

Surveillance Study and Typing of Staph Organisms in Burn Unit

M.D. Thesis in
Medical Microbiology and Immunology

Submitted By

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List of Abbreviation

Abbreviation	
agr	accessory gene regulator
AIP	auto-inducing peptide
BTBSA	burned total body surface area
CA-MRSA	community-acquired methicillin resistant <i>S. aureus</i>
ccrAB and ccrC	recombinases for the cassette
CDC	The Centers for Disease Control and Prevention
CDSs	protein coding sequences
CGD	chronic granulomatous disease
ClfA	Clumping factor A
ClfB	Clumping factor B
Cna	Collagen binding protein
<i>coa</i>	Coagulase gene
CoNS	Coagulase negative staphylococci
<u>ESBL</u>	extended-spectrum b-lactamase
FnbpA	Fibronectin binding protein A
FnbpB	Fibronectin binding protein B
GISA	Glycopeptide intermediate staph aureus
HICPAC	Hospital Infection Control Practices Advisory Committee
Hlg	Gamma-hemolysin
HIV	human immunodeficiency virus
hVISA	heterogeneous vancomycin-intermediate <i>S. aureus</i>
Ica operon	Intracellular adhesion operon
ICU	intensive care unit
igG	Immunoglobulin G
ISA	Invasive staph aureus
IS	Insertion sequences
LFTs	liver function tests
Luk	Leukocidin
MHA	Muller-Hinton agar
MICs	Minimal inhibitory concentrations
MIDs	Multiplex identifiers
MLST	multilocus sequence typing
MLVF	Multilocus Variable-Number Tandem-Repeat
MOD-SA	Modified staph aureus strain
MSA	Mannitol salt agar

MSCRAMMs	microbial surface components recognizing adhesive matrix molecules
MSSA	methicillin sensitive <i>S. aureus</i>
NADPH	nicotinamide adenosine dinucleotide phosphate
NCCLS	the National Committee for Clinical Laboratory Standards
NETs	neutrophil extracellular traps
NO	Nitric oxide
oriF	the origin of replication
ORSAB	oxacillin resistance screening agar base
PBPs	penicillin-binding proteins
PFGE	Pulsed-field gel electrophoresis
Pls	Plasma-sensitive surface protein
PNAG	poly-N-acetylglucosamine
PVL	Panton-Valentine leukocidin
RE	Restriction enzyme
RM	Restriction modification
ROI, RNI	<u>Reactive oxygen and nitrogen intermediates</u>
RT-PCR	<u>Real time-polymerase chain reaction</u>
SaPIs	genes for superantigen toxins
<u>SCC</u>	Staphylococcal cassette chromosome
SCCcap1	Staphylococcal cassette chromosome capsule genes
SCN	severe congenital neutropenia
SCVs	Staphylococcal foodborne disease
SFD	Staphylococcal foodborne disease
siRNAs	small interfering RNAs
<i>Spa</i>	protein A gene
ssr	short sequence repeats
SSTI	Staph soft tissue infection
TSST	toxic shock syndrome toxin
<u>VISA</u>	vancomycin- intermediate <i>S. aureus</i>
VRE	<i>vancomycin-resistant enterococci</i>
VRSA	<u>Vancomycin resistant staph.aureus</u>

Abstract

Staphylococcus aureus (*S. aureus*) and methicillin resistant *S. aureus* (MRSA) are one of the leading causes of infections among burn patients.

Methods: Burn sites of 400 patients were swabbed and cultured on conventional culture media. MRSA isolates were identified using ORSAB and RT-PCR.

Results: *S.aureus* (40%) and MRSA (26%) are the most organisms infect burn wound.

Conclusion: every burn institution should determine the specific pattern of burn wound microbial colonization, and the antimicrobial sensitivity profiles, improving the overall infection-related morbidity and mortality.

Key words: burn infections, *S.aureus*, MRSA, ORSAB media, RT-PCR.

REVIEW

Chapter 1

Staphylococcus aureus

Staphylococcus aureus (*S. aureus*) is a Gram-positive spherical bacterium approximately 1 μm in diameter. Its cells form grape-like clusters, since cell division takes place in more than one plane. It is often found as a commensal associated with skin, skin glands, and mucous membranes, particularly in the nose of healthy individuals. It has been estimated that approx. 20–30% of the general population are *S. aureus* carriers (**Heyman, 2004**).

Enriched medium, *S. aureus* forms “golden yellow” colonies. On sheep blood agar plates, colonies of *S. aureus* often cause β -hemolysis. The golden pigmentation of *S. aureus* colonies is caused by the presence of carotenoids and has been reported to be a virulence factor protecting the pathogen against oxidants produced by the immune system (**Liu et al., 2005**).

Staphylococci are facultative anaerobes capable of generating energy by aerobic respiration, and by fermentation which yields mainly lactic acid. *Staphylococcus* spp. is catalase-positive, a feature differentiating them from *Streptococcus* spp., and they are oxidase-negative and require complex nutrients, e.g., many amino acids and vitamins B, for growth. *S. aureus* is very tolerant of high concentrations of sodium chloride, up to 1.7 molar (**Plata et al., 2009**).

Another feature of the *Staphylococcus* genus is the cell wall peptidoglycan structure that contains multiple glycine residues in the crossbridge, which causes susceptibility to lysostaphin. *S. aureus* produces coagulase which interacts with prothrombin in the blood causing plasma to coagulate by converting fibrinogen into fibrin. Coagulase test is used to distinguish *S. aureus* from other members of the genus, which are collectively designated as coagulase-negative staphylococci (**Ryan & Ray., 2004**).

