterile saline or broth to obtain turbidity optically comparable to that of the 0.5 McFarland standard. This results in a suspension containing approximately 1.5×10^8 CFU/ml.

(B) Direct Colony Suspension Method: As a convenient alternative method, the inoculum was prepared by making a direct broth or saline suspension of isolated colonies selected from a 16 to 24 hour agar plate, such as blood agar or nutrient agar and adjusted to match the 0.5 McFarland turbidity standard.

3.6.1.4 Inoculation of Test Plates

1. Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab.

2. The dried surface of a Mueller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step the rim of the jar was swabbed.
(3) The lid was kept ajar for 3 to 5 minutes to allow for any excess surface moisture to be absorbed before applying the drug-impregnated disks.

3.6.1.5 Application of Disks to Inoculated Agar Plates

(1) As soon as possible and not more than 15 minutes after the inoculation of plates, the antibiotic disks were applied so that diffusion and growth could proceed simultaneously. The disks were applied with the help of forceps. To ensure complete contact of the disk with the agar surface, the disks were pressed down with slight pressure.

(2) Nine disks were applied on each agar plate arranged at least 15 mm from the edge of the plate and apart from each other by a distance of 15 to 20 mm.

(3) The plates were inverted and placed in an incubator set to 35 °C within 15 minutes after the disks were applied. The incubation period was maintained for 18 to 24 hours.

3.6.1.6 Reading Plates and Interpreting Results

(1) After 18 to 24 hours of incubation, each plate was examined. The zones of inhibition were uniformly circular due to a confluent lawn of growth. The diameters of the zones of complete inhibition were measured, including the diameter of the disk. The zones were measured to the nearest whole millimeter, using a ruler, which was held on the back of the inverted petriplate. The petriplates were held a few inches above a black, nonreflecting background and illuminated with reflecting light.

(2) Discrete colonies growing within a clear zone of inhibition were subcultured, re-identified, and retested.

(3) The sizes of the zones of inhibition were interpreted by referring to standard tables in NCCLS documents and the staphylococci were reported as either susceptible, intermediate or resistant to the agents that have been tested.
3.6.1.7 Detection of Methicillin-resistant Staphylococci (MRS)

For best recognition of MRS strains, the following points were considered.

- Besides, methicillin disks the 1μg oxacillin disks were tested for methicillin/oxacillin resistance.
- The inoculum was prepared using the direct colony suspension method rather than the inoculum growth method.
- Tests to detect MRS were incubated for a full 24 hours (rather than 16 to 18 hours) at ≤35 °C.

3.6.1.8 Control Strains

To control the precision and accuracy of the disk test procedures the control strains *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 12228 were obtained from Hi-Media Pvt. Ltd. Mumbai, India. Working control cultures were stored on tryptic soy agar at 4 to 8 °C and subcultured weekly. For prolonged storage these stock cultures were maintained at -20 °C in tryptic soy broth with 15% glycerol. The quality control strains were tested by the standard disk diffusion test procedures using the same antimicrobial agents that were used to test clinical isolates of staphylococci.

### 3.7 Determination of Minimum Inhibitory Concentrations (MIC)

Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an anti-microbial that will inhibit the visible growth of a microorganism after overnight incubation and minimum bactericidal concentrations (MBCs) as the lowest concentration of anti-microbial that will prevent the growth of an organism after subculture on to antibiotic free media. MICs are used by diagnostic laboratories mainly to confirm resistance, but most often as research tool to determine the *in vitro* activity of new antimicrobials, and data from such studies have been used to determine MIC break points. MBC determinations are taken less frequently and their major use has been reserved for isolates from the blood of patients with endocarditis.
The range of antibiotic concentrations used for determining MICs is universally accepted to be in serial twofold dilutions indexed to the base 1 (e.g., 1, 2, 4, 8, 16 mg/ml, etc). Other dilution schemes have also been used, including use of as few as two widely separated or "breakpoint" concentrations between the usual values (e.g., 4, 6, 8, 12, 16 mg/ml). The method described below was used in the present study to check the \textit{in vitro} activity of few antimicrobials against methicillin-resistant \textit{Staphylococcus aureus} isolates.\textsuperscript{346}

\subsection*{3.7.1. PREPARATION OF ANTIBIOTIC STOCK SOLUTIONS}

The selected antibiotics were purchased from Ranbaxy Laboratories Ltd. Secunderbad, and Hi-Media Laboratories Pvt. Ltd., Mumbai. \textit{Inorganic Lab, Department of Chemistry A.M.U. Aligarh} supplied few newly synthesized antimicrobial agents, the activity of these agents was assayed against MRSA isolates. The powders were stored as recommended by the manufacturer or at \( \leq -20 \, ^\circ\text{C} \) in a desiccator, when the desiccator was removed from the refrigerator or freezer, it was allowed to come to room temperature before being opened to avoid condensation of water. Stock solutions were prepared at 20 times the highest MIC range, by weighing the desired amount of powder on an analytical balance. The antimicrobial agent was dissolved in water, buffer or specific solvent, as indicated (Table 3.4). Either of the following formulae was used to determine the amount of powder or diluent needed for a standard solution:

\[ \text{Weight (mg)} = \frac{\text{Volume (ml)} \times \text{Concentration (\( \mu \text{g} / \text{ml} \))}}{\text{Assay Potency (\( \mu \text{g} / \text{mg} \))}} \]

or

\[ \text{Volume (ml)} = \frac{\text{Weight (mg)} \times \text{Assay Potency (\( \mu \text{g} / \text{mg} \))}}{\text{Concentration (\( \mu \text{g} / \text{ml} \))}} \]

or

\[ \text{Volume (ml)} = \frac{\text{Weight of powder (mg)} \times \text{Activity (\%)}}{\text{Desired Concentration (\( \mu \text{g} / \text{ml} \))}} \times 100 \]
Example: to prepare a stock solution containing 1,280 \( \mu g/ml \) of antimicrobial agent with antimicrobial powder that has a potency of 750 \( \mu g/mg \), 182.6 mg of antimicrobial powder should be dissolved in 107.0 ml of diluent, provided that the actual weight of powder is 182.6 mg:

\[
\text{Volume (ml)} = \frac{182.6 \, \text{mg} \times 750 \, \mu g/mg}{1280 \, \mu g/ml} = 107.0 \, \text{ml}
\]

\((\text{Desired concentration})\)

Small volumes of the sterile stock solutions were dispensed into sterile polypropylene vials carefully sealed, and stored at \(-70\) °C but never at a temperature warmer than \(-20\) °C. Vials were thawed as needed and used the same day.

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Solvent</th>
<th>Diluents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin, clavulanic acid, sulbactam, and ticarcillin</td>
<td>Phosphate buffer, pH 6.0, 0.1 mol/liter</td>
<td>Phosphate buffer, pH 6.0, 0.1 mol/liter</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Phosphate buffer, pH 8.0, 0.1 mol/liter</td>
<td>Phosphate buffer, pH 6.0, 0.1 mol/liter</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>95% ethanol</td>
<td>Broth media</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>Saturated solution of sodium bicarbonate</td>
<td>Water</td>
</tr>
<tr>
<td>Cefepime</td>
<td>Phosphate buffer, pH 6.0, 0.1 mol/liter</td>
<td>Phosphate buffer, pH 6.0, 0.1 mol/liter</td>
</tr>
<tr>
<td>Cefotetan</td>
<td>Dimethylsulfoxide</td>
<td>Water</td>
</tr>
<tr>
<td>Cefpodoxime</td>
<td>0.10% aqueous sodium bicarbonate</td>
<td>Water</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>Sodium carbonate</td>
<td>Water</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>Phosphate buffer, pH 6.0, 0.1 mol/liter</td>
<td>Water</td>
</tr>
</tbody>
</table>
Chloramphenicol and Erythromycin

Cinoxacin and nalidixic acid

Enoxacin, norfloxacin and ofloxacin

Imipenem

Moxalactam (diammonium salt)

Nitrofurantoin

Rifampin

Sulfonamides

Trimethoprim

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Preparation Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol and Erythromycin</td>
<td>95% ethanol Water</td>
</tr>
<tr>
<td>Cinoxacin and nalidixic acid</td>
<td>0.5 volume water, then NaOH, 0.1 mol/liter, drop wise to dissolve Water</td>
</tr>
<tr>
<td>Enoxacin, norfloxacin and ofloxacin</td>
<td>0.5 volume water, then NaOH, 0.1 mol/liter, drop wise to dissolve Water</td>
</tr>
<tr>
<td>Imipenem</td>
<td>Phosphate buffer, pH 7.2, 0.01 mol/liter</td>
</tr>
<tr>
<td>Moxalactam (diammonium salt)</td>
<td>0.04N (0.04 mol/liter HCl (let sit for 1.5 to 2h) Phosphate buffer, pH 6.0, 0.1 mol/liter</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>Phosphate buffer, pH 8.0, 0.1 mol/liter</td>
</tr>
<tr>
<td>Rifampin</td>
<td>Methanol Phosphate buffer, pH 8.0, 0.1 mol/liter</td>
</tr>
<tr>
<td>Sulfonamides</td>
<td>0.5 volume of hot water and minimal amount of NaOH, 2.5 mol/liter, to dissolve Water</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>0.05N (0.05 mol/liter) Water (may require lactic or hydrochloric acid, 10% of final volume</td>
</tr>
</tbody>
</table>

3.7.2 PREPARATION OF TEST DILUTIONS

Test dilutions were generally prepared from the antibiotic stock solutions as a series of doubling dilutions (Table 3.5). For the sake of standardization and comparison of results the antimicrobial was diluted in order to provide doubling dilutions above and below a baseline concentration of 1 μg/ml, i.e., 0.007, 0.01, 0.03, 0.06, 0.12, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128; etc.

3.7.3 INOCULUM PREPARATION FOR DILUTION TESTS

A standardized inoculum for the agar dilution and broth dilution methods was prepared by either growing microorganisms to the turbidity of the 0.5 McFarland standard or suspending colonies directly to achieve the same
density. Cultures adjusted to the 0.5 McFarland standard contain approximately 1 to $2 \times 10^5$ CFU/ml with most species, and the final inoculum required is $10^4$ CFU per spot of 5 to 8 mm in diameter which was obtained by diluting 0.5 McFarland suspension to 1:10 in sterile broth or saline so as to get a concentration of $10^7$ CFU/ml, and subsequently inoculated 1 to 2 µl of the diluted suspension on the agar surface. In case of broth dilution method (macrodilution and microdilution) the adjusted inoculum suspension was diluted so that, after inoculation, each tube or well contained approximately $5 \times 10^5$ CFU/ml. This was achieved by preparing antibiotic ranges one step higher than the final dilution range required, i.e. if a final dilution range of 0.5, 1, 2, 4 and 16 µg/ml is required then a range of 1, 2, 4, and 16 and 32 mg/ml was prepared to compensate for the addition of an equal volume of inoculum.

Table 3.5 Scheme for Preparing Dilutions of Antimicrobial Agents to be used in Agar Dilution Susceptibility Tests

<table>
<thead>
<tr>
<th>Antimicrobial solution</th>
<th>Volume (ml)</th>
<th>Distilled water (ml)</th>
<th>Intermediate concentration (µg/ml)</th>
<th>Final concentration at 1:10 dilution in agar or broth (µg/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Step</td>
<td>Source</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5120 Stock</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5120 Step 1</td>
<td>1</td>
<td>5120</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5120 Step 1</td>
<td>1</td>
<td>2560</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1280 Step 3</td>
<td>1</td>
<td>1280</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1280 Step 3</td>
<td>1</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1280 Step 3</td>
<td>1</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>160 Step 6</td>
<td>1</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>160 Step 6</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>160 Step 6</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>20 Step 9</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>20 Step 9</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>20 Step 9</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>2.5 Step 12</td>
<td>1</td>
<td>1.25</td>
</tr>
</tbody>
</table>

*The final concentration is based on the addition of 2 ml of antibiotic solution plus water to 18 ml of melted agar or on the addition of 0.5 ml of antibiotic solution plus water to 4.5 ml of Muller-Hinton broth.
3.7.4 AGAR DILUTION PROCEDURE

The agar dilution method for determining antimicrobial susceptibility is a well-established technique. The antimicrobial agent is incorporated into the agar medium with each plate containing a different concentration of the agent. The inocula can be applied rapidly and simultaneously to the agar surfaces using an inoculum-replicating apparatus. Most replicators currently available transfer 32 to 36 inocula to each plate.

Dilutions prepared as indicated in Table 3.5 were incorporated in melted agar allowed to cool to 45 °C to 50 °C. The antibiotic was added to this agar in a ratio of 1/10, i.e., 2 ml of antibiotic solution plus 18 ml of melted agar when using 9 cm round petri plates. The plates were then set on a horizontal flat surface and allowed to harden undisturbed for at least 10 minutes. Before usage the plates were dried for 10 minutes in an incubator. Two control plates, prepared under the same conditions but without the antibiotics, were prepared and included in every MIC determination.

3.7.4.1 Inoculating Agar Dilution Plates

(1) The tubes containing the adjusted and diluted bacterial suspensions (10^7 CFU/ml) were arranged in order in a rack.

(2) The agar plates were marked for orientation of the inoculum spots.

(3) A 1- to 2-μl aliquot of each inoculum was applied to the agar surface with standardized loops or pipettes.

(4) A growth control plate (no antimicrobial agent) was inoculated first and then, starting with the lowest concentration, the plates containing the different antimicrobial concentrations were inoculated. A second growth control plate was inoculated last to ensure that there was no contamination or significant antimicrobial carry over during the inoculation.

(5) A sample of each inoculum was streaked on a suitable agar plate and incubated overnight to detect cultures and to provide freshly isolated colonies in case retesting proved necessary.
3.7.4.2 Incubating Agar Dilution Plates

The inoculated plates were allowed to stand at room temperature until the moisture in the inoculum spots was absorbed into the agar, i.e., until the spots were dry, but not more than 30 minutes. The plates were inverted and incubated at ≤35 °C for 16 to 24 hours.

3.7.4.3 Determining Agar Dilution End Points

(1) The plates were placed on a dark, non-reflecting surface to determine the end points. The MIC was recorded as the lowest concentration of antimicrobial agent that completely inhibited growth, disregarding a single colony or a faint haze caused by the inoculum.

(2) If two or more colonies persisted in concentrations of the agent beyond an obvious end point, or if there was no growth at lower concentrations but growth at higher concentrations, the culture purity was checked and test repeated.

3.7.5 MACRODILUTION PROCEDURE

(1) Sterile 13-× 100-mm test tubes were used to conduct the test.

(2) A control tube containing broth without antimicrobial agent was used for each test.

(3) The tubes were closed with cotton plugs.

(4) The final twofold dilutions of antibiotic were prepared volumetrically in the broth by taking 1 ml of diluted antibiotic solution (Table 3.5) and 1 ml of adjusted inoculum. The adjusted inoculum was prepared by diluting the 0.5 McFarland Suspension (1×10^8 CFU/ml) to 1:10 to yield 10^7 CFU/ml, and adjusted to 10^6 CFU/ml by mixing 100 ml of diluted suspension and 900 ml of Mueller-Hinton broth. Within 15 minutes after the inoculum has been standardized as described above, 1 ml of the adjusted inoculum was added to each tube already containing 1 ml of antimicrobial agent in the dilution series (and a positive control tube containing only broth), and each tube was mixed. This resulted in a 1:2 dilution of each antimicrobial concentration and a 1:2 dilution of the inoculum (5×10^8 CFU/ml).
(5) The inoculated macrodilution tubes were incubated at \( \leq 35^\circ C \) for 16 to 24 hours in an ambient air incubator.

(6) The MIC was determined as the lowest concentration of antimicrobial agent that completely inhibited growth of the organism in the tube detected by the unaided eyes. The amount of growth in the tubes containing the antibiotic was compared with the amount of growth-control tubes (no antibiotic) used in each set of tests while determining the growth end points.

### 3.7.6 MICRODILUTION PROCEDURE

This method is called "microdilution", because it involves the use of small volumes of broth dispensed in sterile, plastic microdilution trays that have round or conical bottom wells. Each well should contain 0.1 ml of broth.

(1) A growth control well (without antibiotic) and a negative (uninoculated) well were used for each test.

(2) The final two-fold dilutions of antibiotics were prepared by taking 50 \( \mu l \) of diluted antibiotic solution (Table 3.5) and 50\( \mu l \) of adjusted inoculum. The adjusted inoculum was prepared by diluting the 0.5 McFarland Suspension \((1 \times 10^8 \text{ CFU/ml})\) to 1:10 to yield \(10^7 \text{ CFU/ml}\), and adjusted to \(10^6 \text{CFU/ml}\) by mixing 5\( \mu l \) of diluted suspension and 45 \( \mu l \) of Mueller-Hinton broth. Within 15 minutes after the inoculum has been standardized as described above, 50 \( \mu l \) of adjusted inoculum was added to each well already containing 50 \( \mu l \) of antimicrobial agent in the dilution series (and a positive control well containing only broth). This resulted in a 1:2 dilution of each antimicrobial concentration and a 1:2 dilution of the inoculum \((5 \times 10^5 \text{ CFU/ml})\).

(3) To prevent drying, each tray was sealed in a plastic bag, with plastic tape, or with a tight-fitting plastic cover before it was incubated.

(4) The inoculated microdilution trays were incubated at 35 \( ^\circ C \) for 16 to 24 hours in an ambient air incubator.

(5) The MIC was determined as the lowest concentration of antimicrobial agent that completely inhibited growth of the organism in the
microdilution wells containing antibiotic as compared to the amount of growth in the growth-control wells (no antibiotic) detected by the unaided eyes.

3.7.7 DETECTION OF METHICILLIN/OXACILLIN RESISTANT STAPHYLOCOCCI

Historically resistance to the antistaphylococcal \( \beta \)-lactamase-stable penicillins has been referred to as "methicillin resistance", thus the acronyms "MRSA" (for methicillin - resistant \( S. \) aureus) or "MRS" (for methicillin - resistant staphylococci), are still commonly used even though methicillin is no longer the agent of choice for testing or treatment. For detection of MRS, following precautions were taken:

1. Oxacillin being the preferred agent to test, for methicillin/oxacillin resistance, was used in addition to methicillin to detect the resistance.