Methods of S.aureus typing:

1- pulsed field gel electrophoresis:

Pulsed field gel electrophoresis (PFGE) was one of the first genome-based typing methods for MRSA. PFGE is often considered the gold standard for typing MRSA isolates in epidemiological studies. Although this method is known to be highly discriminatory, it is technically demanding and time-consuming, it has a low throughput, and its technical instability has adverse effects on reproducibility (*Babouee et al., 2011*).

PFGE involves embedding organisms in agarose, lysing the organisms in situ, and digesting the chromosomal DNA with restriction endonucleases (*SmaI*) that cleave infrequently. Slices of agarose containing the chromosomal DNA fragments are inserted into the wells of an agarose gel, and the restriction fragments are resolved into a pattern of discrete bands in the gel by an apparatus that switches the direction of current according to a predetermined pattern. The DNA restriction patterns of the isolates are then compared with one another to determine their relatedness (*Tenover et al., 1995*).

2- MLST(Multilocus Sequence Typing):

(MLST) is a sequence-based genotyping method based on polymorphisms (each variant is termed an allele) in seven housekeeping genes (loci) (*arcC, aroE, glpF, gmk, pta, tpi, and yqiL*) in *S. aureus* providing unique allelic profiles known as sequence types (*Larsen et al., 2012*).

DNA was extracted from bacterial cultures. Housekeeping genes of interest were amplified by PCR using primers from standardized MLST schemes, but with universal tails at the 5' end to allow the addition of 454 sequencing-specific nucleotides and isolate-specific multiplex identifiers (MIDs) in a second PCR round. The MLST target genes were amplified by RT-PCR (*Boers et al., 2012*).

3- Spa typing:

Recently, DNA sequencing of the polymorphic X, or short sequence repeat (SSR), region of the protein A gene (*spa*) has been proposed as an alternative to current techniques for the typing of *S. aureus*. The polymorphic X region consists of a variable number of 24-bp repeats and is located immediately upstream of the region encoding the C-terminal cell wall attachment sequence. The diversity of the SSR region seems to arise from deletion and duplication of the repetitive units and also by point mutation. While the biological function is not known, the protein A domain encoded by the X region may serve to extend the N-terminal immunoglobulin G binding portion of the protein through the cell wall. The existence of well-conserved regions flanking the X region coding sequence in *spa* allows the use of primers for PCR amplification and direct sequence typing (*shopsin et al., 1999*).

chromosomal DNA purified from each isolate as a template. PCR amplification of the SSR region of the *spa* gene. Sequences were determined by electrophoresis with the DNA sequencer (*shopsin et al., 1999*).

4- Coagulase gene (coa) repeat region nucleotide sequences:

Coagulase gene (*coa*) short sequence repeat region sequencing was used to measure relatedness among a collection of temporally and geographically diverse methicillin-resistant *S. aureus* isolates. The coagulase protein is an important virulence factor of *S. aureus*. Like *spa*, *coa* has a polymorphic repeat region that can be used for differentiating *S. aureus* isolates. The variable region of *coa* is comprised of 81-bp tandem (SSRs) that are variable in both number and sequence, as determined by restriction fragment length polymorphism analysis of PCR products (*Shopsin et al., 2000*).

chromosomal DNA purified from each isolate as a template. PCR amplification of the SSR region of the *coa* gene. Sequences were

determined by electrophoresis with the DNA sequencer (*shopsin et al., 2000*).

5- Ribotyping :

The amplification of the genomic 16S-23S rRNA spacer region was first described as a reliable technique for typing *Pseudomonas cepacia*, *S. aureus*, *Enterococcus faecium*, *Escherichia coli*, and *Enterobacter* spp. In the present study .scientists explored the natural polymorphism of the genomic 16S-23S rRNA region from *S. aureus* as a genotyping tool. The primer pairs were tested to check for the size of amplicons produced, and best visualization upon agar gel electrophoresis and ethidium bromide (*Oliveira and Ramos., 2002*).

whole-cell DNA was digested with a restriction enzyme (RE). DNA fragments were separated by horizontal agarose gel electrophoresis and transferred to a nylon membrane. Hybridization was performed with plasmid pKK3535, containing an rDNA operon of *Escherichia coli* .The DNA probe was labeled with biotin-7-dATP . Hybrids were revealed on the membrane by using the nonradioactive nucleic acid detection system. To select REs giving a high level of discrimination between isolates (*Blanc et al., 1994*).

6- SCCmec element typing:

SCCmec elements, detected in almost all MRSA strains, belong to particular type of the staphylococcal mobile genetic elements coding for meticillin-resistance and designated as staphylococcal cassette chromosome *mec* (*Katayama et al., 2000*).

first *SCCmec* element was identified in Japanese *S. aureus* strain, N315 in 1999 and shortly after two additional *SCCmec* from MRSA strains were determined . Based on detailed structural analysis these three *SCCmec* elements were classified as types I to III . In time, both new types of *SCCmec*, such as *SCCmecIV* . *SCCmecV* , *SCCmecVI* , *SCCmecVII* , *SCCmecVIII* , *SCCmecIX*, *SCCmecX* , *SCCmecXI* (*McCarthy and Lindsay, 2010*).

PCR based *SCCmec* typing methods: Two different approaches were applied in this methods; one was focused on analysis of these regions, whereas the other determine mainly *mec* class and *ccr* type (*Oliveira and de Lencastre, 2002*).

Real-time PCR based *SCCmec* typing methods: A multiplex scheme based on a real-time PCR targeting the *ccrB* regions of *SCCmec* types I to IV (*Francois et al., 2004*).