2. \( \text{NaCl} \) (2% w/v; 0.34 mol/l) was added to the medium for both broth and agar dilution for testing of the penicillinase stable penicillins.

3. The inoculum was prepared using the direct method of colony suspension rather than the inoculum growth method.

4. Tests to detect MRS were incubated for a full 24 hours (rather than 16 to 20 hours) at a 33 °C to 35 °C (never exceeded 35 °C).

5. If the MIC test result was in doubt with a possible methicillin-resistant \( S. \) aureus, additional confirmatory test was performed such as the oxacillin-salt agar-screening test as described below.

3.7.7.1 Oxacillin Screening Plates

The oxacillin salt-agar screening-plate procedure was used in addition to the dilution methods for the detection of MRSA. The test was performed by inoculating an \( S. \) aureus isolate onto Mueller-Hinton agar that was supplemented with \( \text{NaCl} \) (4% w/v; 0.68 mol/l) and that contained, 6 \( \mu \)g oxacillin/ml. The agar was either inoculated as a spot or streak using a cotton swab that was dipped into a direct colony suspension equivalent to a 0.5 McFarland standard. The plate was incubated no higher than 35 °C for
24 hours and examined carefully with transmitted light for evidence of small colonies (>1 colony) or a light film of growth, indicated oxacillin resistance.\textsuperscript{346}

3.7.8 **DETERMINATION OF REDUCED SUSCEPTIBILITY TO VANCOMYCIN**

The occurrence of strains of \textit{S. aureus} and coagulase-negative staphylococci with decreased susceptibility to vancomycin (MICs 4 to 8 \textmu g/ml) was reported from different parts of the world.\textsuperscript{203,395,410} The exact mechanism of resistance that results in these elevated MICs is unknown although it likely involves alterations in the cell wall and hyper expression of penicillin binding proteins (PBPs). To date, all these \textit{S. aureus} strains appear to have developed from MRSA.

In order to recognize strains with vancomycin MICs of 4 to 8 \textmu g/ml, MIC testing was performed or the vancomycin agar screen test was used to detect these isolates, incubated the plates for a negative quality control strain, such as \textit{S. aureus} ATCC 29213, was used to ensure specificity.

### 3.8 PROTOCOL FOR ISOLATION OF PLASMIDS

The protocol employed for isolation of plasmids from staphylococci is a modified form of Birnboim and Takahashi methods.\textsuperscript{54,300,448} This protocol separates plasmid from chromosomal DNA by taking advantage of the fact that most plasmids exist in supercoiled state and hence are more resistant to denaturation in alkaline solution than is chromosomal DNA. Exposure of these DNA preparations to alkaline solutions denatures the chromosomal and nicked plasmid DNA, and this denatured DNA segregates with the cell wall pellet following neutralization. Supercoiled plasmid remains in solution and can be precipitated with salt and alcohol. The buffers used in this protocol were as follows:

**Buffer A (pH 8.0) (10X):**

- 4000 mol/liter Tris-acetic acid
- 20 mmol Ethylenediaminetetra acetic acid (EDTA)

**Buffer B (pH 5.5):**

- 3 mol/liter Sodium acetate/acetic acid.
Buffer C (pH 8.0):

- 10 mmol/liter Tris-acetic acid
- 2 mmol/liter disodium EDTA

Lysostaphin Solution (pH 6.9):

- 12 units/mL Lysostaphin
- 100 mmol/liter NaCl
- 40 mmol/liter Tris - NaOH
- 50 mmol/liter disodium EDTA

Lysing Solution:

- 4% Sodium dodecyl Sulphate (SDS)
- 100 mmol/liter Tris
- 0.4 N NaOH

Lysing solution was prepared by taking 4% SDS/100 mmol/liter Tris and mixing it with equal volume of freshly prepared 0.4 N NaOH.

TE Buffer (pH 8.0):

- 50 mmol/liter Tris
- 10 mmol/liter EDTA

(1) Took culture as a broth culture grown overnight in 3 to 5 ml of Nutrient broth or Luria-Bertani (LB) at 35 °C.

(2) Harvested cells by centrifugation at room temperature at 4000 rpm for 5 minutes.

(3) Resuspended cells in 200 µl of either Buffer A or Lysostaphin solution with 100 µg/ml RNase. Transferred to 1.5-ml polypropylene eppendorf tube and incubated for 15 minutes at 35 °C.

(4) Added Lysing solution (400 µl) and gently inverted 5 to 10 times. Allowed to stand at room temperature for 5 minutes.

(5) Added 300 µl of cold (4 °C) buffer B and gently inverted 10 to 20 times.
(6) Maintained at 0 °C for 20 minutes, centrifuged at room temperature for 15 minutes, and then maintained at 0 °C for 10 minutes.

(7) Centrifuged @ 12000 rpm for 10 minutes at 0 °C and then transferred the supernatant to a fresh tube.

(8) Extracted with an equal volume of chloroform/isoamyl alcohol (24:1).

(9) Transferred 500 μl of the aqueous phase to a fresh tube. Added 1 ml of cold 95% ethanol and mixed by inversion. Placed on ice for 30 minutes.

(10) Centrifuged @ 12000 rpm at 0 °C for 30 minutes.

(11) Washed pellet with 70% ethanol.

(12) Resuspended in 70 μl of TE Buffer.

(13) Separated 10 μl on an agarose gel to determine plasmid content.

3.8.1 AGAROSE GEL SEPARATION

After plasmid isolation, resulting samples were separated on agarose gels and visualized under UV illumination after staining with ethidium bromide.\textsuperscript{300,389} Buffers used were as follows:

**Loading buffer:** For 10 ml solution took,

5 ml Glycerol

0.5 ml of 1% Bromophenol blue

0.5 ml of 1% Xylene cyanol

4 ml water

**Running Buffer (50X) (Tris/Acetate/EDTA (TAE) Buffer):** For 1 liter solution,

242 g/liter Tris base (2 mol/liter)

28.55 ml Glacial acetic acid (1 mol/liter)

37.2 g/liter EDTA (0.1 mol/liter)

(1) Added 0.7% solid agarose to TAE buffer.

(2) Heated the agarose/buffer mixture until dissolved.
(3) Allowed the agarose to cool, with stirring, to 50 °C to 55 °C.

(4) Added 30 μl of ethidium bromide (1.5mg/ml)/100ml of agarose.

(5) Poured agarose into a gel tray with an inserted comb and allowed polymerization for 30 to 45 minutes.

(6) Removed the well comb and placed the gel within an electrophoresis chamber. Filled both cathode and anode chambers with running buffer (1X) to cover the top of the gel by at least 1 mm.

(7) Loaded the sample with Loading Buffer (mixed 5 μl of sample with /μl of Loading Buffer), into the wells.

(8) Ran the gel at a constant voltage for the desired period of time.

(9) Visualized the gel under UV light and photographed.

3.9 TRANSFORMATION

Transformation involves the uptake and expression of DNA from a donor strain by a recipient strain and has been shown for both chromosomal and plasmid markers in S. aureus.\textsuperscript{288,289} The ability of a strain to be transformed is described as competence and depends on a number of factors, which have been well defined. The conditions under which transformation occurs are very similar to those required for the uptake of free phage DNA by transfections.\textsuperscript{432} The competence of a strain is dependent on the presence of a high concentration of divalent ions. Ba\textsuperscript{2+}, Mg\textsuperscript{2+}, Ca\textsuperscript{2+} and Rb\textsuperscript{2+} ions all enhance competence but Ca\textsuperscript{2+} ions are most commonly used in labs.

3.9.1 PREPARATION OF COMPETENT STAPHYLOCOCCAL CELLS FOR HEAT SHOCK TRANSFORMATION (RbCl\textsubscript{2} METHOD)

Competent \textit{E. coli} cells were prepared by RbCl\textsubscript{2} method, using transformation buffer and all the solutions and tubes were chilled on ice prior to use, sterilized by autoclaving wherever applicable.\textsuperscript{300}

\textbf{Transformation buffer 1 (Tfb1):} For 100 ml solution took,

- 30 mM KCl (0.29 g)
- 100 mM RbCl\textsubscript{2} (1.2 g)
- 10 mM CaCl\textsubscript{2}.2H\textsubscript{2}O (0.14 g)
50 mM MnCl₂·4H₂O (0.98 g)

Glycerol (15 ml)

H₂O (85 ml)

Adjusted pH to 5.8 with 1-2 µl of diluted acetic acid. Kept unexposed to air for more than a couple of minutes to avoid formation of reddish brown precipitate of MnO₂. Tfb1 was filter sterilized.

**Transformation buffer 2 (Tfb2):**

10 mM MOPS (0.21 g)

75 mM CaCl₂·H₂O (1.1 g)

10 mM RbCl₂ (0.12 g)

Glycerol (15 ml)

H₂O (85 ml)

Adjusted pH to 6.5 with IM KOH and filter sterilized.

(1) Inoculated an LB plate (no antibiotic) with DH5 cells from frozen stock.

(2) Incubated overnight at 37 °C.

(3) Picked a large, well-isolated colony and inoculated 80 ml of pre-warmed LB containing 20 mM MgCl₂ (added 1.6 ml of 1 M Mg Cl₂). Grown at 37 °C until OD₆₀₀ reached 0.55-0.6.

(4) Harvested the cells in SS 34 tubes at 4000 rpm at 4 °C for 15 minutes. Discarded the supernatant.

(5) Resuspended cells in (2/5)th original volume (32 ml) Tfb1. Kept on ice for 5 minutes and spun down the cells as in step 4. Discarded the supernatant.

(6) Now suspended cells in 1/25th original volume (3.2 ml) Tfb2. Kept the cell suspension on ice for 15 minutes and eliquoted desired volumes (50 µl) in eppendorf tubes and stored frozen in -70 °C freezer.
3.9.2 PREPARATION OF COMPETENT STAPHYLOCOCCAL CELLS USING CALCIUM CHLORIDE

The following protocol is derived from Reference 389

**Transformation buffer (pH 8.0):**

- 50 mmol/liter CaCl₂
- 10 mmol/liter Tris-HCl

1. Inoculated 1 ml of LB broth with an isolated colony from an appropriate staphylococci sensitive strain.
2. Inoculated 100 ml of LB broth with 1 ml of overnight culture.
3. Incubated for 2 to 3 hours at 37 °C with shaking.
4. Placed the culture on ice for 5 to 10 minutes.
5. Pelleted the culture at 7000 rpm for 5 minutes at 4 °C.
6. Gently resuspended cells in 20 ml of sterile, ice-cold transformation buffer. Placed on ice for 20 minutes.
7. Centrifuged for 5 minutes at 7000 rpm.
8. Carefully resuspended the pellet in 2 ml of ice-cold transformation buffer.
9. Allowed the cells to remain on ice for 1 to 2 hours. Stored at 4 °C for 12 to 24 hours.
10. For long term storage, cells were immediately separated into 200 µl aliquots in 1.5 ml polypropylene tubes, snap frozen in dry ice/alcohol and stored at −70 °C. They were thawed on ice prior to use.

3.9.3 PROTOCOL FOR TRANSFORMATION

1. Took the competent cells from −70 °C freezer.
2. Thawed the aliquot on ice and added 50 ng of DNA (i.e. 2-10 µl of plasmid)
3. Kept the mixture on ice for 30 minutes.
4. For heat shock kept the mixture in 42 °C water bath for 45 seconds.
(5) Again kept it on ice for 5 minutes.

(6) Added 500 \( \mu l \) of autoclaved LB broth in inoculation hood.

(7) Incubated @ 37 °C for 1 hour.

(8) Plated 100-200 \( \mu l \) of broth with the help of spreader. For each transformation inoculated two agar plates one with marker antibiotic and one without antibiotic.

(9) Incubated the plates @ 37 °C for 12 hours.

(10) Transformants were grown in 3 ml of broth and examined for the presence of plasmids.

3.10 PLASMID CURING PROTOCOL

The spontaneous loss of a particular trait or phenotypic characteristic in a strain may be the first indication that the character is plasmid determination, though such loss does not necessarily mean that the genes responsible are plasmid borne. Chromosomal mutations in other bacterial species occur at frequencies, which might be mistaken for plasmid loss. However, point mutations are often reversible whereas loss of an entire plasmid is irreversible. There are various agents that have been found to eliminate resistance determinants from bacteria; these include acridine orange, acriflavin, ethidium bromide, rifampicin, sodium dodecyl sulphate, novobiocin, guanidine hydrochloride etc.\(^{135}\) In the present study the protocol for plasmid curing was followed from Reference 149.

(1) Inoculated a single colony of staphylococcal strain having known resistance markers in 2 ml of Mueller-Hinton broth.

(2) Added 0.1 ml of the overnight culture to Mueller-Hinton broth containing a range of doubling concentrations from 128 to 0.25 mg/ml of ethidium bromide.

(3) After 18 to 24 hours of incubation, cultures, which contained a concentration of agent sub-inhibitory to cell growth, were plated on Mueller-Hinton agar plates.
After 16 to 18 hours of incubation, colonies were tested on antibiotic plates to detect antibiotic segregants.

Rate of cure was the number of antibiotic sensitive colonies per number of colonies screened expressed as percentage.

3.11 CONJUGATION

A number of reports have shown that antibiotic resistance can be transferred from one strain to another through conjugation. As staphylococci are often found in close proximity to one another under natural conditions, this form of transfer may have contributed to the spread of antibiotic resistance in vivo. Mating experiments are designed to detect DNA transfer events that require cell-to-cell contact. Most frequently, we detect the transfer of plasmids. Mating experiments can also detect the transfer of specific segments of chromosomal DNA, via either transformation process, mobilization of the chromosome by plasmids, or processes yet to be defined. Mating experiments performed on DNase-containing media (to exclude transformation) or in experiments designed to allow the passage of bacteriophage (to exclude transduction) are required to be certain of the mechanism of transfer. In present study following mating experiments were employed to detect the transfer of antibiotic resistance markers.389

3.11.1 BROTH MATINGS (Mixed Culture Method)

1. Inoculated 5 ml of Luria-Bertani (LB) (Gram negative) or brain-heart infusion (BHI) (Gram - Positive) broth with a single colony from a recipient culture. Inoculated 5 ml of broth with a single colony of donor culture and incubated both overnight at 37 °C.

2. Set up three flasks with 4.5 ml of broth. Into one (mating flask), inoculated 50 ml of the donor overnight culture and 500 μl of the recipient overnight culture. Into the second (recipient control flask), inoculated 500 μl of the recipient overnight culture. Into the third, inoculated 50 μl of the donor overnight culture.

3. Incubated at 37 °C either 4 hours or overnight, without shaking.
Inoculated selective agar plates with 100 µl from the mating mixture and control flasks.

Serial dilutions were performed on the mating mixtures to determine the number of donor and recipient CFU.

Transfer frequency was expressed as transconjugants per recipient CFU.

3.11.2 FILTER MATINGS

Set up overnight cultures as described in broth matings.

Placed three sterile nitrocellulose disks on a non-selective agar plate.

Inoculated tubes with mating mixtures as described in broth matings.

Placed 100 µl of each mixture into a sterile 1.5 ml, polypropylene tube, mixed, transferred 100 µl to a sterile nitrocellulose filter, and allowed the filter to dry at room temperature.

Placed plates in an incubator overnight at 37 °C.

Picked up filters with sterile forceps and placed into test tubes with 1 to 2 ml of sterile 0.9% saline Vortex-mixed vigorously.

Inoculated 100-µl aliquots from each tube onto selective agar plates.

Inoculated plates selective for donor and recipient CFU determination.

Calculated the frequency of transfer as the number of transconjugants per recipient cell.

3.12 PFGE PROTOCOL FOR FINGERPRINTING OF MRSA

PFGE typing of clinical and carrier MRSA strains isolated during our study was performed as described previously.6,34,450

3.12.1 PRECAUTIONS

Gloves and safety spectacles should be worn throughout the procedure.

Ethidium bromide is teratogenic and should not be handled if pregnant.

Unless an enclosed gel documentation system is used, UV protective visors should be worn when visualizing and photographing gels.
3.12.2 METHOD OF DNA PREPARATION

Genomic DNA was extracted from logarithmic-phase MRSA cultures grown in nutrient broth. Bacterial suspension equivalent to a McFarland standard 3 (approx. 9x10^8 cells/ml) of 0.5 ml was prepared with normal saline and mixed with an equal volume of 2% low melting point (LMP) agarose and allowed to solidify in plug moulds. Then the bacteria were lysed by incubation of the agarose plugs at 37 °C for 12 h in 1ml of lysostaphin solution (Sec. 3.8) and 1ml of lysing solution (Section 3.8). The agarose plugs were then washed three times with 10 ml of Tris EDTA (TE) buffer (Sec. 3.8) for 30 min at room temperature and transferred to a tube containing TE buffer placed in a refrigerator at 4 °C until use.

DNA from the S. aureus strain NCTC 8325 used as molecular size marker was also prepared following the same protocol.

3.12.3 DIGESTION (MRSA strains and NCTC 8325)

For restriction endonuclease digestion, approximately 1 to 1.5 mm of a plug was cut and incubated with 250 μl of restriction buffer containing 20U of Sma-I (Bangalore Genei Pvt. Ltd. India) at 25 °C for 4 h.

3.12.3 PFGE

After DNA digestion, the agarose plugs were incubated with 1 ml of TE buffer at 37 °C for 1 h. The plugs were then inserted into 1% agarose gel in 0.5X TBE buffer, and restriction fragments were separated using a contour-clamped homogeneous electric field system (CHEF-DRII; Bio-Rad, Laboratories). Electrophoresis was performed using the following conditions:

Block 1: initial switch time - 5 sec; final switch time - 15 sec; run time – 10 h; volatage – 200V or 6 V/cm. Block 2: initial switch time - 15 sec, final switch time - 60 sec. run time –13 h, voltage – 20V or 6 V/cm.

3.12.4 STAINING, VISUALIZING AND PHOTOGRAPHING GEL, AND STORING IMAGE

- Stain gel for 30-45 min in ethidium bromide (1 μg/ml).
- View under UV transillumination and photograph using gel documentation system.
**Important:** Zoom to maximum to include the whole width (20 cm) of the gel and save the image as tagged image file format (TIFF) file for analysis with BioNumerics version 2.5 software (Applied Maths, Kortrijk, Belgium). Using the BioRad camera gel documentation system this correspond to a file size of 768 x 574 pixels. This allows a resolution of tracks = 500 pixels or 100 dpi.