7- Multilocus Variable-Number Tandem-Repeat (MLVF):

MLVF analyzes the variation in the number of tandem repeats in seven genes (*clfA*, *clfB*, *sdrC*, *sdrD*, *sdrE*, *spa*, and *sspA*) by multiplex PCR and has been reported to be highly discriminatory and reproducible (*Holmes et al., 2010*).

8- Capsular typing:

Among those factors considered for typing, capsular polysaccharides expressed by *S. aureus* are one of them, since they are also important in the pathogenesis of staphylococcal infections. Most *S. aureus* isolates are encapsulated and so far eleven capsular serotypes have been described. Of these, types 5 and 8 predominate in approximately 75% of the clinical isolates. Though the method for capsular typing was described two decades ago, during the recent years typing of *S. aureus*, based on its capsular polysaccharide types is been increasingly adopted (*Paul-Satyaseela et al., 2011*).

suspended in 1 mL saline. Clumping occurred within 10 s for positive reactions. For every isolate both the type-8 and -5 antisera were used to exclude cross-reactivity. The prototype strains for the capsular types 5 and 8, their respective mutants, along with the necessary protocols (*Paul-Satyaseela et al., 2011*).

9- Phage typing:

For decades, bacteriophage typing was the standard method for typing of *S. aureus*. Phage typing is still widely used today, despite a number of drawbacks. Drawbacks include limited typeability of isolates, limited technical reproducibility of results, and variable expression of

epidemiological determinants, resulting in limited biological reproducibility (*Zadoks R. N. et al., 2002*).

strains of *S. aureus* were selectively isolated on mannitol salt agar and the mannitol-fermenting colonies were subcultured into 1 mL Nutrient Broth. The tubes were incubated at 37°C until the turbidity reached McFarland's standard of 0.5. This inoculum was spread on the Nutrient Agar and allowed to air dry. The conventional set of 23 phages were filled in the labeled wells of the Perspex block of the Lidwell's phage-typing apparatus and spotted at ×1 RTD. Next day the plates were examined against a dark background (*Paul-Satyaseela et al., 2011*).

10- Plasmid Profile Analysis:

Analysis of bacterial plasmids was the first molecular technique used for the epidemiological investigation of MRSA. This technique consists in the extraction of plasmid DNA and subsequent separation of this DNA by electrophoresis in agarose gels. It is an easily executed and interpreted technique, however it has several limitations, especially inherent to the fact that plasmids are mobile extrachromosomal elements that can be spontaneously lost or readily acquired by bacteria (*Trindade P. A. et al, 2002*).

Consequently, epidemiologically related isolates can display different plasmid profiles. Moreover, many plasmids carry resistance determinants contained in transposons that can be readily lost or acquired, quickly altering the composition of plasmid DNA. The reproducibility of the generated profiles can be affected by the fact that plasmids exist in different spatial conformations (supercoiled, nicked, and linear), which possess different migration velocities when submitted to agarose gel electrophoresis (*Trindade P. A. et al, 2002*).

Both the reproducibility and discriminatory power of plasmid profile analysis can be substantially enhanced by carrying out enzymatic restriction of the plasmids. The majority of MRSA isolates carry plasmids, but when these are absent the isolates are considered nontypeable. Another limitation is the number of plasmids present in these isolates, usually one or two, which leads to poor discrimination between them (*Trindade P. A. et al, 2002*).

Biochemical Reaction:

The ability of the staphylococci to ferment sugars varies greatly according to the strain employed. For this reason it is not possible to classify them on this basis with anything like the same precision as, for example, the coliform group of bacilli. As a rule the golden cocci have the greatest fermentative power, the white are less active. There is a wealth of literature on the fermentative capacities of the staphylococci, with a corresponding difference of opinion amongst the various authors as to the importance of the different sugars (*Wilson and Miles, 2007*).

Thus, lay stress on the reactions in maltose, lactose, glycerol and mannitol. On the other hand, come to the conclusion that the only sugar of differential value is lactose. Working with *S.aureus*, these authors found some strains formed acid from glucose, (63%), from maltose, and (49%) from lactose ; salicin, inulin and raffinose were rarely fermented, mannitol and dulcitol never (*Wilson and Miles, 2007*).

With these findings most authors disagree, particularly with regard to mannitol, which is generally held to be fermented by *S.aureus*, and frequently by *S.albus*. It is quite clear, however, that it is impossible to dogmatize on the reactions of any one strain. The scientists examined 121 aureus and albus strains on a large number of sugars, found that very few agreed in giving identical results (*Wilson and Miles, 2007*).

Similarly with litmus milk the reactions are variable. Studying 180 aureus and albus strains, scientists found that 75 strains produced acid, clot and peptonization, 60 strains produce acid, generally clot, and no peptonization, 22 strains produce alkali and peptonization, 16 strains produce alkali but no peptonization, while 7 strains produced no change (*Wilson and Miles, 2007*).

These findings are in agreement with those of other authors, except with regard to peptonization, which is less commonly reported. The proteolytic activity of staphylococci is not very strong. Some strains are fibrinolytic, and some, particularly those of canine origin, can digest coagulated horse serum. Lipase production has been reported by some scientists (*Wilson and Miles, 2007*).

The methyl-red test is generally positive with the aureus strains, negative with the citreus strains. The Voges-Proskauer reaction is given by most strains of *S. aureus* (**Wilson and Miles, 2007**).

Most strains of staphylococci reduce nitrates to nitrites. On the contrary, it is found that, though 49 out of 50 aureus strains reduced nitrates, only 23 out of 152 albus strains were able to do so. Hydrogen sulphide is stated by scientists to be formed in small quantity by the pyogenic staphylococci, in greater quantity by *S. albus*. We have been unable to confirm this (**Wilson and Miles, 2007**).

S. aureus infections:

S. aureus is a commensal and a pathogen. The anterior nares are the major site of colonization in humans. About 20–30% of individuals are persistent carriers of *S. aureus*, which means they are always colonized by this bacterium, and 30% are intermittent carriers (colonized transiently) (**Wertheim et al., 2005**).

Colonization significantly increases the risk of infections since it provides a reservoir of the pathogen from which bacteria are introduced when host defense is compromised. Patients with *S. aureus* infections are usually infected with the same strain that they carry as a commensal (**Plata et al., 2009**).

S. aureus is one of the main causes of hospital- and community-acquired infections which can result in serious consequences (**Diekema et al., 2001**).

Nosocomial *S. aureus* infections affect the bloodstream, skin, soft tissues and lower respiratory tracts. *S. aureus* can be a cause of central venous catheter-associated bacteremia and ventilator assisted pneumonia. It also causes serious deep-seated infections, such as endocarditis and osteomyelitis (**Schito, 2006**).

In addition to the infections listed above, *S. aureus* is often responsible for toxin-mediated diseases, such as toxic shock syndrome, scalded skin syndrome and staphylococcal foodborne diseases (SFD). Hospitalized patients are particularly exposed to *S. aureus* infections due to their compromised immune system and frequent catheter insertions and injections (*Lindsay & Holden, 2004*).

The Sentry Surveillance Program investigating worldwide *S. aureus* infections during a two-year period has revealed that this pathogen is the leading cause of bloodstream, lower respiratory tract and skin/soft tissues infections in all regions surveyed. The importance of this human pathogen, apart from its ability to cause life-threatening infections, is its remarkable potential to develop antimicrobial resistance (*Diekema et al., 2001*).

Virulence factors:

S. aureus is equipped with a great variety of virulence factors, which include both structural and secreted products participating in pathogenesis of infection:

(i) Attachment-improving agents:

S. aureus carries numerous surface proteins named “microbial surface components recognizing adhesive matrix molecules” (MSCRAMMs) that mediate attachment to host tissues and initiate colonization leading to an infection (*Gordon & Lowy., 2008*).

Fibronectin binding proteins A and B (FnbpA and FnbpB) participate in attachment of bacterial cells to an extra-cellular matrix component, fibronectin, and to plasma clot (*Plata K et al., 2009*).

Plasma-sensitive surface protein (Pls), once processed by plasmin, participates in binding to both fibrinogen and fibronectin. Protein A is a hallmark of *S. aureus* which is encoded by the *spa* gene and is a cell wall-associated protein that binds to the Fc domain of immunoglobulin G (IgG).

Protein A binds IgG in “wrong orientation” on the surface of *S. aureus* cells which is thought to disrupt opsonization and phagocytosis (*Hauck, Ohlsen, 2006*).

Collagen binding protein, Cna, is necessary for adherence of *S. aureus* to collagenous tissues and cartilage and it has been shown that antibodies against Cna block the bacteria attachment to those tissues (*Plata et al., 2009*).

Clumping factor A and B (ClfA and ClfB) mediate clumping and adherence of bacteria cells to fibrinogen in the presence of fibronectin. Clumping factors are thought to play a significant role in wound and foreign body infections and it has been shown that clfA mutant is less virulent than the wild type isogenic strain (*Plata et al., 2009*).

Protein A also exhibits an ability to bind to von Willebrand factor, a protein present at sites of damage of endothelium, and as a result, it can play a role in adherence and induction of endovascular diseases by *S. aureus* (*Hartleib et al., 2000*).

Implanted biomedical device-related *S. aureus* infections depend on the pathogen's ability to attach to the surface of the biomaterial and consequently to form a mucoid biofilm. Biofilms are complex bacterial populations which are surface-attached and enclosed in a polysaccharide matrix, composed of poly-N-acetylglucosamine (PNAG). PNAG production depends on proteins encoded by the *ica* (intracellular adhesion) operon (*Fitzpatrick et al., 2005*).

Biofilm-associated bacteria, unlike their planktonic counterparts, are resistant to the host immune responses and to antimicrobials, which often complicates treatment. It was reported that 60% of *S. aureus* strains were able to produce biofilm (*Arciola et al., 2001*).

Recent studies indicated that among clinical isolates of *S. aureus*, only between 45% and 70% (depending on the type of infection) strains were able to form biofilm. Those studies also suggested that no correlation exists between biofilm production and the type of staphylococcal infection (*Grinholc et al., 2007*).

However, contradictory results obtained by other investigators suggested that all *S. aureus* strains possess the *icaADBC* genes. In addition, there is a regulatory gene called *icaR* that, together with the *icaA* promoter, is subject to a multitude of regulatory effects linking *ica* gene expression to virulence regulator (*Rohde et al., 2001*).

(ii) Exotoxins:

One of the important characteristics of *S. aureus* is its capability to secrete toxins that disrupt membranes of host cells. Cytolytic toxins form β -barrel pores in the cytoplasmic membranes and cause leakage of the cell's content and lysis (*Foster, 2005*).

S. aureus secretes several cytolysin toxins, among them alpha-hemolysin, beta-hemolysin, gamma-hemolysin, leukocidin, and Panton-Valentine leukocidin (PVL) (*Kaneko & Kamio, 2004*).

Alpha-hemolysin, encoded by the *hla* gene, inserts into eukaryotic membrane and oligomerizes into a β -barrel that forms a pore which causes osmotic cytolysis. Alpha-hemolysin is particularly cytolytic toward human platelets and monocytes (*Menestrina et al., 2001*).

PVL is classified as a bicomponent cytolysin because it is dependent on two secreted proteins (LukF-PV and LukS-PV) that insert into the host's cytoplasmic membrane and hetero-oligomerize to form a pore (*Kaneko & Kamio, 2004*).