### 3.13 POLYACRYLAMIDE GEL ELECTROPHORESIS

In order to study the whole-cell protein banding patterns of MRSA isolates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 12% acrylamide - 0.15% bisacrylamide in the running gel (13 x 18 cm). The stacking gel was composed of 5% acrylamide-0.07% bisacrylamide, 0.15 M Tris hydrochloride (pH 8.8), 0.1% SDS, 0.05% TEMED, and 0.1% ammonium persulfate.

Bacterial suspensions of 5 µl equivalent to a McFarland 0.5 standard were added to 45 µl sample loading buffer containing 0.05M Tris hydrochloride (pH 6.8), 1.6% SDS, 0.04% bromophenol blue, and 27% glycerol and were boiled for 30 min.

Samples were loaded to the gel lanes and run through the stacking gel at 75 mV and then electrophoresed through the running gel at 120 mV for 45 to 60 min past the time that the leading edge had passed the bottom of the running gel. The gels were stained with Coomassie brilliant blue and destained overnight with methanol-acetic acid-water (30:5:65), and photographed using Bio-Rad gel documentation system.

### 3.14 STATISTICAL ANALYSIS

Statistical analysis was done with the help of Windows based Graphical Software SPSS-11. Comparison between two groups of data was performed using Student's *t* test. Comparison of percentages between groups was made with the Chi-square Test using Asymptotic Exact Test mode.
4.1 BIOCHEMICAL CHARACTERISTICS

Of the 850 clinical isolates of Staphylococci, 513 were identified as S. aureus, 312 as S. epidermidis and 25 as other coagulase-negative Staphylococci (other CNS) on the basis of standard biochemical tests (Table 4.1). Pigmentation in different shades of cream-yellow, yellow, yellow-orange was observed in 484 stains (56.9%) on nutrient agar after the 48 h incubation while as 366 (43.1%) showed no pigmentation (Chi-square, 0.0; degree of freedom, 5, asymptotic significance, 1.0). Although all clinical isolated studied grew on mannitol salt agar but only 480 (56.5%) isolates showed heamolysis on 5% sheep blood agar plates after 18-24 h incubation at 35-37 °C. Of the 513 clinical isolates of S. aureus, all were positive for staphylocoagulase, mannitol fermentation and DNase but only 499 (58.7%) showed the presence of clumping factor (slide coagulase test). Out of 850 only 22 strains (2.6%) were negative for alkaline phosphatase and 25 were positive for novobiocin resistance. So 25 strains were classified as other CNS. As for as the β-lactamase test is concerned, 762 (89.6%) strains were positive and 88 (10.4%) were negative for this enzyme in the present study (Chi-square, 0.0; degree of freedom, 5, asymptotic significance, 1.0). 80% (40/50) isolates from normal eyes were β-lactamase +ve as compared to 83.3% (100/220) of clinical strains isolated from diseased eyes. Nose of hospital workers contributed 88.8% (111/125) of staphylococci positive to β-lacatamase as compared to 90% (90/100) isolated from nose of patients. In case of other clinical sources of patients like pus, urine, blood, CSF, semen etc. the staphylococcal isolates positive to β-lactamase were shown to be 92.5% (421/455).

4.2 AGE AND GENDER DISTRIBUTION

Of the 750 subjects studied the male: female ratio was 52.5:47.5, of whom 175 were normal hospital workers (Table 4.2). The age range was 5 to 75
years. Of these 12 cases of mastitis belonged exclusively to females and 18 cases of prostatitis to men. Of patients having wound infections (n=122), the highest prevalence of staphylococci (27%; 33/122) was found in the age group of 45-64 years and so was the case with patients having urinary tract infections (32.6%; 29/89). (Chi-square, 3.286; degree of freedom, 10; asymptotic significance, 0.794) In this study staphylococcal conjunctivitis was found equally prominent in the age group of 5-14 (23.1%; 12/52) and 45-64 (23.1%; 12/52) years. Of the 175 normal hospital workers, the highest number of carrier staphylococci (48%; 84/175) was isolated from the subjects having age between 25 and 44 years while as the age group 5-14 years contributed none. (Chi-square, 3.143; degree of freedom, 9.0; asymptotic significance, 0.958). In the present study 52 subjects were lacking the information regarding their age and so were categorized as unknown age group.

4.3 FREQUENCY OF STAPHYLOCOCCAL ISOLATES AMONG CLINICAL SOURCES

Of the 850 clinical strains of staphylococci studied, 513 (60.3%) were Staphylococcus aureus and 337 (39.7%) coagulase-negative staphylococci (Table 4.3). Of these 180 (35.1%) were methicillin-resistant Staphylococcus aureus (MRSA) and 76 (22.5%) were methicillin-resistant coagulase-negative staphylococci (MRCNS). The highest number of S. aureus strains was isolated from pus (22.7%; 115/513) followed by urine (14.8%, 76/513) and blood (11.3%; 58/513). Likewise, the highest number of coagulase-negative staphylococcal isolates was found in urine (22.8%, 77/337) followed by conjunctivitis (16.3%; 55/337) and pus (10.7%; 36/337). Of the 225 nasal swabs of hospital workers and patients studied, 175 (34.1%) were S. aureus and 50 (14.8%) were coagulase-negative staphylococci, while as from ocular swabs (n=50) of hospital workers all the strains studied were coagulase-negative staphylococci. Of the 180 MRSA strains the highest number was found in pus (35.5%; 64/180) followed by urine (16.1%; 29/180) and blood (9.4%; 17/180). (Chi-square, 3.0; degree of freedom, 8.0; asymptotic significance, 0.934). In case of nasal swabs of hospital workers and patients (n=225) the frequency of MRSA isolates was 28.9% (52/180) that is
equivalent to MRSA carrier rate of 23.1% (52/225). Of the 76 MRCNS studied the highest number was obtained from urine (25.0%; 19/76) followed by conjunctiva (18.4%; 14/76) and nasal swabs of patients having ocular infections (14.5%; 11/76), while as 10.5% (8/76) of MRCNS were isolated from ocular swabs of hospital workers. (Chi-square, 1.5; degree of freedom, 8.0; asymptotic significance, 0.993)

**4.4 RESISTANCE PROFILES OF STAPHYLOCOCCAL ISOLATES**

The resistance patterns of staphylococcal isolates (n=850) to 18 antimicrobial agents were shown in Table 4.4. Vancomycin was shown to be the most effective drug. In case of *Staphylococcus aureus* (n=513), the highest resistance was observed to penicillin G (92.0%) followed by ampicillin (80.5%) and co-trimoxazole (50.0%). Though the highest resistance in coagulase-negative staphylococci (n=337) was shown to penicillin G (89.9%) but it was lesser by (2.1%) as compared to its resistance in *Staphylococcus aureus*, followed by the same pattern as to ampicillin (71.5%) and co-trimoxazole (40.3%).

As far as the clinical versus carrier staphylococcal isolates are concerned the general observation was that the resistance in carrier isolates was lesser than clinical strains in case of all antibiotics. In clinical (n=338) and carrier (n=175) isolates of *S. aureus* the highest resistance was found to penicillin G (92.6% versus 90.60%) followed by ampicillin (81.4% versus 78.8%) and co-trimoxazole (51.8% versus 46.3%). respectively. The least resistance followed to vancomycin and fusidic acid was shown to chloramphenicol (4.7% versus 3.4%), tetracycline (12.7% versus 7.1%) and amikacin (13.9% versus 10.3%) respectively in clinical and carrier isolates of *S. aureus*. Almost similar trend was observed in clinical (n=237) and carrier (n=100) isolates of coagulae-negative staphylococci, with the exception that in carrier isolates zero resistance to amikacin was seen.

**4.5 RESISTANCE PATTERNS OF METHICILLIN-RESISTANT AND METHICILLIN-SENSITIVE STAPHYLOCOCCAL ISOLATES**

The comparative resistance patterns of methicillin-resistant and methicillin-sensitive staphylococcal isolates were shown in Table 4.5. The susceptibility of 180 MRSA and 76 MRCNS strains to 17 antimicrobial agents showed that
vancomycin was the most effective drug. Resistance to penicillin and cephalaxin in case of MRSA, penicillin, cephelexin, ampicillin, cefazolin in case of MRCNS was shown to be 100%. Although methicillin resistant-staphylococci are clinically thought to be resistant to all β-lactams and cephalosporins but these in vitro studies showed that 1.1%, 5.6% and 3.9% MRSA strains showed susceptibilities to ampicillin, cefazolin, cephotoxime and imipenem respectively. No MRCNS strain showed susceptibility to any of the β-lactam or cephalosporin antibiotics assayed except imipenem. There was no appreciable difference of resistance to amikacin (36.1% versus 35.5%) and gentamicin (84.4% versus 85.5) between MRSA and MRCNS isolates respectively. But chloramphenicol and clindamycin resistance varied by 6% and 11.3% in isolated strains of MRSA and MRCNS respectively.

In case of 333 MSSA and 261 MSCNS isolates, vancomycin, methicillin and amikacin were shown to be most effective drugs as compared to penicillin G, ampicillin and co-trimoxazole, which showed least effectiveness. There was a significant decrease in resistance frequency against 17 antimicrobials in case of methicillin sensitive staphylococcal isolates. The appreciable difference of resistance to ampicillin (70.6% versus 63.2%); cephotoxime (10.2% versus 3.8); ciprofloxacin (8.1% versus 15.7%); and roxithromycin (6.0% versus 0.4%), was observed respectively among MRSA and MRCNS strains in the present study.

In addition the comparative resistance patterns of carrier staphylococcal isolates from eyes and nose of patients and hospital workers, depicted in Table 4.6, revealed that the resistance shown to chloramphenicol, (15.6%, nasal MRSA versus ocular MRSE; 5%, nasal MRSE versus ocular MRSE; 1.6%, nasal MSSA versus ocular MSSE; 0.2%, nasal MSSE versus ocular MSSE); ciprofloxacin (11.3%, nasal MRSA versus ocular MRSE; 10% nasal MRSE versus ocular MRSE; 12.7%, nasal MSSE versus ocular MSSE; 3.2% nasal MSSE versus ocular MSSE); and gentamicin (4.8%, nasal MRSA versus ocular MRSE; 27.5%, nasal MRSE versus ocular MRSE; 3.8%, nasal MSSE versus ocular MSSE; 0.6% nasal MSSE versus ocular MSSE); was
higher in case of both methicillin-resistant and methicillin-sensitive ocular isolates as compared to nasal isolates.

4.6 COMPARATIVE RESISTANCE PATTERNS OF METHICILLIN-RESISTANT AND METHICILLIN-SENSITIVE STAPHYLOCOCCAL CLINICAL AND CARRIER ISOLATES

All the 18 antimicrobials assayed against both clinical and carrier MRSA and MSSA isolated depicted that clinical isolates were showing a bit higher frequency of resistance than carrier isolates with few exceptions. As shown in Table 4.7 and Fig 4.7, the resistance to amikacin, with respect to clinical and carrier MRSA was 36.7% versus 34.6%; cefazolin (93.0% versus 48.11%); chloramphenicol (11.7% versus 9.6%); and tetracycline (30.5% versus 28.8%), respectively. However, in case of resistance to ampicillin (98.4% versus 100.0%); co-trimoxazole (96.9% versus 100.0%); and gentamicin (85.2% versus 82.7%), the values were fractionally less with respect to clinical and carrier MRSA isolates, respectively. In case of MRCNS clinical and carrier isolates the resistance to A, Cz, Cp, Ce, I and P was almost having the same frequency, while resistance frequency to other antibiotics was outweighed by clinical MRCNS to carrier MRNS, with few exceptions, respectively. The same trend was observed when clinical and carrier MSSA and MSCNS were observed for their frequency of resistance to antibiotics assayed.

4.7 COMPARATIVE MULTI-DRUG RESISTANCE PATTERNS OF METHICILLIN-RESISTANT AND METHICILLIN-SENSITIVE STAPHYLOCCAL CLINICAL AND CARRIER ISOLATES

Resistance to 4 antibiotics or more, was observed in both S. aureus and Coagulase-negative staphylococci (Table 4.8, Fig 4.9). In case of MRSA isolates, no strain showed resistance to 5 or less than 5 antibiotics, while as all MRCNS strains showed resistance to 8 or more than 8 antibiotics (Table 4.8, Fig 4.9). As for as the clinical and carrier MRSA are concerned, in the present study, both types of strains showed resistance to 6 or more than 6 antibiotics assayed (Table 4.9, Fig 4.10). In case of clinical and carrier MSSA isolates, no strain showed resistance to more than 7 antibiotics.
Almost same trend of multidrug resistance patterns was observed in case of clinical and carrier, MRCNS and MSCNS isolates from eye and other clinical sources in health and diseases (Table 4.9, Fig 4.11).

### 4.8 ANTIBIOTIC RESISTANCE PROFILES OF MRSA ISOLATES IN AN ORTHOPEDIC SURGICAL WARD

In this study, 61 isolates turned to be MRSA, were isolated in an Orthopaedic Surgical Ward from wound infections (n=56); their nose (n=50); and nose of hospital workers attending wound infections (n=50) according to the method described in Section 3.3 of Chapter 3. As depicted in Table 4.10, only 3 MRSA isolates (JMR-8, 31, 56) showed resistance to minimum of 6 antibiotics; and one strain showed resistance to maximum of 16 antibiotics (JMR-41), and two strains showed resistance to maximum of 17 antibiotics (JMR-28, 60) assayed in the present study. Antibiotic resistance patterns, 3, 24, and 36 were the most common profiles found by 4 strain each in this study. Some strains from Table 4.10 were selected for studying antibiotic resistance transfer mechanisms.
Table 4.1 Biochemical Characteristics of Staphylococci (n=850) Isolated from Eyes and Other Clinical Sources in Health and Disease

<table>
<thead>
<tr>
<th>Clinical Source</th>
<th>Colony Characteristics</th>
<th>Biochemical Characteristics</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pigmentation</td>
<td>Hemolysis</td>
<td>Mannitol Salt Agar growth</td>
</tr>
<tr>
<td>Normal eye (n=50)</td>
<td>+</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>Diseased eye (n=120)</td>
<td>25</td>
<td>95</td>
<td>27</td>
</tr>
<tr>
<td>Nose of hospital workers (n=125)</td>
<td>119</td>
<td>6</td>
<td>115</td>
</tr>
<tr>
<td>Nose of patients (n=100)</td>
<td>45</td>
<td>55</td>
<td>48</td>
</tr>
<tr>
<td>Other clinical sources* (n=455)</td>
<td>295</td>
<td>160</td>
<td>290</td>
</tr>
<tr>
<td>Total (n=850)</td>
<td>484</td>
<td>366</td>
<td>480</td>
</tr>
</tbody>
</table>

*aPigmentation in different shades of cream-yellow, yellow, yellow-orange or orange was observed on nutrient agar and blood agar after the 48 h incubation.

*bHemolysis was seen on 5-10% sheep blood agar.

*cClinical sources include pus, urine, blood, cerebrospinal fluid, semen, catheter tip etc.
Table 4.2 Age and Gender Distribution of Staphylococcal Infected/Colonized Subjects (n=750) According to Clinical Diagnosis