PVL exhibits a high affinity toward leukocytes and is mostly associated with community-acquired methicillin resistant *S. aureus* (CA-MRSA)

which causes severe necrotizing pneumonia and contagious skin infections (*Foster, 2005*).

Other bicomponent toxins, gamma-hemolysin (Hlg) and leukocidin (Luk), are cytotoxic toward erythrocytes and leukocytes, respectively (*Kaneko & Kamio, 2004*).

(iii) Superantigenic exotoxins:

S. aureus generates a group of powerful immuno-stimulatory proteins implicated in gastroenteritis and toxic shock syndrome. They are resistant to heat denaturation and proteases. These toxins have the ability to cross-link MHC class II molecules located on antigen-presenting cells with T-cell receptors, forming a trimolecular complex. Formation of the complex induces intense T-cell proliferation in an antigen-independent manner resulting in massive cytokine production and release which causes capillary leak, epithelial damage and hypotension (*Baker & Acharya, 2004*).

The primary function of superantigens is thought to weaken the host's immune system sufficiently to allow the pathogen to propagate and the disease to progress. The staphylococcal enterotoxins A, B, C, D, E, G, Q are responsible for staphylococcal foodborne diseases and toxic shock syndrome, while TSST-1 (toxic shock syndrome toxin-1) is the cause of toxic shock syndrome. The superantigen toxins are typically encoded by mobile genetic elements (*Novick, 2003*).

Small-colony variants:

Small-colony variants (SCVs) represent a subpopulation of naturally occurring, slowly growing *S. aureus* with distinct phenotype and pathogenetic features. SCVs have been reported to cause recurrent, persistent infections many years after the initial infection had been cured (*Plata et al., 2009*).

Very often they (obligate intracellular) reside inside human cells avoiding host defenses and antimicrobial chemotherapeutics. SCVs are defective in their electron transport pathways and usually form non-pigmented, non-hemolytic colonies on agar (*Kaneko & Kamio, 2004*).

The small-colony variants display marked auxotrophisms for thymidine, menadione and/or hemin. They exhibit reduced rate of metabolism and are less virulent, but due to their low growth and reduced cell wall synthesis, they are more tolerant of β -lactam antibiotics than the wild-type parents. Their low membrane potential makes them also resistant to aminoglycoside antibiotics (*Proctor et al., 2006*).

Regulation of genes involved in virulence:

The genes coding for virulence factors are regulated in a tightly coordinated manner that is synchronized with the biological cycle of *S. aureus*. The production of factors involved in virulence is controlled by quorum sensing mechanism. In *S. aureus*, genes coding for surface proteins are down regulated during early stages of the growth whereas genes that encode secreted proteins are up regulated in late exponential phase (*Plata et al., 2009*).

This pattern of gene expression in which surface proteins involved in adhesion and defense against host's immune system (protein A, coagulase, fibronectin binding proteins, among many others) are synthesized before production of secreted hemolysins, cytotoxins, proteases and other degradative enzymes seems to reflect a strategy of *S. aureus* in which the pathogen first establishes itself in the host and only then attacks it. This regulation is, in large part, due to the accessory gene regulator (*agr*) two component system (*Novick & Geisinger, 2008*).

The *agr* locus consists of two divergent transcription units RNAII and RNAIII driven by two promoters, P2 and P3, respectively. The P2 transcript, RNAII, contains four cistrons: *agrA*, *agrB*, *agrC* and *agrD*. The sensor *AgrC*, and the response regulator, *AgrA*, comprise the two component system that responds to auto-inducing peptide (AIP). This peptide is present in the extracellular environment and drives transcription from both P2 and P3 promoters (*Plata et al., 2009*).

RNAIII stimulate the expression of post-exponentially synthesized extracellular toxins and enzymes and represses synthesis of exponential phase surface proteins. RNAIII acts primarily as an antisense RNA for translational activation of certain mRNAs or binds to the ribosome binding site in the case of repressed mRNAs, preventing ribosome binding and inducing fast mRNA degradation by endoribonuclease III (*Boisset et al., 2007*).

Organization of the *S. aureus* genome:

The first genome sequences of *S. aureus* strains Mu50 and N315 were published in 2001. At present, complete genomic sequences of ten *S. aureus* strains are available, and the genomes of several others have been partially determined (*Diep et al., 2006*).

The genome of *S. aureus* is a circular chromosome that is 2.8–2.9 Mbp in size; with a G+C content of about 33%. The chromosome encodes approximately 2700 CDSs (protein coding sequences) as well as structural and regulatory RNAs. It has been proposed that the *S. aureus* genome is composed of the core genome, accessory component and foreign genes (*Plata et al., 2009*).

The core genes are present in more than 95% of isolates, represent 75% of any *S. aureus* genome and determine the backbone of the genome. The organization of the core component is highly conserved and the identity of individual genes between isolates is 98–100%. The majority of core genes are associated with fundamental functional categories of housekeeping functions and central metabolism (*Plata et al., 2009*).

The accessory component includes genetic regions present in 1–95% of isolates and accounts for about 25% of any *S. aureus* genome. It typically consists of mobile genetic elements that have or previously had the ability of horizontal transfer between strains. These genetic elements include pathogenicity islands, genomic islands, prophages, chromosomal cassettes and transposons (*Lindsay & Holden, 2004*).

Pathogenicity islands

The family of staphylococcal pathogenicity islands that carry genes for superantigen toxins (*SaPIs*) are 15–20 kb elements located at constant positions in the chromosome. *SaPIs* possess certain bacteriophage-related attributes: genes coding for integrases, helicases and terminases, and flanking direct repeats (*Novick, 2003*).