<table>
<thead>
<tr>
<th>Clinical Diagnosis</th>
<th>Age (y)</th>
<th>5-14 n=63 (%)</th>
<th>15-24 n=133 (%)</th>
<th>25-44 n=224 (%)</th>
<th>45-64 n=182 (%)</th>
<th>&gt;65 n=96 (%)</th>
<th>Unknown* n=52 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Wound infection</td>
<td>8 (12.7)</td>
<td>5 (7.9)</td>
<td>16 (12.0)</td>
<td>14 (10.5)</td>
<td>16 (7.1)</td>
<td>7 (3.1)</td>
<td>17 (9.3)</td>
</tr>
<tr>
<td>n=122 (16.2)</td>
<td></td>
<td></td>
<td>n=67 (50.4)</td>
<td>Female n=66 (49.6)</td>
<td>n=116 (51.8)</td>
<td>n=108 (48.2)</td>
<td>n=101 (55.5)</td>
</tr>
<tr>
<td>Postoperative</td>
<td>1 (1.6)</td>
<td>2 (3.2)</td>
<td>2 (1.5)</td>
<td>1 (0.7)</td>
<td>2 (0.9)</td>
<td>8 (3.6)</td>
<td>4 (2.2)</td>
</tr>
<tr>
<td>infection</td>
<td></td>
<td></td>
<td>n=35 (4.7)</td>
<td></td>
<td>n=35 (4.7)</td>
<td>n=35 (4.7)</td>
<td>n=35 (4.7)</td>
</tr>
<tr>
<td>Bacteraemia</td>
<td>1 (1.6)</td>
<td>1 (1.6)</td>
<td>-</td>
<td>2 (1.5)</td>
<td>4 (1.8)</td>
<td>2 (0.9)</td>
<td>7 (3.8)</td>
</tr>
<tr>
<td>n=25 (3.3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n=25 (3.3)</td>
<td>n=25 (3.3)</td>
<td>n=25 (3.3)</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>5 (7.9)</td>
<td>1 (1.6)</td>
<td>1 (0.7)</td>
<td>-</td>
<td>1 (0.4)</td>
<td>1 (0.4)</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td>n=21 (2.8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n=21 (2.8)</td>
<td>n=21 (2.8)</td>
<td>n=21 (2.8)</td>
</tr>
<tr>
<td>Septicaemia</td>
<td>-</td>
<td>-</td>
<td>1 (0.7)</td>
<td>1 (0.7)</td>
<td>1 (0.4)</td>
<td>2 (0.9)</td>
<td>2 (1.1)</td>
</tr>
<tr>
<td>n=15 (2.0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n=15 (2.0)</td>
<td>n=15 (2.0)</td>
<td>n=15 (2.0)</td>
</tr>
<tr>
<td>Urinary tract</td>
<td>-</td>
<td>1 (1.6)</td>
<td>4 (3.0)</td>
<td>5 (3.8)</td>
<td>10 (4.5)</td>
<td>15 (6.7)</td>
<td>14 (7.7)</td>
</tr>
<tr>
<td>infections</td>
<td></td>
<td></td>
<td>n=89 (11.9)</td>
<td></td>
<td>n=89 (11.9)</td>
<td>n=89 (11.9)</td>
<td>n=89 (11.9)</td>
</tr>
<tr>
<td>Mastitis</td>
<td>-</td>
<td>-</td>
<td>2 (1.5)</td>
<td>-</td>
<td>8 (3.6)</td>
<td>-</td>
<td>2 (1.1)</td>
</tr>
<tr>
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<td></td>
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<td>n=12 (1.6)</td>
<td>n=12 (1.6)</td>
<td>n=12 (1.6)</td>
</tr>
<tr>
<td>Prostatitis</td>
<td>-</td>
<td>-</td>
<td>1 (0.7)</td>
<td>-</td>
<td>5 (2.2)</td>
<td>-</td>
<td>9 (4.9)</td>
</tr>
<tr>
<td>n=18 (2.4)</td>
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<td>n=18 (2.4)</td>
<td>n=18 (2.4)</td>
<td>n=18 (2.4)</td>
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<tr>
<td>Conjunctivitis</td>
<td>7 (11.1)</td>
<td>5 (7.9)</td>
<td>5 (3.8)</td>
<td>4 (3.0)</td>
<td>1 (0.4)</td>
<td>4 (1.8)</td>
<td>7 (3.8)</td>
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<td>n=52 (6.9)</td>
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<td>n=52 (6.9)</td>
<td>n=52 (6.9)</td>
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<tr>
<td>Corneal ulcer</td>
<td>1 (1.6)</td>
<td>-</td>
<td>2 (1.5)</td>
<td>-</td>
<td>2 (0.9)</td>
<td>1 (0.4)</td>
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<tr>
<td>n=20 (2.7)</td>
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<td>n=20 (2.7)</td>
<td>n=20 (2.7)</td>
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<tr>
<td>Endophthalmitis</td>
<td>-</td>
<td>-</td>
<td>1 (0.7)</td>
<td>-</td>
<td>1 (0.4)</td>
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<td>2 (1.1)</td>
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<tr>
<td>n=11 (1.5)</td>
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<td>n=11 (1.5)</td>
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<td>n=11 (1.5)</td>
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<tr>
<td>Other*</td>
<td>8 (12.7)</td>
<td>13 (20.6)</td>
<td>11 (8.3)</td>
<td>13 (9.8)</td>
<td>12 (5.3)</td>
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<td>n=110 (14.7)</td>
<td>n=110 (14.7)</td>
<td>n=110 (14.7)</td>
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<tr>
<td>Normal*</td>
<td>-</td>
<td>-</td>
<td>20 (15.0)</td>
<td>18 (13.5)</td>
<td>56 (25.0)</td>
<td>46 (20.5)</td>
<td>16 (8.8)</td>
</tr>
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<td>n=175 (23.3)</td>
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<td>n=175 (23.3)</td>
<td>n=175 (23.3)</td>
<td>n=175 (23.3)</td>
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<tr>
<td>Unknown*</td>
<td>-</td>
<td>4 (6.3)</td>
<td>3 (2.2)</td>
<td>6 (4.5)</td>
<td>6 (2.7)</td>
<td>5 (2.2)</td>
<td>8 (4.4)</td>
</tr>
<tr>
<td>n=45 (6.0)</td>
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<td></td>
<td>n=45 (6.0)</td>
<td>n=45 (6.0)</td>
<td>n=45 (6.0)</td>
</tr>
</tbody>
</table>
*Age not recorded for 52 subjects.

*bAbscess or osteomyelitis, cystitis, peritonitis, endocarditis, toxic shock syndrome, keratitis, etc.

*cStrains isolated from nose of hospital workers attending wound infections (n=50), nose of hospital workers (n=75) and normal eyes of hospital workers (n=50) respectively in Orthopaedic Surgical Ward of J.N Medical College, A.M.U., Aligarh, S.K. Institute of Medical Sciences Kashmir and Surgical Wards of Institute of Ophthalmology, A.M.U. Aligarh.

dClinical diagnosis not recorded for 45 subjects.
### Table 4.3 Frequency of Staphylococcal Isolates among the Clinical Sources

<table>
<thead>
<tr>
<th>Clinical Source</th>
<th>No. of Staphylococcal Isolates n=850 (100%)</th>
<th>No. of <em>Staphylococcus aureus</em> Isolates N=513 (60.3%)</th>
<th>No. of Coagulase negative Staphylococcal Isolates n=337 (39.7%)</th>
<th>No. of Methicillin-resistant <em>Staphylococcus aureus</em> Isolates n=180 (35.1%)</th>
<th>No. of Methicillin-resistant Coagulase-negative Staphylococcal Isolates n=76 (22.5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conjunctiva</td>
<td>76 (8.9)</td>
<td>21 (4.1)</td>
<td>55 (16.3)</td>
<td>5 (2.8)</td>
<td>14 (18.4)</td>
</tr>
<tr>
<td>Corneal scrapings</td>
<td>29 (3.4)</td>
<td>6 (1.2)</td>
<td>23 (6.8)</td>
<td>2 (1.1)</td>
<td>5 (6.6)</td>
</tr>
<tr>
<td>Vitreous humour</td>
<td>15 (1.8)</td>
<td>3 (0.6)</td>
<td>12 (3.6)</td>
<td>1 (0.5)</td>
<td>3 (3.9)</td>
</tr>
<tr>
<td>Pus*</td>
<td>151 (17.8)</td>
<td>115 (22.4)</td>
<td>36 (10.7)</td>
<td>64 (35.5)</td>
<td>8 (10.5)</td>
</tr>
<tr>
<td>Urine</td>
<td>153 (18.0)</td>
<td>76 (14.8)</td>
<td>77 (22.8)</td>
<td>29 (16.1)</td>
<td>19 (25.0)</td>
</tr>
<tr>
<td>Blood</td>
<td>70 (8.2)</td>
<td>58 (11.3)</td>
<td>12 (3.6)</td>
<td>17 (9.4)</td>
<td>2 (2.6)</td>
</tr>
<tr>
<td>Cerebrospinal fluid</td>
<td>14 (1.6)</td>
<td>4 (0.8)</td>
<td>10 (3.0)</td>
<td>2 (1.1)</td>
<td>4 (5.3)</td>
</tr>
<tr>
<td>Semen</td>
<td>18 (2.1)</td>
<td>18 (3.5)</td>
<td>-</td>
<td>3 (1.7)</td>
<td>-</td>
</tr>
<tr>
<td>Catheter tip</td>
<td>18 (2.1)</td>
<td>16 (3.1)</td>
<td>2 (0.6)</td>
<td>2 (1.1)</td>
<td>-</td>
</tr>
<tr>
<td>Other*</td>
<td>31 (3.6)</td>
<td>21 (4.1)</td>
<td>10 (3.0)</td>
<td>3 (1.7)</td>
<td>2 (2.6)</td>
</tr>
<tr>
<td>Nasal swabs*</td>
<td>225 (26.5)</td>
<td>175 (34.1)</td>
<td>50 (14.8)</td>
<td>52 (28.9)</td>
<td>11 (14.5)</td>
</tr>
<tr>
<td>Ocular swabs*</td>
<td>50 (5.9)</td>
<td>-</td>
<td>50 (14.8)</td>
<td>-</td>
<td>8 (10.5)</td>
</tr>
</tbody>
</table>

*Strains isolated from wound exudate, ulcer, abscess etc.
*Sputum or tracheal aspirate, ear fluid, drained fluid, tissue sample, vaginal swabs etc.
*Strains isolated from nose of patients having wound infections and hospital workers attending wound infections n=(50+50), nose of hospital workers (n=75) and nose of patients having eye infections (n=50) respectively in Orthopaedic Surgical Ward of J.N. Medical College, A.M.U. Aligarh, S.K. Institute of Medical Sciences Kashmir and Microbiology Section, Institute of Ophthalmology, A.M.U., Aligarh.
*Strains isolated from normal eyes of hospital workers of Institute of Ophthalmology, A.M.U., Aligarh.
Table 4.4 Resistance Profiles of Staphylococcal Isolates (n=850) from Eyes and Other Clinical Sources in Health and Disease

<table>
<thead>
<tr>
<th>Antimicrobial Agent (n=18)</th>
<th>Staphylococcus aureus n=513 (%)</th>
<th>Clinical S. aureus n=338 (%)</th>
<th>Carrier S. aureus n=175 (%)</th>
<th>Coagulase-negative Staphylococci (CNS) n=337 (%)</th>
<th>Clinical CNS n=237 (%)</th>
<th>Carrier CNS n=100 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>65 (12.7)</td>
<td>47 (13.9)</td>
<td>18 (10.3)</td>
<td>27 (8.0)</td>
<td>27 (11.4)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>413 (80.5)</td>
<td>275 (81.4)</td>
<td>138 (78.8)</td>
<td>241 (71.5)</td>
<td>183 (77.2)</td>
<td>58 (58.0)</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>190 (37.0)</td>
<td>131 (38.8)</td>
<td>59 (33.7)</td>
<td>80 (23.7)</td>
<td>64 (27.0)</td>
<td>16 (16.0)</td>
</tr>
<tr>
<td>Cephalixin</td>
<td>237 (46.2)</td>
<td>165 (48.8)</td>
<td>72 (41.1)</td>
<td>86 (25.5)</td>
<td>68 (28.7)</td>
<td>18 (18.0)</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>207 (40.3)</td>
<td>145 (42.9)</td>
<td>62 (35.4)</td>
<td>78 (23.2)</td>
<td>68 (28.7)</td>
<td>16 (16.0)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>22 (4.3)</td>
<td>16 (4.7)</td>
<td>6 (3.4)</td>
<td>17 (5.0)</td>
<td>12 (5.1)</td>
<td>5 (5.0)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>103 (20.1)</td>
<td>81 (24.0)</td>
<td>22 (12.6)</td>
<td>75 (22.2)</td>
<td>56 (23.6)</td>
<td>19 (19.0)</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>79 (15.4)</td>
<td>60 (17.7)</td>
<td>19 (10.9)</td>
<td>71 (21.1)</td>
<td>56 (23.6)</td>
<td>15 (15.0)</td>
</tr>
<tr>
<td>Co-trimoxazole&lt;sup&gt;a&lt;/sup&gt;</td>
<td>256 (50.0)</td>
<td>175 (51.8)</td>
<td>81 (46.3)</td>
<td>136 (40.3)</td>
<td>104 (43.9)</td>
<td>32 (32.0)</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>18 (3.5)</td>
<td>14 (4.1)</td>
<td>4 (2.3)</td>
<td>12 (3.6)</td>
<td>8 (3.4)</td>
<td>4 (4.0)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>172 (33.5)</td>
<td>122 (36.1)</td>
<td>50 (28.6)</td>
<td>90 (26.7)</td>
<td>72 (30.4)</td>
<td>18 (18.0)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>136 (26.5)</td>
<td>97 (28.7)</td>
<td>39 (23.3)</td>
<td>68 (20.2)</td>
<td>54 (22.8)</td>
<td>14 (14.0)</td>
</tr>
<tr>
<td>Methicillin&lt;sup&gt;b&lt;/sup&gt;</td>
<td>180 (35.1)</td>
<td>128 (37.9)</td>
<td>52 (29.7)</td>
<td>76 (22.5)</td>
<td>63 (26.6)</td>
<td>13 (13.0)</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>472 (92.0)</td>
<td>313 (92.6)</td>
<td>159 (90.9)</td>
<td>303 (89.9)</td>
<td>222 (93.7)</td>
<td>81 (81.0)</td>
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<tr>
<td>Rifampin</td>
<td>97 (18.9)</td>
<td>68 (20.1)</td>
<td>29 (16.6)</td>
<td>41 (12.2)</td>
<td>33 (13.9)</td>
<td>8 (8.0)</td>
</tr>
<tr>
<td>Roxithromycin</td>
<td>146 (24.6)</td>
<td>103 (30.5)</td>
<td>43 (24.6)</td>
<td>53 (15.7)</td>
<td>48 (20.2)</td>
<td>8 (8.0)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>61 (11.9)</td>
<td>43 (12.7)</td>
<td>18 (10.3)</td>
<td>24 (7.1)</td>
<td>21 (8.9)</td>
<td>3 (3.0)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>


<sup>b</sup>Atleast one antimicrobial agent assayed from a particular class of antibiotics.

<sup>c</sup>The intermediate resistant category was included in sensitive category.

<sup>d</sup>(trimethoprim/sulphamethoxazole).

<sup>e</sup>Methicillin resistance was confirmed by Oxacillin Salt-agar screening – plate procedure.
Table 4.5 Comparative Resistance Patterns * of Methicillin-resistant and Methicillin-sensitive Staphylococcal Isolates (n=850) from Eyes and other Clinical Sources in Health and Disease

<table>
<thead>
<tr>
<th>Antimicrobial Agent (n=18)⁵</th>
<th>Staphylococcus aureus</th>
<th>Congulase-negative Staphylococci</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MRSA N=180 (%)</td>
<td>MSSA n=333 (%)</td>
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<tr>
<td>Amikacin</td>
<td>65 (36.1)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>178 (98.9)</td>
<td>235 (70.6)</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>170 (94.4)</td>
<td>20 (6.0)</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>180 (100.0)</td>
<td>57 (17.1)</td>
</tr>
<tr>
<td>Cephotaxime</td>
<td>173 (96.1)</td>
<td>34 (10.2)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>20 (11.1)</td>
<td>2 (0.6)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>76 (42.2)</td>
<td>27 (8.1)</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>65 (36.1)</td>
<td>14 (4.2)</td>
</tr>
<tr>
<td>Co-trimoxazole¹</td>
<td>176 (97.8)</td>
<td>80 (24.0)</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>16 (8.9)</td>
<td>2 (0.6)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>152 (84.4)</td>
<td>20 (6.0)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>126 (70.0)</td>
<td>10 (3.0)</td>
</tr>
<tr>
<td>Methicillin</td>
<td>180 (100.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>180 (100.0)</td>
<td>292 (87.7)</td>
</tr>
<tr>
<td>Rifampin</td>
<td>90 (50.0)</td>
<td>7 (2.1)</td>
</tr>
<tr>
<td>Roxithromycin</td>
<td>126 (70.0)</td>
<td>20 (6.0)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>54 (30.0)</td>
<td>7 (2.1)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>


⁵Atleast one antimicrobial agent assayed from a particular class of antibiotics.

The intermediate resistant category was included in either resistant or sensitive depending upon the closeness of diameter of zone of inhibition to either of the respective categories.

¹Trimethoprim/sulphamethoxazole
Table 4.6 Comparative Resistance Patterns * of Methicillin-resistant and Methicillin-sensitive Staphylococcal Isolates (n=225) from Nose and Eyes of Patients and Hospital Workers

<table>
<thead>
<tr>
<th>Antimicrobial Agent (n=18)</th>
<th>Resistant Staphylococci (%)</th>
<th>Resistant Staphylococci (%)</th>
<th>Resistant Staphylococci (%)</th>
<th>Resistant Staphylococci (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nasal&lt;sup&gt;a&lt;/sup&gt; Staphylococcus aureus (n=175)</td>
<td>Nasal&lt;sup&gt;a&lt;/sup&gt; Staphylococcus epidermidis (n=50)</td>
<td>Ocular&lt;sup&gt;a&lt;/sup&gt; Staphylococcus epidermidis (n=50)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRSA n=52 (%)</td>
<td>MSSA n=123 (%)</td>
<td>MRSE n=5 (%)</td>
<td>MSSE n=45 (%)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>18 (34.6)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>52 (100.0)</td>
<td>86 (69.9)</td>
<td>5 (100.0)</td>
<td>20 (44.4)</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>51 (98.1)</td>
<td>8 (6.5)</td>
<td>5 (100.0)</td>
<td>2 (4.4)</td>
</tr>
<tr>
<td>Cephalaxin</td>
<td>52 (100.0)</td>
<td>20 (16.3)</td>
<td>5 (100.0)</td>
<td>3 (6.7)</td>
</tr>
<tr>
<td>Cephotaxime</td>
<td>50 (96.1)</td>
<td>12 (9.7)</td>
<td>5 (100.0)</td>
<td>2 (4.4)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>5 (9.6)</td>
<td>1 (0.8)</td>
<td>1 (20.0)</td>
<td>1 (2.2)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>20 (38.7)</td>
<td>2 (1.6)</td>
<td>2 (40.0)</td>
<td>5 (11.1)</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>17 (32.7)</td>
<td>2 (1.6)</td>
<td>2 (40.0)</td>
<td>4 (8.9)</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>52 (100.0)</td>
<td>29 (23.6)</td>
<td>5 (100.0)</td>
<td>10 (22.2)</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>3 (5.8)</td>
<td>1 (0.8)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>43 (82.7)</td>
<td>7 (5.7)</td>
<td>3 (60.0)</td>
<td>4 (8.9)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>36 (69.2)</td>
<td>3 (2.4)</td>
<td>4 (80.0)</td>
<td>2 (4.4)</td>
</tr>
<tr>
<td>Methicillin</td>
<td>52 (100.0)</td>
<td>0 (0.0)</td>
<td>5 (100.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>52 (100.0)</td>
<td>107 (87.0)</td>
<td>52 (100.0)</td>
<td>34 (75.5)</td>
</tr>
<tr>
<td>Rifampin</td>
<td>26 (50.0)</td>
<td>3 (2.4)</td>
<td>3 (60.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Roxithromycin</td>
<td>36 (69.2)</td>
<td>7 (5.7)</td>
<td>1 (20.0)</td>
<td>1 (2.2)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>15 (28.8)</td>
<td>3 (2.4)</td>
<td>1 (20.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>

<sup>b</sup>Atleast one antimicrobial agent assayed from a particular class of antibiotics.
<sup>c</sup>Strains isolated from nose of patients having wound infections and hospital workers attending wound infections n=(50+50), and nose of hospital workers (n=75) and nose of patients having eye infections n=50 respectively in Orthopaedic Surgical Ward of J.N. Medical College, A.M.U., Aligarh, and S. K. Institute of Medical Sciences Kashmir and at Microbiology Section, Institute of Ophthalmology A.M.U., Aligarh.
<sup>d</sup>Strains isolated from nose of patients having eye infections (n=50)
<sup>e</sup>Strains isolated from normal eyes of hospital workers of Institute of Ophthalmology A.M.U., Aligarh.
Table 4.7 Comparative Resistance Patterns\(^{a}\) of Methicillin – resistant and Methicillin-snsitive Staphylococcal Clinical and Carrier Isolates

<table>
<thead>
<tr>
<th>Antimicrobial Agents (n=18)(^{b})</th>
<th>Staphylococci aureus (n=513)</th>
<th>Resistant Staphylococci (%)</th>
<th>Coagulase-negative Staphylococci (n=377)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clinical(^{c}) (n=338)</td>
<td>Carrier(^{d}) (n=175)</td>
<td>Clinical(^{e}) (n=237)</td>
</tr>
<tr>
<td></td>
<td>MRSA N=128 (%)</td>
<td>MSSA n=210 (%)</td>
<td>MRSA n=52 (%)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>47 (36.7)</td>
<td>0 (0.0)</td>
<td>18 (34.6)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>126 (98.4)</td>
<td>149 (70.9)</td>
<td>52 (100.0)</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>119 (93.0)</td>
<td>12 (5.7)</td>
<td>51 (48.1)</td>
</tr>
<tr>
<td>Cephalaxin</td>
<td>128 (100.0)</td>
<td>37 (17.6)</td>
<td>52 (100.0)</td>
</tr>
<tr>
<td>Cephotaxime</td>
<td>123 (96.1)</td>
<td>22 (10.5)</td>
<td>50 (96.1)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>15 (11.7)</td>
<td>1 (0.5)</td>
<td>5 (9.6)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>56 (43.7)</td>
<td>25 (11.9)</td>
<td>20 (38.7)</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>48 (37.5)</td>
<td>12 (5.7)</td>
<td>17 (32.7)</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>124 (96.9)</td>
<td>51 (24.3)</td>
<td>52 (100.0)</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>12 (9.2)</td>
<td>2 (0.9)</td>
<td>4 (7.7)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>109 (85.2)</td>
<td>13 (6.2)</td>
<td>43 (82.7)</td>
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<tr>
<td>Imipenem</td>
<td>90 (70.3)</td>
<td>7 (3.3)</td>
<td>36 (69.2)</td>
</tr>
<tr>
<td>Methicillin</td>
<td>128 (100.0)</td>
<td>0 (0.0)</td>
<td>52 (100.0)</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>128 (100.0)</td>
<td>183 (88.1)</td>
<td>52 (100.0)</td>
</tr>
<tr>
<td>Rifampin</td>
<td>64 (50.0)</td>
<td>4 (1.9)</td>
<td>26 (50.0)</td>
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<tr>
<td>Roxithromycin</td>
<td>90 (70.3)</td>
<td>13 (6.2)</td>
<td>36 (69.2)</td>
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<tr>
<td>Tetracycline</td>
<td>39 (30.5)</td>
<td>4 (1.9)</td>
<td>15 (28.8)</td>
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<tr>
<td>Vancomycin</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>


\(^{b}\)At least one antimicrobial agent was assayed from a particular class of antibiotics.

\(^{c}\)Strains isolated from clinical sources of patients (n=338) having different types of diseases/infections

\(^{d}\)Strains isolated from nose of the patients having wound infection and the hospital workers attending wound infections n=(50+50), and nose of hospital workers (n=75) respectively in Orthopedic Surgical Ward of J.N. Medical College, A.M.U. Aligarh and S.K. Institute of Medical Sciences, Kashmir

\(^{e}\)Strains isolated from symptomatic eyes and other clinical sources of the patients having different diseases/infections.

\(^{f}\)Strains isolated from nose of the patients having eye infections (n=50) and eyes of the hospital workers (n=50) at Microbiology Section, Institute of Ophthalmology, J.N. Medical College, A.M.U., Aligarh.
Table 4.8 Multidrug Resistance in Staphylococci (n=850) Isolated from Eyes and Other Clinical Sources in Health and Disease

<table>
<thead>
<tr>
<th>No. of Antimicrobial Agents(^a)</th>
<th>S. aureus n=513 (%)</th>
<th>Coagulase-negative Staphylococci n=337 (%)</th>
<th>Resistant(^b) Staphylococci (%)</th>
<th>MRSA n=180 (%)</th>
<th>MSSA n=333 (%)</th>
<th>MRCNS n=76 (%)</th>
<th>MSCNS n=261 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>41 (8.0)</td>
<td>34 (10.1)</td>
<td>0 (0.0)</td>
<td>41 (12.3)</td>
<td>0 (0.0)</td>
<td>34 (13.0)</td>
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<tr>
<td>1</td>
<td>59 (11.5)</td>
<td>62 (18.4)</td>
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<td>59 (17.7)</td>
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<td>62 (23.7)</td>
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</tr>
<tr>
<td>2</td>
<td>157 (30.6)</td>
<td>105 (31.2)</td>
<td>0 (0.0)</td>
<td>157 (47.1)</td>
<td>0 (0.0)</td>
<td>105 (40.2)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>19 (3.6)</td>
<td>46 (13.6)</td>
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<td>19 (5.2)</td>
<td>0 (0.0)</td>
<td>46 (17.6)</td>
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</tr>
<tr>
<td>4</td>
<td>30 (5.8)</td>
<td>4 (1.2)</td>
<td>0 (0.0)</td>
<td>30 (9.0)</td>
<td>0 (0.0)</td>
<td>4 (1.5)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>17 (3.3)</td>
<td>2 (0.6)</td>
<td>0 (0.0)</td>
<td>17 (5.1)</td>
<td>0 (0.0)</td>
<td>2 (0.8)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>10 (1.9)</td>
<td>4 (1.2)</td>
<td>3 (1.7)</td>
<td>7 (2.1)</td>
<td>0 (0.0)</td>
<td>4 (1.5)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>8 (1.6)</td>
<td>4 (1.2)</td>
<td>5 (2.8)</td>
<td>3 (0.9)</td>
<td>0 (0.0)</td>
<td>4 (1.5)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>26 (5.1)</td>
<td>1 (0.3)</td>
<td>26 (14.4)</td>
<td>0 (0.0)</td>
<td>1 (1.3)</td>
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<td>9</td>
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<td>4 (1.2)</td>
<td>10 (5.5)</td>
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<td>4 (5.3)</td>
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<tr>
<td>10</td>
<td>33 (6.4)</td>
<td>3 (0.9)</td>
<td>33 (18.3)</td>
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<td>3 (3.9)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
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<td>6 (3.3)</td>
<td>0 (0.0)</td>
<td>12 (15.8)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>18 (3.5)</td>
<td>15 (4.4)</td>
<td>18 (10.6)</td>
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<td>15 (19.7)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>13</td>
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<td>14 (4.1)</td>
<td>14 (7.8)</td>
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<td>14 (18.2)</td>
<td>0 (0.0)</td>
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</tr>
<tr>
<td>14</td>
<td>4 (0.8)</td>
<td>3 (0.9)</td>
<td>4 (2.2)</td>
<td>0 (0.0)</td>
<td>3 (3.9)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>39 (7.6)</td>
<td>7 (2.1)</td>
<td>39 (21.7)</td>
<td>0 (0.0)</td>
<td>7 (9.2)</td>
<td>0 (0.0)</td>
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</tr>
<tr>
<td>16</td>
<td>4 (0.8)</td>
<td>5 (1.5)</td>
<td>4 (2.2)</td>
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<td>5 (6.6)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>18 (3.5)</td>
<td>12 (3.6)</td>
<td>18 (10.6)</td>
<td>0 (0.0)</td>
<td>12 (15.8)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Multidrug resistance assayed for 18 antimicrobial agents representing different classes of antibiotics against Staphylococcal isolates

\(^b\)Number of staphylococcal isolates resistant to multidrugs was determined in accordance to Performance Standards for Antimicrobial Disk Susceptibility Tests NCCLS (1998, 1999, 2000, 2002).

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### Figure 4.9 Multidrug Resistance of Methicillin-resistant and Methicillin-sensitive Staphylococcal Clinical and Carrier Isolates

<table>
<thead>
<tr>
<th>No. of Antimicrobial Agents&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Resistant&lt;sup&gt;b&lt;/sup&gt; Staphylococci (%)</th>
<th>Coagulas negative Staphylococci (CNS) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clinical (n=513)</td>
<td>Carrier (n=175)</td>
</tr>
<tr>
<td></td>
<td>MRSA (n=128)</td>
<td>MSSA (n=210)</td>
</tr>
<tr>
<td></td>
<td>MRSA (n=52)</td>
<td>MSSA (n=123)</td>
</tr>
<tr>
<td>0</td>
<td>0 (0.0)</td>
<td>25 (11.9)</td>
</tr>
<tr>
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<td>0 (0.0)</td>
<td>38 (18.1)</td>
</tr>
<tr>
<td>2</td>
<td>0 (0.0)</td>
<td>100 (47.6)</td>
</tr>
<tr>
<td>3</td>
<td>0 (0.0)</td>
<td>10 (4.8)</td>
</tr>
<tr>
<td>4</td>
<td>0 (0.0)</td>
<td>20 (9.5)</td>
</tr>
<tr>
<td>5</td>
<td>0 (0.0)</td>
<td>10 (4.8)</td>
</tr>
<tr>
<td>6</td>
<td>2 (1.6)</td>
<td>5 (2.4)</td>
</tr>
<tr>
<td>7</td>
<td>4 (3.1)</td>
<td>2 (0.9)</td>
</tr>
<tr>
<td>8</td>
<td>15 (11.7)</td>
<td>11 (21.1)</td>
</tr>
<tr>
<td>9</td>
<td>8 (6.2)</td>
<td>2 (3.8)</td>
</tr>
<tr>
<td>10</td>
<td>20 (15.6)</td>
<td>13 (25.0)</td>
</tr>
<tr>
<td>11</td>
<td>5 (3.9)</td>
<td>1 (1.9)</td>
</tr>
<tr>
<td>12</td>
<td>10 (7.8)</td>
<td>8 (15.4)</td>
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<td>13</td>
<td>10 (7.8)</td>
<td>4 (7.7)</td>
</tr>
<tr>
<td>14</td>
<td>3 (2.3)</td>
<td>1 (1.9)</td>
</tr>
<tr>
<td>15</td>
<td>31 (24.2)</td>
<td>8 (15.4)</td>
</tr>
<tr>
<td>16</td>
<td>4 (3.1)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>17</td>
<td>16 (12.5)</td>
<td>2 (3.8)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Multidrug resistance assayed for 18 antimicrobial agents representing different classes of antibiotics against Staphylococcal isolates

<sup>b</sup> Number of staphylococcal isolates resistant to multidrugs was determined in accordance to Performance Standards for Antimicrobial Disk Susceptibility Tests NCCLS (1998, 1999, 2000, 2002).
Table 4.10 Antibiotic Resistance Profiles of 61 Strains of MRSA Isolated in an Orthopaedic Surgical Ward

<table>
<thead>
<tr>
<th>Pattern</th>
<th>Antibiotic Resistance Profiles of MRSA</th>
<th>No. of resistance Markers</th>
<th>Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A,Cz,Ce,Cp,Co,G,I,M,P,R,Ro,T</td>
<td>12</td>
<td>JMR-1</td>
</tr>
<tr>
<td>2</td>
<td>Ak,A,Cz,Ce,Cp,Cf,Cd,Co,G,I,M,P,R</td>
<td>13</td>
<td>JMR-2</td>
</tr>
<tr>
<td>3</td>
<td>Ak,A,Cz,Ce,Cp,Co,G,I,M,P,R</td>
<td>11</td>
<td>JMR-,3,5,5,35,59</td>
</tr>
<tr>
<td>4</td>
<td>A,Cz,Ce,Cp,Cd,Co,G,I,M,P,T</td>
<td>11</td>
<td>JMR-4</td>
</tr>
<tr>
<td>5</td>
<td>A,Cz,Ce,Cp,Cd,Co,G,M,P</td>
<td>9</td>
<td>JMR-5</td>
</tr>
<tr>
<td>6</td>
<td>A,Cz,Ce,Cp,Cf,Cd,Co,G,M,P,R,Ro</td>
<td>12</td>
<td>JMR-,6,38,51</td>
</tr>
<tr>
<td>7</td>
<td>A,Cz,Ce,Cp,Cf,Co,M,P,R, Ro</td>
<td>10</td>
<td>JMR-7</td>
</tr>
<tr>
<td>8</td>
<td>Ce,Cd,Co,M,P,R</td>
<td>6</td>
<td>JMR-8</td>
</tr>
<tr>
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<td>A,Cz,Ce,Cp,Cd,Co,M,P,R</td>
<td>10</td>
<td>JMR-9</td>
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<tr>
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<td>Ak,A,Cz,Ce,Cp,Cf,Co,G,M,P,R,Ro,T</td>
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<td>Ak,A,Cz,Ce,Cp,Co,G,M,P,R, Ro</td>
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<td>JMR-,11,53</td>
</tr>
<tr>
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<td>Ak,A,Cz,Ce,Cf,Cd,Co,G,I,M,P,R,Ro</td>
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<td>JMR-12</td>
</tr>
<tr>
<td>13</td>
<td>A,Cz,Ce,Cp,Cd,Co,G,M,P,R</td>
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<td>JMR-13</td>
</tr>
<tr>
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<td>A,Cz,Ce,Cp,Co,M,P,R</td>
<td>8</td>
<td>JMR-14</td>
</tr>
<tr>
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<td>JMR-,15,58</td>
</tr>
<tr>
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<td>A,Cz,Ce,Cp,Cf,Co,G,M,P</td>
<td>9</td>
<td>JMR-16</td>
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<tr>
<td>17</td>
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<td>JMR-20</td>
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<tr>
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<td>JMR-21</td>
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<tr>
<td>22</td>
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<td>JMR-22</td>
</tr>
<tr>
<td>23</td>
<td>A,Cz,Ce,Cp,Co,G,M,P,R</td>
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<td>JMR-23</td>
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<tr>
<td>24</td>
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<td>JMR-25</td>
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<td>JMR-,26,52</td>
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<tr>
<td>27</td>
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<td>JMR-30</td>
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<tr>
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<td>JMR-41</td>
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</tr>
</tbody>
</table>
Figure 4.1 Distribution of Staphylococcal Isolates Among Infected/Colonized Subjects According to Clinical Diagnosis

Figure 4.2 Distribution of Staphylococcal Isolates Among the Clinical Sources
Figure 4.3 Comparative Resistance Profiles of *Staphylococcus aureus* and Coagulase-negative Staphylococcal Isolates from Eyes and Other Clinical Sources in Health and Disease.

Figure 4.4 Comparative Resistance Patterns of Methicillin-resistant *Staphylococcus aureus* (MRSA) and Methicillin-sensitive *Staphylococcus aureus* (MSSA)
Figure 4.5 Comparative Resistance Patterns of Methicillin-resistant and Methicillin-sensitive Coagulase-negative Staphylococcal Isolates from Eyes and Other Clinical Sources in Health and Disease

Figure 4.6 Comparative Resistance Patterns of Methicillin-resistant Staphylococcus aureus and Methicillin-resistance Coagulase-negative Staphylococcal Isolates from Eyes and Other Clinical Sources in Health and Disease
Figure 4.7 Comparative Resistance Patterns of Methicillin-resistant and Methicillin-sensitive *Staphylococcus aureus* Clinical and Carrier Isolates

Figure 4.8 Comparative Resistance Patterns of Methicillin-resistant and Methicillin-sensitive Coagulase-negative *Staphylococcal* Clinical and Carrier Isolates
Figure 4.9 Occurrence of Multidrug Resistance in Staphylococcal Isolates (n=850) from Eyes and Other Clinical Sources in Health and Disease

Figure 4.10 Occurrence of Multidrug Resistance in Methicillin-resistant and Methicillin-sensitive Staphylococcus aureus Clinical and Carrier Isolates
Figure 4.11 Occurrence of Multidrug Resistance in Methicillin-resistant and Methicillin-sensitive Coagulase-negative Staphylococcal Clinical and Carrier Isolates
4.9 SDS-PAGE WHOLE-CELL PROTEIN PROFILES OF MRSA STRAINS

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 12% acrylamide – 0.15% bisacrylamide in the running gel (13 by 18 cm) (Bangalore Genei Pvt. Ltd. India). The staking gel was composed of 5% acrylamide – 0.07% bisacrylamide. Electrophoresis was carried out according to the method described in Materials and Methods (Section 3.13). In Fig 4.12 lanes 1 and 20 were loaded with protein markers. Whole cell protein samples of MSSA strains were loaded in lanes 2-10, while as MRSA whole cell protein samples were loaded in lanes 11-19. Here the aim of experiment was to see the difference of protein banding patterns between MRSA and MSSA, but as evident from the figure through visual analysis there was not found any difference, however there may be difference in the level of expression of some proteins as is seen in few lanes like 15 and 16 but it seems to be non-significant. In Fig 4.13 the similar protein banding patterns of 42 MRSA strains representing 42 antibiograms of 61 MRSA isolates collected from wound infections of patients (n=56); their nose (n=50); and nose of hospital workers (n=50) in an Orthopedic Surgical Ward, were shown. Here all the strains showed the same patterns as that of control MRSA (ATCC .43300). Similarity of whole cell protein banding patterns indicated that the method is not applicable for typing MRSA strains.

4.10 PFGE PROFILES OF MRSA

Chromosomal DNA from MRSA isolates was prepared in agarose blocks and was cleaved with SmaI by method of Bannerman et al. The samples were run according to the protocol described in Materials and Methods (Section 3.12). These 61 MRSA strains were collected from wound infections (n=56); their nose (n=50); and nose of hospital workers attending wound infections (n=50) in an Orthopedic Surgical Ward. As shown in figure 4.14, the 14 common methicillin – resistant S. aureus (MRSA) patterns were identified among 61 stains of MRSA isolated in the Orthopedic Surgical Ward. The patterns were designated by the letters A to N based on difference in banding patterns. The banding type A was shown by highest number of MRSA strains (n=7), while as type I was shown by only one
MRSA isolate (Table 4.10b). These results indicated that typing can be performed effectively through molecular techniques such as PFGE patterns but not through antibiograms as 61 strains showed 42 antibiograms which were narrowed down to only 14 types by PFGE. Moreover, it is evident that the MRSA strains which display a common antibiogram can not necessarily show the same PFGE pattern, for example, JMR-3, 55, 33, 59 showed antibiogram 3 (Table 4.10) but JMR-33 showed PFGE pattern L and JMR-59 showed PFGE pattern H although both JMR-3 and JMR-55 showed the same PFGE pattern A.