The archetype of this family, *SaPI1*, codes for toxic shock syndrome toxin TSST and is excised and induced to replicate as well as transduced at high frequency by phage 80 α . DNA of *SaPI1* is encapsulated into 80 α phage-like particles for transfer (*Ruzin et al., 2001*).

Another member of *SapI* family, *SapI3*, encodes enterotoxin B and is thought to be mobilized and encapsulated by phage 29 (*Novick, 2003*).

Members of the *SaPI* family have been found in almost all strains of *S. aureus* sequenced so far (*Diep et al., 2006*).

In addition to *SaPIs*, *S. aureus* strains contain Genomic islands carry genes coding for about half of the *S. aureus* toxins and virulence factors, and greatly contribute to the pathogenicity of this species (*Gill et al., 2005*).

They are found in all sequenced strains in the same locations and some of the genes carried by them are highly conserved. They encode their own integrase and usually are spontaneously excised from the host chromosome (*Baba et al., 2002*).

Members of this family of genomic islands include, but are not limited to *vSa1* (carrying enterotoxin genes *seb*, *tsst*, *ear*), *vSa2* (containing genes encoding enterotoxin (*sec*) and TSST (*Gill et al., 2005*)).

Additionally they carry a cassette encoding a restriction-modification system and genes encoding leukocidin (*lukDE*) (*Baba et al., 2002*).

Prophages:

Prophages of *S. aureus* can be classified into three groups based on the size of their genome. Class I includes phages with genomes of less than 20kb, class II has a genetic material of approximately 40kb and class III of more than 125 kb (*Kwan et al., 2005*).

Prophages are thought to play an important role in evolution and pathogenicity of *S. aureus* and very often offer means for the horizontal transfer of genetic information. Each of the *S. aureus* strains sequenced so far contains between one and three prophages, most of them carry virulence determinants exemplified by enterotoxins A, G, K, exfoliative toxin, staphylokinase and Panton-Valentine Leukocidin (*Diep et al., 2006*).

Insertion sequences and transposons:

Insertion sequences (IS) carry at least one gene coding for a transposase which participates in the recombination required for transposition. Most IS elements also contain short inverted terminal repeats acting as transposase binding sites (*Baba et al., 2004*).

Insertion elements are randomly scattered throughout the genome of *S. aureus*, both in coding and non-coding regions. In MRSA, *S. aureus* N315 and Mu50 strains, eight copies of IS1181 have been found (*Gill et al., 2005*).

Transposons are larger transposable genetic elements that, in addition to a transposase gene, carry other genes which very often are antibiotic resistance determinants. *S. aureus* is the host to more than ten transposons, the majority of which carry antibiotic resistance genes (*Baba et al., 2004*).

Plasmids:

Plasmids, defined as extrachromosomal genetic elements bearing only non-essential genes which, however, may provide a benefit to the host under special environmental conditions, often encode factors determining resistance to antibiotics or heavy metals, virulence factors and proteins facilitating survival in the presence of unusual nutrients (*Wegrzyn, 2005*).

Plasmids of *S. aureus* have been categorized into three classes. Class I plasmids are of the size of 1–5 kb and occur in high copy number (15–50 per cell). They usually carry a single antibiotic resistance determinant. The class II plasmids are of intermediate size and occur in intermediate copy number (15–5 per cell), and they usually code for β -lactamase and confer resistance to inorganic ions. The last group of staphylococcal plasmids, class III, consists of large conjugative plasmids (40–60 kb). Class III plasmids (copy number: 1–5 per cell) carry multiple resistance determinants exemplified by resistance to trimethoprim, gentamycin and ethidium bromide (*Plata et al., 2009*).

The plasmids often can serve as means by which antibiotics resistance is transmitted. Moreover, the conjugative plasmids encode their own conjugative horizontal transfer mechanism by *tra* genes that offer an advantage by which transfer of extrachromosomal genetic information to other bacteria occurs (*Holden et al., 2004*).

Staphylococcal cassette chromosome (SCC):

SCC elements are mobile genetic elements that integrate at the same site on the *S. aureus* chromosome. The most notorious of these are SCC*mec* elements that carry the *mec* region encoding methicillin resistance, but alternatively they can also carry other sets of genes, such as capsule genes (SCC*cap1*) (*Luong et al., 2002*).

SCC*mec* elements were probably acquired horizontally from *coagulase-negative staphylococci* (CoNS), such as *S. haemolyticus*. However, it is not known exactly how they moved as they do not appear to encode a physical transfer mechanism and are often too large to be transferred by

bacteriophages. Epidemiological data suggest that transfer of *SCCmec* from CoNS is probably a rare event and has possibly occurred only a handful of times (*Katayama et al., 2001*).

However, at least four different versions of *SCCmec* genes are found in *S.aureus*: *SCCmec* types I to IV. A recent estimate of the number of times that *SCCmec* genes has been acquired by *S. aureus* suggests that at least 20 separate events have occurred. Because some of the elements consist of many different transposons, insertion sequences and plasmids, it is possible that re-arrangements occur in *S. aureus* and that this is an on-going process (*Robinson and Enright, 2003*).

Type IV *SCCmec* genes has appeared most recently and is associated with community strains. This class of element appears to have spread much more rapidly than the other versions, which could be due to its smaller size allowing transduction to take place (*Fey et al., 2003*).

SCCmec elements are currently found in approximately half of the common CC types defined by MLST. This suggests that there might be some barrier preventing elements from moving to certain lineages. This could potentially be due to phage immunity or restriction modification (RM), where foreign DNA is recognized and digested by restriction enzymes. RM genes are wide spread in *S. aureus*, and sometimes carried on mobile genetic elements. This raises the possibility that if *SCCmec* spreads to new CC types, MRSA rates could increase further as these strains are selected for over their MSSA (methicillin sensitive staph.aureus) equivalents (*Robinson and Enright, 2003*).