Table 4.10b PFGE Patterns of 61 MRSA Isolates

<table>
<thead>
<tr>
<th>Banding Pattern</th>
<th>Strains</th>
<th>Number of Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>JMR-1, 2, 3, 4, 6, 38, 55</td>
<td>7</td>
</tr>
<tr>
<td>B</td>
<td>JMR-5, 14, 18</td>
<td>3</td>
</tr>
<tr>
<td>C</td>
<td>JMR-9, 13, 19, 26, 52</td>
<td>5</td>
</tr>
<tr>
<td>D</td>
<td>JMR-16, 25, 29</td>
<td>3</td>
</tr>
<tr>
<td>E</td>
<td>JMR-24, 46, 48, 39</td>
<td>4</td>
</tr>
<tr>
<td>F</td>
<td>JMR-31, 56</td>
<td>2</td>
</tr>
<tr>
<td>G</td>
<td>JMR-37, 50, 54, 15, 58</td>
<td>5</td>
</tr>
<tr>
<td>H</td>
<td>JMR-21, 40, 42, 44, 59, 61</td>
<td>6</td>
</tr>
<tr>
<td>I</td>
<td>JMR-8</td>
<td>1</td>
</tr>
<tr>
<td>J</td>
<td>JMR-17, 20, 23, 27, 30, 32</td>
<td>6</td>
</tr>
<tr>
<td>K</td>
<td>JMR-11, 12, 28, 41, 53, 60</td>
<td>6</td>
</tr>
<tr>
<td>L</td>
<td>JMR-22, 33, 34, 35, 36</td>
<td>5</td>
</tr>
<tr>
<td>M</td>
<td>JMR-43, 45, 51</td>
<td>3</td>
</tr>
<tr>
<td>N</td>
<td>JMR-7, 10, 47, 49, 57</td>
<td>5</td>
</tr>
</tbody>
</table>

The epidemiology of carrier MRSA is evidenced in Fig 4.15 where molecular typing by PFGE of Smal-digested DNA from MRSA strains isolated from eight patients having post operative wound infections admitted in Orthopedic Surgical Ward, was performed. The staphylococcal strains were isolated
from pus, skin and nose of the patients as described in section 3.3 of Chapter 3. Here again the samples were run according to the protocol described in Materials and Methods. The banding patterns were interpreted visually according to the following guidelines: 1) Isolates with banding patterns appearing identical in sizes and number of bands were considered to represent the same type. This type was designated with one English capital letter, for example, type A. 2) Isolates with banding patterns that differed from the main pattern by up to three bands were considered to represent subtypes within the same main type and were designated with different number (A1, A2, A3, etc). 3) Isolates with banding patterns that differed from the main pattern by four or more bands were considered to be of a different type and designated with other English capital letters (types B, C, D, etc.).

PFGE patterns are indistinguishable between MRSA from the nasal cavity (A1, A1), and pus (A1, A1) but the pattern is different for MRSA from the skin (B, E) nearby wounds for cases 1 and 8, respectively. PFGE patterns are indistinguishable between MRSA from the nasal cavity (C1) and pus (C1) and closely related to that of MRSA from the skin (C2) for case 2 (Fig. 4.15). PFGE patterns are indistinguishable between MRSA from the skin (C4) and pus (C4) but closely related to that of MRSA from the nasal cavity (C2) for case 7. The patterns of MRSA isolates from the pus, skin and nasal cavity are indistinguishable for cases 3 (A2, A2, A2), 4 (C3, C3, C3), 5 (C3, C3, C3) and 6 (D, D, D). These results indicated that self-infection through colonization needs to be taken into consideration and the appropriate measures should be followed to minimize the role of carrier isolates in post operative infections. Furthermore the bacteria that normally colonize the human body (the resident microflora) could act as reservoirs for resistance genes, which could then be transferred to pathogens during their temporary colonization of the same site, and need to be focused while treating infections.

4.11 PLASMID-DETERMINED RESISTANCE TRANSFER

In order to study the transfer of resistance genes between different species of bacteria, transformation of plasmid DNA from Staphylococcus aureus (JMR-10) (A^R, G^R, Ak^R) (Table 4.10) to Escherichia coli (DH5α) (A^S, G^S, Ak^S)
was performed according to the protocol described in Materials and Methods (Section 3.9). Plasmid DNA was isolated from JMR-10 strain and transformed to DH5α. Transformation was tested by antibiotic sensitivity tests as described elsewhere. The \textit{E. coli} (DH5α), which was earlier sensitive to Ampicillin (A), Gentamicin (G) and Amikacin (Ak) now acquired resistance to these three antibiotics after transformation of plasmid pJMR-10 into it. Again plasmid was isolated from transformed \textit{E. coli} (DH5α) and the isolated plasmid preparation from \textit{S. aureus} (JMR-10) and transformed \textit{E. coli} (DH5α) were loaded in 0.7% agarose gel. Horizontal electrophoresis was performed in TAE buffer (pH 8.2) for 6 h (Fig 4.16) as described in Materials and Methods (Section 3.8.1). From the Fig. 4.16 it is clear that the 4.3 kb plasmid from \textit{S. aureus} (Lane A) was transformed to \textit{E. coli} (Lane C).

Minimum inhibitory concentrations of A, G, Ak on \textit{S. aureus} strain JMR-10 and \textit{E. coli} transformant were determined as described in Chapter 3 (Section 3.7). The results indicated (Table 4.11) that the MIC for Ak was higher in \textit{E. coli} than \textit{S. aureus} and may be due to the fact that Ak resistance gene was very efficiently expressed in \textit{E. coli}.

For resistance transfer to be studied through cell – to – cell contacts between clinical and carrier \textit{S. aureus} strains, plasmid preparations were electrophoresed in 0.7% agarose in TAE buffer (pH 8.2), for 6 h as described in chapter 3 (Section 3.8.1). The conjugation was performed through mixed culture tests as described in Broth Matings in Materials and Methods (Section 3.11.1). In our study Filter Matings experiments did not yield significant transconjugants and that is why we focused on Broth Matings only. Here \textit{S. aureus} strains were isolated from a patient having post operative ocular infection admitted in the Post Operative Ward of Institute of Ophthalmology. JN-49 strain (\(A^R, P^R, Cf^R, E^R, G^S, T^S\)) was isolated from the nose of the same patient having post operative ocular infection and JS-105 (\(T^R, E^R, G^R, A^S, P^S, Cf^S\)) was isolated from ocular swab. Plasmids were electrophoresed (Fig. 4.18) after isolation from JN-49 (Lane A), JS-105 (Lane B), and transconjugant JNS-1 (Lane E). Here transconjugants were screened as (\(A^R, T^R\)). The transconjugant JNS-1 was subjected to curing treatment with ethedium bromide as described in Chapter 3 (Section 3.)
Three types of cured transconjugents were obtained. As shown in fig 4.18 cured transconjugant JNS-IA (Lane C), having 38 Kb plasmid showed resistance pattern as $G^R$, $E^R$, $Cf^R$, $T^S$, $A^S$, $P^S$. Similarly, cured transconjugant JNS-IB (Lane D), having no plasmid depicted resistance profile as $E^R$, $Cf^R$, $T^S$, $G^S$, $A^S$, $P^S$. Likewise, cured transconjugant JNS-IC (Lane F), having 4.4 Kb plasmid, displayed resistance pattern as $T^R$, $Cf^R$, $E^R$, $G^S$, $A^S$, $P^S$. The conjugation experiments clearly showed that resistant markers G and T got transferred from clinical $S. aureus$ (JS-105) to carrier $S. aureus$ (JN-49). Moreover the ciprofloxacin ($Cf$) and erythromycin ($E$) resistance seemed to be chromosomal mediated as evidenced in Lane D.

In order to study the transfer of resistance traits from $S. epidermidis$ to $S. aureus$, conjugational experiment was performed through Broth Matings as described in Materials and Methods (Section 3.). The plasmids were isolated as described in chapter 3 from $S. epidermidis$ (JS-15) $S. aureus$ (JS-79) (hemolytic) and transconjugant $S. aureus$ (IS-79T). $S. aureus$ (JS-79) was isolated from normal eye of a hospital worker and $S. epidermidis$ (JS-15) was isolated from ocular swab from a patient having eye infection, in the Institute of Ophthalmology. Transconjugants were selected first on mannitol salt agar and letter on blood agar mixed with 64 μg/ml gentamicin. The transconjugant hemolytic colonies grew on gentamicin containing blood agar plates. As shown in the figure 4.19, $S. aureus$ (JS-79), having no plasmid (Lane A), was sensitive to gentamicin, tetracycline and erythromycin ($G^S$, $T^S$, $E^S$), while as $S. epidermidis$ (JS-15), having 38 kb, 4.4 kb, and 2.5 kb plasmids (Lane B) showed resistance pattern as $G^R$, $T^R$, $E^R$ as per NCCLS recommendations. There was a two-fold increase in the MIC of transconjugant $S. aureus$ (JS-769T) to Gentamicin, while its MIC level reduced to half against chloramphenicol and lowered by 16-folds against penicillin as compared to the donor $S. epidermidis$ (Table 4.12). After curing experiments with ethedium bromide, the three types of cured transconjugants (Lanes D, E, and F in Fig 4.19) showed that the 38 kb, 4.4 kb and 2.5 kb plasmids carry resistance traits against gentamicin, tetracycline and erythromycin respectively.
Effect of calcium chloride and temperature on the conjugation transfer frequency was performed according to the protocol described in Materials and Methods. Transfer frequency was expressed as the number of transconjugants per recipient cell. Transfer of resistance was studied between donor *S. epidermidis* (JS-15) \((G^R, E^R, T^R)\) and recipient *S. aureus* (JS-79) \((G^S, E^S, T^S)\) with respect to resistance markers G, E and T. At 37 °C transfer frequency was found to be higher with respect to G than E and T (Table 4.13). Furthermore, the effect of calcium chloride and 30 °C temperature was found to be having no significant effect on conjugation frequency, while at 22 °C, the temperature found in the human nose and eye environment, was observed to be having a profound effect on conjugation frequency. With respect to all the three resistance markers here studied, it was found that the transfer frequency dropped by more the 10,000 times. The results seemed to be supporting the clinical significance of lower temperature prevailing in the nose and eye, the two sites most exposed to the external environment.

4.12 STAPHYLOCOCCAL β-LACTAMASE ASSAY

In our study, 762 out of 850 staphylococcal strains turned to be β-Lactamase- positive by iodometric tests as described in Chapter 3 (Section 3.5.3.8.1). In order to see the effect of β-Lactamase on the activity of various penicillins and caphalosporins, a methicillin-resistant *S. aureus* strain (JMR-60) was selected for isolation of β-Lactamase. MRSA (JMR-60) was having the highest number of resistance markers (Table 4.10). Two liters of nutrient broth was inoculated with 200 ml of an 18 to 24 h old culture containing \(7.5 \times 10^7\) cells per ml. After 3 to 4 h of incubation with shaking at 37 °C, the cells were harvested by centrifugation at 10,000 rpm for 10 min in a Sorvall RC 2B centrifuge at 4 °C. The cell pellet was suspended in approximately 20 ml of 0.05 M phosphate buffer, pH 7.0, recentrifuged, and resuspended in the same volume of fresh buffer. Cells were disrupted by sonication at 4 °C and the material was again centrifuged at 10,000 rpm for 30 min. The supernatant was collected and tested for β-Lactamase activity using appropriate antibiotics. The assays were performed...
spectrophotometrically by measuring the change in absorbance at the appropriate wavelength for each substrate by using Hitachi 228 Spectrophotimeter with a heated attachment at 30 °C. Hydrolysis assays were carried out in 0.5 ml of 10 mM phosphate buffer, pH 7.4, at 30 °C. Antibiotic powders with known potencies were kindly provided by Hi-Media, India. The wavelengths were 235 nm for penicillin G (P), ampicillin (A), and amoxycillin (Ax), 263 nm for methicillin (M), Oxacillin (Ox) and cloxacillin (Cx), 254 nm for cephalaxin and cefazolin, 257 nm for cefotaxime and 274 nm for cephalosporin C as described elsewhere. All hydrolysis assays were performed three times. Value of 100 was taken arbitrarily as the rate of hydrolysis of penicillin G by β-Lactamase (Table 4.14). Similarly the concentration of β-Lactamase in the supernatant (Crude lysate) was arbitrarily taken as 100 (Figure 4.20). Likewise, the concentration of 100 mg of an antibiotic solution was arbitrarily taken as 100. Upon hydrolysis the unhydrolysed antibiotic was converted to % residual concentration and plotted against % of stock enzyme solution. The results indicated that methicillin is the most stable penicillin and cephotaxime is the most stable cephalosporine to staphylococcal β-Lactamase.

4.13 ACTIVITY OF A SYNTHETIC COMPOUND AGAINST MRSA

The emergence of MRSA and now VRSA has intensified the search for alternative therapies for the treatment of infections caused by these organisms. Keeping in view the above fact, activity of a new synthetic compound was determined against MRSA strains by broth macrodilution and microdilution testing as described in section 3.7 of Chapter 3. The proposed antimicrobial agent along with control was dissolved in dimethylsulfoxide (DMSO). First Mueller-Hinton agar plates were prepared and wells were bored with well borer under aseptic conditions. Plates were swabed with methicillin-resistant Staphylococcus aureus, JMR-60 (Table 4.10), as it showed resistance to maximum number of antibiotics. In plates A, B, C, and D, the well-1 was loaded with the parent compound 2-mercapto benzimidazole (MBI), which was the basic substrate for synthesis of Nickel-ligand-MBI-complex, the proposed antimicrobial agent (Fig 4.21). Similarly well-2 was loaded with a ligand derived from MBI without complexing to a
metal; well-3 was loaded with a copper complex of MBI (Cu-MBI); and well-4 was loaded with the proposed antimicrobial derived from complexing MBI and ligand with Nickel. Like-wise, well-5 was loaded with the solvent DMSO as control. In plate A, the concentration of each tested agent was 10 mg/ml, while as in the plates, B, C, and D, the concentration of the tested agents was 20 mg/ml, 30 mg/ml and 40 mg/ml, respectively. From the figure-4.21 it is clear that the proposed antimicrobial agent, Ni-ligand-MBI complex (well-4) is having the highest activity against MRSA as compared to the parent compound (well-1), ligand alone (well-2), and Cu-MBI complex (well-3). MIC of the proposed antimicrobial agent (Ni-Ligand-MBI-complex) by broth micro- and macrodilution testing was determined to be > 64 \( \mu \text{g/ml} \) as compared to MBI (MIC, 1536 \( \mu \text{g/ml} \)); ligand (MIC, 1024 \( \mu \text{g/ml} \)) and Cu-MBI-complex (MIC, 512 \( \mu \text{g/ml} \)).
Figure 4.12 SDS-PAGE Protein Profiles of MRSA and MSSA Isolates. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 12% acrylamide – 0.15% bisacrylamide in the running gel (13 by 18cm) (Bangalore Genei Pvt. Ltd. India). The staking gel was composed of 5% acrylamide – 0.07% bisacrylamide. Electrophoresis was carried out according to the method described in Materials and Methods (Section 3.). In Fig 4.12 lanes 1 and 20 were loaded with protein markers. Whole cell protein samples of MSSA strains were loaded in lanes 2-10, while as MRSA whole cell protein samples were loaded in lanes 11-19.
Figure 4.14 Pulsed-field Gel Electrophoresis (PFGE) Profiles of MRSA. The preparation of chromosomal DNA for pulsed-field gel electrophoresis was performed as described by Bannerman et al. DNA restriction was done with Smal according to the manufacturer’s recommendation (Bangalore Genei Pvt. Ltd. India). Gels for PFGE were run in a CHEF-DR II apparatus (Bio-Rad). The 14 common methicillin-resistant S. aureus (MRSA) patterns were identified among 61 strains of MRSA isolated in an Orthopaedic Surgical Ward. These strains were collected from wound infections (n=56); their nose (n=50); and nose of the hospital workers attending wound infections (n=50). Lanes 1, 9 and 17: S. aureus 8325 (molecular weight marker).
Figure 4.15 Molecular typing by PFGE of Smal-digested DNA from MRSA Clinical & Carrier Isolates from Eight Patients. The preparation of chromosomal DNA for pulsed-field gel electrophoresis was performed as described by Bannerman et al.\textsuperscript{34} DNA restriction was done with \textit{Sma}I according to the manufacturer's recommendation (Bangalore Genei Pvt. Ltd. India). Gels for PFGE were run in a CHEF-DR II apparatus (Bio-Rad). PFGE patterns are indistinguishable between MRSA from the nasal cavity and pus but the pattern is different for MRSA from the skin nearby wound for cases 1 and 8. PFGE patterns are indistinguishable between MRSA from the nasal cavity and pus and closely related to that of MRSA from the skin for case 2. PFGE patterns are indistinguishable between MRSA from the skin and pus but closely related to that of MRSA from the nasal cavity for case 7. The patterns of MRSA isolates from the nasal cavity, skin, and pus are indistinguishable for cases 3, 4, 5, & 6.
Figures 4.18 Plasmids Transferred in Conjugation Through Mixed Culture Test (MCT) Experiments (Broth Matings). Horizontal electrophoresis was performed with 0.7% agarose in TAE buffer (pH 8.2), for 6 h as described in Materials and Methods (Section 3.8.1).

Lane C, Cured transconjugant, JNS-1A ($G^R,E^R,Cf^R,T^S,A^S,P^S$), having 38 kb plasmid;
Lane D, Cured transconjugant, JNS-1B ($E^R,Cf^R,T^S,G^S,A^S,P^S$), having no plasmid;
Lane E, Transconjugant JNS-1 ($T^R,Cf^R,E^R,G^R,A^R,P^R$), having 38 kb, 4.4 kb and 2.8 kb plasmid;
Lane F, Cured Transconjugant JNS-1C ($T^R,Cf^R,E^R,G^S,A^S,P^S$), having 4.4 kb plasmid;

Results: Transconjugants were screened as ($A^R,T^R$). The conjugation experiment clearly shows that resistance markers G and T got transferred from clinical *S. aureus*, JS-105 to Carrier *S. aureus*, JN-49. Moreover, the Ciprofloxacin (Cf) and erythromycin (E) resistance seem to be chromosomal mediated as evidenced in Lane D.
Horizontal electrophoresis was performed with 0.7% agarose in TAE buffer (pH 8.2), for 6 h as described in Materials and Methods (Section 3.8.1).