Host defence and pathogenesis in staphylococcal infection:

Classically, neutrophils represent the major host defence cells against this organism, yet recent work suggests that staphylococcal actions render granulocytes ineffectual. Restoring their potency may offer the key to reversing failures of innate immunity (*Anwar et al., 2009*).

Approximately 30% of the population is colonized with *S. aureus* either chronically or intermittently, although this is of no pathological consequence per se. Colonization is, however, linked intimately to disease as it is a major risk factor for invasive infection, and it is partly

these carriage rates which help *S. aureus* to thrive as an opportunist(*Nouwenet al., 2008*).

Typically, *S. aureus* exploits vulnerable populations such as the elderly, immunosuppressed or debilitated. Major risk factors include breaches of the skin barrier, often by trauma, intravenous drug use or medical instrumentation and impaired mucosal immunity, for example, due to cystic fibrosis, artificial ventilation or post-influenza infection. These deficiencies provide bacterial access to local tissue and to the bloodstream, facilitating dissemination of infection. Local infections may be highly destructive in situ, while haematogenous spread results in deep-seated invasive disease including septic arthritis, osteomyelitis, pneumonia and endocarditis (*Anwar et al., 2009*).

Mechanism of killing of Neutrophil: they are highly efficient at killing phagocytosed pathogens. They engage a complex cascade of cellular events to eradicate pathogens via oxidative and non-oxidative mechanisms. Following bacterial phagocytosis, the nicotinamide adenosine dinucleotide phosphate (NADPH) enzyme complex and nitric oxide (NO) synthase immediately generate reactive oxygen and nitrogen intermediates (ROI, RNI) within the phagosomal compartment, molecules which are implicated directly in microbicidal activity (*Anwar et al., 2009*).

Lysosomes laden with proteases, cathepsins, defensins and other antimicrobial proteins fuse rapidly with the phagosome, discharging their potent contents into the phagolysosome. The generation of superoxide by the NADPH complex permits activation of granule proteases within the acidified phagolysosome, thus linking oxidative and non-oxidative bactericidal mechanisms (*Segal, 2005*).

Neutrophils are also capable of killing non-phagocytosed pathogens through the formation of neutrophil extracellular traps (NETs), comprising tangles of chromatin and granule proteins which are released by rupture of the neutrophil cell membrane. These structures ensnare

bacteria and kill them by exposure to high local concentrations of antimicrobial molecules (*Fuchs et al., 2007*).

The consequences of neutrophil deficiencies in number or function substantiate their critical bactericidal role, as affected patients succumb to repeated bacterial infections. Evidence provided by patients with genetic defects implicates neutrophils in opposing *S. aureus* specifically (*Spickett, 2008*).

Patients susceptible to recurrent *S. aureus* infection include those with chronic neutropenia such as severe congenital neutropenia (SCN), impaired neutrophil migration such as deficiency in leucocyte adhesion molecules and those with disorders of intracellular killing (*Lakshman & Finn, 2001*).

This latter group includes patients with chronic granulomatous disease (CGD), who exhibit profoundly impaired oxidative killing due to defective assembly of the NADPH oxidase complex and Chediak Higashi patients, in whom degranulation is impaired due to failure of phagolysosome maturation (*Segal et al., 2000*).

Furthermore, experimental work using murine models has attributed roles for specific neutrophil microbicidal proteases to particular pathogens; for example, selectively knocking out neutrophil cathepsin G, but not neutrophil elastase, predisposes to *S. aureus* infection. There are also numerous in vitro studies that support the neutrophil as the key innate effector cell in controlling *S. aureus* infection (*Miller et al., 2007*).

Consistent with the notion that neutrophils are a major resource in the conflict against invading *S. aureus*, the bacterium invests in panoply of virulence determinants to avoid recognition and phagocytosis by neutrophils (*Jongerijs et al., 2007*).

Several secreted and cell-bound proteins act in concert to effectively thwart neutrophil responses at multiple stages including chemotaxis,

opsonization, activation and phagocytosis. These sophisticated mechanisms equip the bacterium with major advantages over neutrophils. Additionally, the acquired immune response is considered weak in the face of this pathogen because the presence of anti-staphylococcal antibodies does not confer protection against further infection (*Gjertsson et al., 2000*).

Despite this, there is a growing body of evidence which suggests that neutrophil defences are of only limited efficacy against staphylococcal insult. Abscess formation is a typical pathology during *S. aureus* infection which comprises bacteria and recruited neutrophils, many of which are merely corpses, walled off by a fibrin mesh. This is clearly a neutrophil-rich site and yet it is often a focus of persistent infection, allowing speculation that an abscess represents a frustrated immune response: it can contain infection but is unable to resolve it. Also, patients rendered neutropaenic acutely through the administration of chemotherapy are susceptible to a broad range of pathogens and *S. aureus*, although important, does not predominate (*Viscoli et al., 2005*).

There is a direct evidence of intracellular survival of *S. aureus* within neutrophils although considered classically an extracellular pathogen, *S. aureus* is known to possess many virulence determinants which protect it from neutrophil microbicides. For example, physical and electrochemical cell wall properties resist the effects of neutrophil defensins and lysozyme, while neutralizing enzymes and carotenoid pigment confer resistance to ROI (*Liu et al., 2005*).