**Lanes 1, 2 & 3**, Carrier *S. aureus*, JN-49 (\(A^R, P^R, C^R, E^R, G^S, T^S\)) having 2.8 kb plasmid;


**Lane 5**, Cured Transconjugant JNS-IC (\(T^R, C^R, E^R, G^S, P^S\)), having 4.4 kb plasmid

**Lane 6**, Plasmid marker, *S. aureus* WBG 4483.
Figures 4.18 Plasmids Transferred in Conjugation Through Mixed Culture Test (MCT) Experiments (Broth Matings). Horizontal electrophoresis was performed with 0.7% agarose in TAE buffer (pH 8.2), for 6 h as described in Materials and Methods (Section 3.8.1).


**Lane B**, Clinical *S. aureus*, JS-105 ($T^R,E^R,G^R,A^S,P^S,Cf^S$), having 38 kb and 4.4 kb plasmid;

**Lane C**, Cured trasnconjugant, JNS-1A ($G^R,E^R,Cf^R,T^S,A^S,P^S$), having 38 kb plasmid;

**Lane D**, Cured trasnconjugant, JNS-1B ($E^R,Cf^R,T^S,G^S,A^S,P^S$), having no plasmid;

**Lane E**, Transconjugant JNS-1 ($T^R,Cf^R,E^R,G^R,A^R,P^R$), having 38 kb, 4.4 kb and 2.8 kb plasmid;

**Lane F**, Cured Transconjugant JNS-1C ($T^R,Cf^R,E^R,G^S,A^S,P^S$), having 4.4 kb plasmid;

**Results**: Tranconjugants were screened as ($A^R,T^R$). The conjugation experiment clearly shows that resistance markers G and T got transferred from clinical *S. aureus*, JS-105 to Carrier *S. aureus*, JN-49. Moreover, the Ciprofloxacin (Cf) and erythromycin (E) resitnace seem to be chromosomal mediated as evidenced in Lane D.
Table 4.12 Minimum Inhibitory Concentrations (MICs) of Antibiotics for Selected Staphylococcal Strains

<table>
<thead>
<tr>
<th>Staphylococcal Strains</th>
<th>MIC (μg/ml) of given antibiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G</td>
</tr>
<tr>
<td>JS-79 (S. aureus)</td>
<td>0.5</td>
</tr>
<tr>
<td>JS-15 (S. epidermidis)</td>
<td>64</td>
</tr>
<tr>
<td>JS-79T (transconjugant S. aureus)</td>
<td>128</td>
</tr>
</tbody>
</table>

*Minimum inhibitory concentrations were determined by NCCLS recommended protocol.*

Table 4.13 Effect of Calcium Chloride and Lower Temperature on transfer Frequency between S. epidermidis Donor (JS-15) and S. aureus Recipient (JS-79)

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Resistance Marker</th>
<th>Transfer Frequencya</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No reagent (37 °C)</td>
</tr>
<tr>
<td>JS-15</td>
<td>JS-79</td>
<td>G</td>
<td>1.35 x 10^-5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>5.30 x 10^-7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>2.70 x 10^-7</td>
</tr>
</tbody>
</table>

*Transfer frequency was expressed as the number of transconjugants per recipient cell.*
Figure 4.19 Agarose Gel Electrophoresis of Crude Plasmid DNA of Donor, Recipient and Transconjugant Staphylococcal Isolates. Horizontal electrophoresis was performed with 0.7% agarose in TAE buffer (pH 8.2), for 6 h as described in Materials and Methods (Section 3.8.1). Lane A, S. aureus recipient, JS-79, having no plasmid but hemolytic; Lane B, S. epidermidis, JS-15 (G^R, T^R, E^R), having 38 kb, 4.4 kb, and 2.5 kb plasmids; Lane C, Transconjugant S. aureus, JS-79T (G^R, T^R, E^R), having 38 kb, 4.4 kb, and 2.5 kb plasmids; Lane D, Cured transconjugant S. aureus, JS-79TA (G^R, E^R), having 38 kb and 2.5 kb plasmids; Lane E, Cured transconjugant S. aureus, JS-79TB (G^R), having 38 kb plasmid; Lane F, Cured transconjugant S. aureus, JS-79TC (G^R, T^R), having 38 kb and 4.4 kb plasmids;

Results: In this study the clinical strain of S. epidermidis, JS-15 has been attributed the resistance traits to specific plasmids based on agarose gel electrophoresis of DNA obtained from transconjugants and selected transconjugants cured for various markers by ethidium bromide. The transconjugant S. aureus, JS-79T, which was resistant to gentamicin, erythromycin, and tetracycline, was used in curing experiments. Furthermore, the effect of calcium chloride and the temperature of 30 °C was found to be having no significant effect on conjugation, while the temperature of 22 °C, the temperature found in the human nose and eye environment, was observed to be having a profound effect on conjugation frequency.
Table 4.14 Rates of Hydrolysis of Various Substrates by Purified Preparations of β-lactamase from Methicillin-resistant S. aureus (MRSA)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rate of hydrolysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin G (P)</td>
<td>100</td>
</tr>
<tr>
<td>Ampicillin (A)</td>
<td>90</td>
</tr>
<tr>
<td>Amoxycillin (Ax)</td>
<td>85</td>
</tr>
<tr>
<td>Methicillin (M)</td>
<td>1.5</td>
</tr>
<tr>
<td>Oxacillin (Ox)</td>
<td>4.5</td>
</tr>
<tr>
<td>Cloxacillin (Cx)</td>
<td>3.5</td>
</tr>
<tr>
<td>Cephalexin (Cp)</td>
<td>3.5</td>
</tr>
<tr>
<td>Cephazolin (Cz)</td>
<td>0.5</td>
</tr>
<tr>
<td>Cephotaxime (Ce)</td>
<td>0.1</td>
</tr>
<tr>
<td>Cephalosporin C (Cc)</td>
<td>5</td>
</tr>
</tbody>
</table>

*Value of 100 was taken arbitrarily as the rate of hydrolysis of penicillin G by β-lactamase. Here the experiment was performed according to the method described in section 4.12.
Figure 4.20 Inactivation of Penicillins (Graph A) and Cephalosporins (Graph B) by β-lactamase. Here the experiment was performed according to the method described in section 4.12. The enzyme was purified from methicillin-resistant *S. aureus*. Symbols: Penicillin G (P); Ampicillin (A); Amoxicillin (Ax); Methicillin (M); Oxacillin (Ox); Cloxacillin (Cx); Cephalexin (Cp); Cephaezolin (Cz); Cephotaxime (Ce); Cephalosporin C (Cc).
A novel metal complex derived from parent compound, 2-mercaptobenzimidazole was synthesized in the Inorganic Chemistry Lab of AMU. The activity and MIC of this compound was determined in this study against MRSA isolates. In plate A, the concentration of each tested agent was 10 mg/ml, while as in the plates B, C, and D, the concentration of the tested agents was, 20 mg/ml, 30 mg/ml, and 40 mg/ml, respectively.

Well 1, Parent Compound, 2-mercaptobenzimidazole (MBI)
Well 2, A ligand derived from (MBI) without complexing to a metal.
Well 3, A Cu-complex of MBI
Well 4, The new antimicrobial agent derived by complexing MBI and ligand with Nickel
Well 5, DMSO solvent as control.

MIC of the new antimicrobial agent was determined to be $\geq 64 \mu$g/ml as compared to MBI (MIC, 1536 $\mu$g/ml); ligand (MIC, 1024 $\mu$g/ml); and Cu-Complex (MIC, 512 $\mu$g/ml).
In the celebrated movie Crouching Tiger, Hidden Dragon, two warriors face each other in a closed courtyard whose walls are lined with a fantastic array of martial-arts weaponry, including iron rods, knives, spears and swords. The older, more experienced warrior grabs one instrument after another from the arsenal and battles energetically and fluidly with them. But one after another, the weapons prove useless, each, in turn, is broken or thrown aside, the shards of an era that can hold little contest against a young, triumphant, upstart warrior who has learned not only the old ways but some that are new.

One of the foundations of the modern medical system is being similarly overcome. Healthcare workers are increasingly finding that nearly every weapon in their arsenal of more than 150 antibiotics is becoming useless. Bacteria that have survived attack by antibiotics have learned from the enemy and have grown stronger; some that have not had skirmishes themselves have learned from others that have. The result is a rising number of antibiotic resistant strains. Infections - including tuberculosis, meningitis and pneumonia - that would once have been easily treated with an antibiotic are no longer so readily thwarted. More and more bacterial infections are proving deadly.

The present study was undertaken with the aim of determining epidemiology of clinical and carrier staphylococci and biochemical studies of their acquisition and dissemination of resistance. The rationale of this study was to assess the prevalence of methicillin-resistant staphylococci from different clinical sites of patients, and nose of the hospital workers. We were particularly interested in analyzing the MRSA nasal colonization rate in patients admitted in an Orthopaedic Surgical Ward and the medical personnel and its relation to frequency of MRSA isolates in wound infections. We also observed the prevalence of MRSE in ocular infections and its relationship with nasal colonization of patients and ocular colonization of
hospital workers. The epidemiology of MRSA was analyzed by SDS – PAGE protein profiles and PFGE DNA finger printing patterns of the isolated strains. The results would be useful for medical personnel who need to eradicate the troublesome organisms from the hospital environment. In addition to the reservoir(s) searched, the susceptibility of both MRSA and MRSE isolates to 17 antimicrobial agents were also performed in order to provide update information on the use of appropriate drug(s) in the treatment of MRS infections and to determine the antimicrobial susceptibility pattern (antibiogram) of these strains to be used as a tool in further epidemiological study. Furthermore, the plasmid profiles of staphylococcal isolates and subsequent transformation and conjugation studies were performed in the present study. The isolation of β-lactamase enzyme and its activity against different classes of antibiotics was studied together with the activity of new antimicrobials against MRSA using standard procedures.

Staphylococci are important causes of human infections but are also found as non-pathogenic microorganisms in human samples. The spectrum of S. aureus infections includes toxic shock syndrome, food poisoning, meningitis, as well as dermatological disorders ranging from minor infections and eczema to blisters and scalded skin syndrome. Recent reports have begun to document infections caused by Staphylococcus epidermidis, such as bacterial endocarditis, prosthetic heart valve endocarditis, bacteraemia, surgical wound infections, intravascular catheters, post-operative endophthalmitis, conjunctivitis and keratitis. Several other coagulase-negative staphylococci (CNS) species have been implicated at low incidence in a variety of infections. The CNS species Staphylococcus saprophyticus was often regarded as a more important opportunistic pathogen than S. epidermidis in human UTIs, especially in young sexually active females. It was considered to be the second most common cause of acute cystitis or pyelonephritis in these patients.

In the present study highest prevalence of S. aureus was found in pus followed by urine and blood. Likewise the highest number of coagulase negative staphylococcal isolates was found in urine followed by conjunctiva
and pus. Besides, a significant number of staphylococcal isolates were observed from the clinical conditions like post operative infections, bacteraemia, pneumonia, corneal ulcer, prostatitis, septicaemia, mastatitis and endophthalmitis.

Methicillin-resistant strains of staphylococci were identified immediately upon the introduction of methicillin into clinical practice. Methicillin-resistant *S. aureus* (MRSA) was initially identified for the first time in 1961 by Jevons.\(^{39,235}\) Since then strains of methicillin-resistant *Staphylococcus aureus* and methicillin-resistant coagulase-negative Staphylococci have spread worldwide\(^{251,316}\) and have become established inside and outside of the hospital environment.\(^{22,325}\) There are reports of emergence and high occurrence of strains resistant to methicillin from various parts of the world.\(^{480}\) Recent studies have documented the increased costs associated with MRSA infection, as well as the importance of colonization pressure.\(^{147,459}\) Already multiresistant to different classes of antibiotics, MRSA had been reported to acquire resistance to gentamicin and related aminoglycosides,\(^{406,420}\) therefore the treatment of infections due to these organisms and their eradication is very difficult. Constant monitoring of these strains is essential in order to control their spread in the hospital environment and transmission to the community.

Of the 513 clinical strains of *S. aureus*, 180 (65.1%) were methicillin-resistant *S. aureus* (MRSA) and out of 337 coagulase–negative staphylococcal isolates 76 (22.5%) were methicillin–resistant (MRCNS). Of the 180 MRSA strains the highest number was found in pus followed by urine and blood. Of the 76 MRCNS studied the highest number was obtained from urine followed by conjunctiva and nasal swabs of patients having ocular infections. The number of MRSA isolates being drastically high in wound infections, this might be due to the fact that orthopedic unit is a fertile environment for MRSA. The open wounds and the frequent dressing changes often necessitate a dressing team or multiple persons plus the inherent immunosupression of the wound patients might lead to MRSA colonization.

The present study confirms that MRSA remains a mainly hospital-acquired infection, but a significant proportion may be acquired in community facilities
like nursing and residential homes. The major reservoir of staphylococci in hospitals are colonized/infected in-patients and colonized hospital workers, with carriers at risk for developing endogenous infection or transmitting infection to health care workers and patients, while transient hand carriage of the organism on the hands of health care workers account for the major mechanism for patient to patient transmission. Low prevalence of MRSA colonization in an adult outpatient population indicated that MRSA carriers most likely acquired the organism through contact with healthcare facilities rather than in the community. These data show that care must be taken when attributing MRSA colonization to the community if detected in outpatients or during the first 24 to 48 hours of hospitalization. The risk to patients in terms of transmission of MRSA seems to be influenced strongly by the proportion of patients with colonization at intensive care unit admission and is associated with severity of illness, length of stay, and exposures to antibiotics and medical devices.

Colonization with *S. aureus* can occur soon after birth and at any given time, the nasal carriage rate in adults is estimated at between 20% and 40%. Healthcare workers have a higher incidence of colonization. The carrier state is clinically important because carriers undergoing surgery will experience more infections than non-carriers. Fierobe et al. has shown a relationship between MRSA postoperative intra-abdominal sepsis and nasal colonization of MRSA. MRSA strains are usually introduced into an institution by an infected or colonized patient or by a colonized healthcare worker, and transfer from one patient to another has led to major epidemics in tertiary care hospitals as well as chronic care facilities. Colonization of anterior nares with MRSA carries a significantly higher risk for infection than does colonization by sensitive strains.

Intranasal 2% mupirocin applied twice daily for five days appears to be the most effective topical agent against MRSA and eliminates 91% of stable carrier states. Rohr et al., have demonstrated significantly reduced MRSA levels from nose, groin and axilla after the treatment of nasal mupirocin combined with octenidine dihydrochloride whole-body wash.
confining the investigation to nasal carriage in healthy state, colonization at other body sites may have remained undetected and the ‘true’ prevalence in this study may have been underestimated. There is, however, overall agreement that sensitivity of nose swabs in detecting MRSA carriage is reasonably high (>70%), and it was decided to confine the investigation to nasal specimens for reasons of accessibility, compliance and consistency with other investigations. The design of the study does not allow one to distinguish between patients who acquired MRSA during their current episode of hospital stay or patients already colonized on arrival to the hospital. Despite this, most of the carriers appeared to have acquired the organisms in hospital settings, as most of the isolates seem to be of the same types of strains as isolated from nose of the hospital workers.

It is imperative we continue with basic infection control principles like hand washing and contact isolation and barrier nursing. There are conflicting reports regarding control of MRSA in hospital settings. Herwaldt and Fekety in their papers justified aggressive measures to counteract this emerging health problem. Our study did not look into the cost effectiveness of MRSA screening, but Papia et al. have shown that a screening programme for identification of MRSA colonization may be cost effective. The benefit of such a screening program may need to be confirmed by a randomized controlled trial.

From this study we suggest that all patients having history of previous hospital admission and patients admitted directly from nursing homes should be screened for MRSA prior to any elective surgical or orthopaedic operative procedures. We believe that patients for routine elective procedures should have a negative result before undergoing the procedure. If surgery cannot be delayed due to medical reason then prophylaxis against MRSA should be given prior to the operation, though we appreciate this may not have much benefit in case of emergency admissions. However we would suggest that in patients with any of the above risk factors admitted for emergency surgery which may involve prosthetic implant, should be screened as well as given MRSA prophylaxis prior to their operations.
It is well documented that MRSA is likely to be multiple resistant (Figure 4.10). Although methicillin-resistant staphylococci are clinically thought to be resistant to all β-lactams and cephalosporins but our in vitro studies showed that 1.1%, 5.6% 3.9% and 30% of MRSA strains showed susceptibilities to ampicillin, cefazolin, cephotaxime and imipenem, respectively. No MRCNS strain showed susceptibility to any of the β-lactam or cephalosporin antibiotics assayed except imipenem. Methicillin resistance gene meca is associated with transposons and insertion sequences, including Tn554 and IS431. IS431 is a common insertion sequence in staphylococcal chromosome and plasmids and can be associated with host of resistance determinants. The ability of IS431 elements through homologous recombination to trap and cluster resistance determinants with similar IS elements explain the multiple drug resistance phenotype that is characteristic of methicillin resistant staphylococci.15,46

Broad-spectrum insusceptibility of all 180 MRSA and 76 MRCNS isolates to common antimicrobial agents were observed. Among all 17 antimicrobial agents used only vancomycin was shown to be consistently effective against all MRSA and MRCNS. We did not find any glycopeptide resistant S. aureus in our study, and vancomycin and teicoplanin remain the drugs of choice, although decreased susceptibility as well as resistance to vancomycin has been reported recently.87,203,410,434.

Of the 850 staphylococcal isolates, surprisingly about 90%, 80% and 50% strains were resistant to Pencillin G, ampicillin and co-trimoxazole, respectively. This might be due to the fact that at present time these agents are tremendously used in the treatment of general infections. On the contrary to the multiple resistance of MRSA, MSSA seemed to be much more susceptible to all tested antimicrobial agents except for pencillin, ampicillin and co-trimoxozable. As far as the clinical versus carrier staphylococcal isolates are concerned the general observation was that resistance in carrier isolates was lesser than clinical strains in case of all antibiotics tested. In the present study it is documented that resistance to chloramphenicol and ciprofloxacin was comparatively higher in ocular isolates as compared to staphylococcal strain isolated from other clinical
sources. This might be due to more pronounced use of these antibiotics for treatment of ocular infections.

The high rates of multiple resistance is a direct consequence of hospital and community use of antibiotics and its concurrent selection of resistant strains. In India, as in many other developing countries, antibiotics are readily available over the counter. A person can buy the antibiotic of their choice or the pharmacist will prescribe one just by describing their symptoms. Patients, therefore, treat themselves with the wrong antibiotic or wrong dosage. Thus, patients may then come to the hospital already harbouring resistant strains. These strains may be the cause of endogenous infections or exogenous infections in other patients. Another important cause of resistance is excessive or inappropriate use of antibiotics in hospitals. The magnitude of the problem of multi-resistance is such that clinicians must be familiar with the causes of antibiotic resistance and the measures for preventing or minimizing the emergence of resistance. This study underscores the need for hospital clinicians to be aware of the common bacterial isolates in their unit and their usual antibiotic susceptibility. This is imperative in order to make rational decisions for the prudent use of antibiotics, particularly for empirical therapy.

PFGE, because of its great discriminatory power and high degree of specimen typeability is accepted as the gold standard for the molecular typing of *S. aureus* isolates. It has successfully been used to study the epidemiology of *S. aureus* nosocomial infection and methicillin resistance. Nevertheless, PFGE is time-consuming and labor intensive, in this study PFGE exhibited superiority as a technique for analyzing epidemiology of *S. aureus*. In the present study it was observed that the 61 strains of MRSA isolated in an Orthopaedic Surgical Ward displayed 42 patterns of antibiogram. These 61 MRSA strains were subjected to PFGE analysis whereby 14 PFGE patterns were observed. These results suggest that most of the MRSA appeared to have been acquired by patients during their current episode of hospital stay. Moreover similarity of PFGE patterns of MRSA isolates from pus, skin and nasal cavity suggest that most MRSA types isolated from pus were derived from the
nasal cavity but some types were derived from the nearby skin and that these microorganisms occasionally cause wound infections.

The association between the administration of antibiotics and the occurrence of MRSA is complex. Since the MRSA status of our sample populations on admission is unclear, it becomes difficult to decide if the antibiotics had (i) selected for resistant strains that were already colonizing on admission, or (ii) supported persistent colonization by excluding competing organisms or by enhancing the adhesion and colonizing capacity of the carriage strains, or (iii) increased the susceptibility of the patients to colonization with MRSA. An association between the use of antibiotics and MRSA has been repeatedly observed in different studies \(^\text{[^59,132,328,364,452]}\) and ciprofloxacin has been identified as a risk factor in outbreak situations. \(^\text{[^266]}\)

MRSA acquisition depends on 2 major and independent determinants: colonization pressure and antimicrobial selective pressure. In case of colonization with distinct multiple clones of MRSA, antimicrobial pressure plays a major role; in the case of colonization with a single dominant clone of MRSA, colonization pressure plays a major role. McGown \(^\text{[^314]}\) proposed a biological model to explain the relationship between antimicrobial use and the emergence of resistance. At the level of individual patient, antimicrobial treatment leads to a large modification in the endogenous flora. The usual result is that susceptible strains are replaced by resistant ones. At the collective level, antimicrobial use in a hospital unit tends to maintain the presence of multidrug – resistant organisms in inpatients, healthcare workers, and the environment. In cases in which basic infection–control practices are inconsistently applied, these pathogens are implicated in the majority of infections. Antimicrobials such as β-lactams and fluoroquinolones, which are ineffective against MRSA and have excellent tissue diffusion, could promote the acquisition of MRSA by increasing the ‘receptiveness’ of the patients and thereby allowing the progression towards colonization and infection.

Our observations in Orthopedic Surgical Ward through antibiograms and PFGE patterns indicated that most MRSA infections resulted from colonization in hospital settings, although the role of community–MRSA has
not been taken into consideration. Therefore the prevention and control of drug-resistant infections in hospital settings requires measures to promote the appropriate use of antimicrobial drugs and prevent the transmission of infections. At present, control policies primarily consist of both infected and colonized (asymptomatic) patients and increased staff hygiene measures (principally handwashing compliance). Both of these are intended to reduce patient-to-patient transmission mediated by transiently colonized health workers. In the strongest form of isolation, patients carrying MRSA are placed in dedicated isolation units (IUs). If operated appropriately with designated staff, such units should be effective at preventing almost all transmission to patients elsewhere in a hospital.

The spread of resistance to antimicrobial agents in *S. aureus* is largely due to the acquisition of plasmids and/or transposons. Although transfer of resistance between staphylococcal strains in the laboratory has been shown to occur via transformation, transduction, and conjugation, only conjugative transfer appears to be significant in vivo. In staphylococci, the conjugative transfer of resistant determinants is usually mediated by conjugative plasmids but has also been shown to occur in the absence of detectable conjugative plasmids. Conjugative plasmids, usually 35 to 50 kb, spread resistance determinants between species and genera. Besides transferring the resistance determinants, they can mobilize non-conjugative plasmids, recombine with nonconjugative plasmids to form new plasmids, or acquire and transfer resistance transposons. Studies with human staphylococcal strains indicate that *Staphylococcus epidermidis* is a reservoir of antibiotic resistance genes that can be transferred to *S. aureus* under *in vitro* and *in vivo* conditions.

*In vitro* studies of drug resistance transfer between clinical and carrier staphylococcal strains, was done in the present study. The results indicated that tetracycline resistance was transferred from clinical to carrier isolates. This reflects that the use of antibiotics in humans to treat infections can promote resistance in normal flora.
Plasmid transfer among bacteria provides a means for dissemination of resistance to multiple antibiotics. Resistance to multiple antibiotics is frequently found with *S. aureus* clinical isolates and is often plasmid mediated. Transduction as a mechanism of gene transfer was first described in *S. aureus* in 1958 by Ritz. In contrast to the *Enterobacteriaceae*, transduction is believed to be virtually the only means of transfer of genetic material between staphylococci cells. Although transformation in staphylococci *in vitro* has been described, practically all cultures of *S. aureus* contain high levels of nuclease, which can be expected to prevent transformation under natural conditions.

Transfer of resistance in mixed broth cultures by *S. aureus* has been described and is dependent on the presence of calcium chloride. In some reports, investigators have attributed the transfer in mixed-culture transfer of resistance as being phage-mediated conjugation. This process requires calcium chloride and is abolished by citrate, and transferring particles are often not detectable in donor culture supernatants. Lacy proposes that the bacteriophage is cell bound, causing alterations in the cell surface of either the donor or recipient and thereby allowing plasmid transfer. However, the process of transfer here characterized is not dependent on the presence of calcium ions.

In light of these data, we conclude that conjugation is the probable mechanism for transfer. Although the role of bacteriophage cannot be completely excluded, our experimental data are more consistent with a conjugal transfer process. Cell-to-cell contact was essential for transfer since resistance transfer could not be shown when donor and recipient cells were not allowed in contact with one another, analogous to transferable plasmids well characterized in streptococci. The transfer process also required viable donor cells and was energy dependent.

In order to see the effect of lower temperature as found in the eye and nose environment (i.e. 22 °C) upon the conjugation process, it was observed that the application of lower temperature is having a profound effect upon conjugation transfer frequency. At a temperature of 30 °C there was a marginal decrease and at 22 °C there was a substantial reduction in transfer.
frequency. But at 20 °C there was hardly any conjugation observed as compared to control (at 37 °C). These results indicate that the natural conditions prevailing in the eye and nose environment do not favour the resistance transfer mechanism significantly.

The resistance often is transferable at interspecies and intergeneric levels. The transfer of plasmids from \textit{S. aureus} to \textit{E. coli} has previously been demonstrated to be possible. However in these studies, the staphylococcal plasmids were modified by \textit{in vitro} manipulation before their introduction into \textit{E.coli} cells. In contrast, the transfer reported here was performed without any alteration of the native plasmid structure. Therefore, these experiments strongly support the hypothesis that genetic material could successfully flow from \textit{S. aureus} to \textit{E. coli} by transformation.

Other Gram-negative bacteria of medical importance may also act as receptors for \textit{S. aureus} plasmids (i.e., bacteria of the genus \textit{Klebsiella} or \textit{Serratia}). After transfer of plasmid DNA from Gram-positive to Gram-negative bacteria, illegitimate recombination is a likely step for stabilization of the exogenous resistance genes.

The MICs for A, G and Ak are shown in Table 4.11. As can be seen, the resistance to A and G were lower in \textit{E. coli} transformants than in \textit{S. aureus}. However, the MIC for Ak was higher in \textit{E. coli}. This result indicates that the Ak resistance gene is very efficiently expressed in \textit{E. coli}.

Although the staphylococcal resistance to methicillin in attributed to the altered PBP\textsubscript{s}, but virtually all the penicillin resistant strains of staphylococci isolated from hospital infections owe their resistance to the production of \(\beta\)-lactamase. The ‘efficiency of \(\beta\)-lactamase depends not only on the maximum velocity, \(V_{\text{max}}\), of hydrolysis of the substrate but also on the Michaelis constant, \(K_m\), of the enzyme, and Pollack has suggested the term ‘physiological efficiency’ (defined as \(K_m/V_{\text{max}}\)) to describe the efficiency of a \(\beta\)-lactamase at destroying penicillin under physiological conditions. In the present study the substrate profile of \(\beta\)-lactamase so extracted from a methicillin-resistant \textit{S. aureus} against different penicillins and caphalosporins indicated that methicillin and
Cephotaxime remain the most stable antibiotics to the staphylococcal β-lactamase. The results indicated that methicillin resistance is independent of β-lactamase production in MRSA.

The recent emergence of MRSA with decreased susceptibility to vancomycin has intensified the search for alternative therapies for the treatment of infections caused by this organism. In this study, the anti-MRSA activity of a synthesized compound in an Inorganic Lab of A.M.U. was determined by \textit{in vitro} testing. The results indicated that the Nickel-metalo complex of 2-mercaptobenzimidazole has good activity against MRSA (MIC, \( \geq \) 64 \( \mu \text{g/ml} \)), and demands to be analyzed for clinical applications in future.

\textbf{5.1 SUMMARY}

The emergence of multidrug-resistant bacteria is a phenomenon of concern to the clinician and the pharmaceutical industry, as it is the major cause of failure in the treatment of infectious diseases. Staphylococci have a record of developing resistance quickly and successfully to antibiotics. This defensive response is a consequence of the acquisition and transfer of antibiotic resistance plasmids and the possession of intrinsic resistance mechanisms. The acquired defense systems by staphylococci may have originated from antibiotic-producing organisms, where they may have been developed and then passed on to other genera. There are reports of emergence and high occurrence of staphylococcus strains resistant to methicillin, especially MRSA, from various parts of the World. These organisms are mostly resistant to multiple antibiotics; therefore the treatment of infections due to these organisms and their eradication is very difficult.

The present study was undertaken with the aim of determining epidemiology of clinical and carrier staphylococci and biochemical studies of their acquisition and dissemination of resistance.

The prevalence of methicillin-resistant staphylococci was determined in 750 subjects infected/colonized with staphylococci providing 850 isolates. Of 850 strains 575 were isolated from clinical specimens, 100 from nasal cultures of hospitalized patients, 125 from nasal and 50 strains were isolated from
ocular swabs of hospital workers. It was shown that 35.1% (180/513) of *Staphylococcus aureus* and 22.5% (76/337) of coagulase-negative staphylococcal isolates were resistant to methicillin. Highest percentage of MRSA (35.5%) was found in pus specimens (n=151) while as urine samples (n=153) contributed 25.0% of methicillin-resistant coagulase-negative staphylococci. 29.7% of methicillin resistant *Staphylococcus aureus* isolates were found in nasal swabs (n=175) while as 10.0% and 16.5% of methicillin-resistant coagulase-negative staphylococci were observed in nasal swabs (n=50) and ocular cultures (n=50) respectively. The results indicated that major reservoir of methicillin resistant staphylococci in hospitals are colonized/infected inpatients and colonized hospital workers, with carriers at risk for developing endogenous infection or transmitting infection to health care workers and patients. Restriction mapping and SDS-PAGE protein profiles of both clinical and carrier methicillin-resistant *Staphylococcus aureus* isolates confirmed the results. The multiple drug resistance of all MRSA (n=180) and MRCNS (n=76) isolates was detected. In case of both methicillin-resistant as well as methicillin-sensitive staphylococcal isolates zero resistance was found to vancomycin followed by fusidic acid while as highest resistance was found to penicillin G followed by ampicillin.

Selective methicillin-resistant staphylococcal isolates were subjected to plasmid isolation and curing treatments to study their mode of resistance. The plasmids were transformed into antibiotic sensitive *Escherichia coli* Strain DH5-α. In one of the experiments plasmid pJMR10 from *Staphylococcus aureus* coding for ampicillin (A'), gentamicin (G') and amikacin (Ak') resistance was transformed into *Escherichia Coli*. Transformation efficiency was about 2 x 10^3 transformants/μg of plasmid DNA. The minimal inhibitory concentrations (MICs) for A and G were lower in *E. coli* than in *S. aureus*. However, the MIC for AK was higher in *E. coli* transformants than in *S. aureus*. In one of conjugation studies transfer of erythromycin resistance was observed between clinical and carrier strains of *Staphylococcus aureus*. Furthermore enzyme mediated resistance particularly through β–lactamase was studied in selected strains. The substrate profile of β – lactamase so
extracted was studied against different classes of pencillins and cephalosporins. The results indicated that methicillin remains the most stable penicillin to the staphylococcal \( \beta \)-lactamase.

The anti-MRSA activities of a synthesized compound from an Inorganic Lab of A.M.U; was determined by \textit{in vitro} testing. The results indicated that the Nickle–metallo, complex of 2-mercaptobenzimidazole has good activity against MRSA (MIC > 64 \( \mu \)g/ml) and demands to be analyzed for clinical applications in future.

The widespread occurrence and dissemination of \( \beta \)-lactamase and PBP mediated resistance leading to multiple antibiotics ineffective, thus increasing the cost of health care, needs to be tackled logistically by wise and judicious use of existing antibiotics and by developing ideal and cost effective antibiotics having least chances of acquiring resistance. Furthermore the hospital acquired MRSA infections through colonization of patients and hospital workers demands appropriate and timely measure to counteract this health problem.

\textbf{5.2 LIMITATIONS AND FUTURE CONSIDERATIONS}

Knowledge and understanding of the growing problem of antimicrobial resistance is a pre-requisite for a planned and coordinated scientific response to this challenge. The emerging threat of antimicrobial resistance demands sustained research that will lead to an increased understanding broadly of: microbial physiology, ecology, genetics, mechanisms of resistance, host factors, and of the impact of variable antimicrobial use patterns, preventive, therapeutic, and growth promoting agents and environmental residues on the emergence and spread of resistant organisms and resistance factors. In this direction research community needs genomics and other powerful technologies to identify targets in critical areas for the development of new rapid diagnosis methodologies, novel therapeutics and interventions to prevent the emergence and spread of resistant pathogens. Various infection control interventions have been recommended for limiting the
spread of MRSA in hospitals. The recommendations have included laboratory surveillance for MRSA, implementation of a variety of barrier precautions, isolation procedure and cohorting, eradication of MRSA from colonized patients and staff, and disinfection of the inanimate environment of infected individuals.

The present study though aimed at determining epidemiology of clinical and carrier staphylococci and biochemical studies of their acquisition and dissemination of resistance, has few limitations. The epidemiological study of MRSA infections in S.K. Institute of Medical Sciences and Institute of Ophthalmology although depict the clear picture of prevailing antibiotic resistance patterns but the source of infections would have been clear if molecular biological typing of both nosocomial and community acquired MRSA and MSSA could have been done. Studies have reportedly found little evidence for sustained community spread of important nosocomial MRSA strains, and most patients carrying MRSA in community prevalence studies have had recent hospital exposure. For MRSA, as with all infectious diseases, ultimate control depends on keeping the mean number of secondary cases caused by each case below one. However, when carriage can persist for a long time, the secondary cases by each case may be distributed over several hospital admissions. Even limited community transmission of MRSA, although difficult to detect may profoundly alter the dynamics. Quantifying such transmission is an important area for future research.

As far as the epidemiological study of MRSA infections in Orthopedic Surgical Ward is concerned, the SDS-PAGE whole cell protein banding though help in identification of strains but its role in epidemiology is not clear. Although the PFGE typing is clearly showing the role of carrier–MRSA in nosocomial and self infections but the study would have been of substantial importance if the carrier state before admission to Orthopedic Surgical Ward would have been typed as control.

PFGE has proven to be robust enough to type strains with great resolution, is highly reproducible, and is considered the 'gold standard' technique for
typing MRSA. However, PFGE is time consuming and requires both specialized electrophoretic equipment and software. So there is the need of a simple technique just like PCR based typing of MRSA that relies upon the length of polymorphisms of the hypervariable region of the staphylococcal methicillin resistance gene (mec) for strain resolution. The near future will provide techniques for typing and for prediction of the epidemic capacity or pathogenicity of MRSA strains. Both structural and transcriptional differences in genomes can be revealed using DNA microarray assays. The availability of complete genome sequences of several strains will result in identification of new areas important for understanding the adaptive fitness of MRSA strains in different environments.

The curing experiments, transfer of resistance markers through conjugation and transformation together with the β-lactamase assay against selected antibiotics reveals a missing experimental-tag, the emphasis on which we believe will be a safe and cost effective measure to combat antimicrobial resistance: the use of consumable herbs of medicinal importance. The extracts from these herbs can be studied as curing agents and/or the inhibitors of antibiotic gene transfer and resistance enzymes. The metabolites so identified can be used in combination with antibiotics for counteracting this health problem.

The substantial decreases in frequency of conjugation at lower temperature particularly at 22 °C, the temperature prevailing in the anterior nares of human beings depicts a biological significance. Moreover, the clinical importance of regular nose washing as experienced by some communities such as Muslims, five times in a day while they offer prayers, needs to be analyzed based on population studies.
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