Both *in vitro* and *in vivo* work has supported that *S. aureus* up-regulates a plethora of virulence factors, including haemolysins, leucotoxins, iron scavengers and stress response genes, when exposed to purified neutrophil derived anti-microbial factors. Moreover, these potent microbicides, including hydrogen peroxide, hypochlorous acid and azurophilic granule proteins, merely exerted bacteriostatic rather than bactericidal effects. It was established that intracellular bacteria remain viable and virulent (*Palazzolo-Balance et al., 2008*).

They described the recovery of viable *S.aureus* from neutrophils isolated from a murine peritonitis model and that infected neutrophils were sufficient to establish infection in a naive mouse (*Gresham et al.,2000*).

Electron microscopy revealed that *S. aureus* strains better able to survive within neutrophils were localized within large vacuoles termed ‘spaciousphagosomes’ and phagosomal membranes sometimes appeared partially degraded, suggestive of an early stage of bacterial escape into the cytoplasm. Notably, neutrophil depletion resulted in improved outcome of infection in this study and others, suggesting that an excess of neutrophils may perversely facilitate infection and the persistence of inflammation (*Mcloughlin et al., 2008*).

The intracellular survival of *S. aureus* within macrophages whereby the bacteria exist ‘silently’ inside phagolysosomes for several days and subsequently escape by inducing spontaneous cell lysis. This process is dependent upon multiple virulence factors, in particular a haemolysin (*Kubica et al., 2008*).

CONS (coagulase negative staphylococci):

The human skin and mucous membranes represent a diverse environment of bacteria, the normal microflora. Probably the most important bacteria of this microflora are members of the genus *Staphylococcus*. The genus *Staphylococcus* is currently divided in 38 species and 17 subspecies, half of which are indigenous to humans. *Staphylococci* generally have a benign or symbiotic relationship with their host. However, they may develop into a pathogen if they gain entry into the host tissue through trauma of the cutaneous barrier, inoculation by needles, or implantation of medical devices (*Heikens et al., 2005*).

In last two decades, CONS have also emerged as significant pathogens, especially in immunocompromised patients, premature newborns, and patients with implanted biomaterials. The most frequently encountered CONS species associated with human infections is *S.epidermidis*, in particular in association within intravascular catheters. In addition, *S.*

epidermidis is the predominant agent of nosocomial bacteremia, prosthetic-valve endocarditis, surgical wounds, central nervous system shunt infections, intravascular catheter-related infections, peritoneal dialysis-related infections, and infections of prosthetic joints (**Heikens et al., 2005**).

The second most frequently encountered CONS species is *S.haemolyticus*. *S. haemolyticus* has been implicated in native-valve endocarditis, septicemia, peritonitis, and wound, bone, and joint infections. Other CONS species are involved in a variety of infections. For example, *S.saprophyticus* is an important pathogen in human urinary tract infections, especially in young, sexually active females, and *S.lugdunensis* has been implicated in arthritis, catheter infections, and prosthetic joint infections (**Heikens et al.,2005**).

Chapter 2

Antibiotic Resistance

The emergence, spread and globalization of antimicrobial resistance:

Antibacterial therapy has only emerged over the last 60 years as a practical proposition and has become one of the pillars of modern medicine. The removal of the scourge of premature death due to bacterial infection is now taken for granted in the developed world, but this is threatened by the development of resistance to antimicrobials. Despite this, we have seen the development, even since their early use, of antibiotic resistance in many bacterial species. The first description of the clinical use of penicillin was contemporaneous with a report of an enzyme (named penicillinase by the authors)—a specific member of the family of beta-lactamases—that destroyed benzylpenicillin and conferred resistance to penicillin (*Hawkey, 2008*).

Surprisingly, penicillinase production in *S.aureus* spread rapidly, and by the late 1940s, 50% of the *S. aureus* in the UK were positive for this trait. This was closely followed by the accumulation of resistance to penicillin, tetracycline and macrolides in the 1950s, creating strains of *S. aureus* that caused considerable problems in the management of nosocomial infection. In contrast, vancomycin has been used for nearly 50 years and yet significant numbers of *S. aureus* isolates with high-level resistance are yet to emerge. Thus, the emergence of resistance to antibiotics is associated with their use, although the precise correlation can be highly variable (*Hawkey, 2008*).

It is clear that the horizontal gene pool, which consists of genes present on a plethora of diverse mobile genetic elements, results in the lateral transfer of genes both among strains of an individual species and among different species of both Gram-negative and Gram-positive bacteria and is the process whereby we arrive at multi-resistant bacteria. The engine driving this process is the selective pressure of antimicrobial use (*Lautenbach et al., 2006*).

The movement of people, and also food (which is increasingly screened for recognized human pathogens such as *Salmonella* and *Campylobacter*, but which is not routinely screened) may explain well the movement of

particular genes that have emerged in certain parts of the world and then spread (*Hawkey, 2008*).

The costs associated with antimicrobial resistance are multiple; inadequate or failed treatment of patients leading to morbidity and mortality is a huge human cost. However, this cost pales into insignificance when compared with the cost of the disruption of the delivery of healthcare services caused by multiple antibiotic-resistant bacteria (cost of isolation, cross-infection control and cancelled procedures) (*Hawkey, 2008*).

Mechanisms of Antibiotic Resistance:

At least 17 different classes of antibiotics have been produced to date. Unfortunately, for each one of these classes at least one mechanism of resistance (and many times more than one) has developed over the years. In fact, in some cases bacteria have been able to develop simultaneous resistance to two or more antibiotic classes, making the treatment of infections caused by these microorganisms extremely difficult, very costly and in many instances associated with high morbidity and mortality (*Levy & Marshall, 2004*).

In general, it can be said that bacterial resistance has its foundation at the genetic level. This means that in most cases of bacterial resistance, changes in the genetic make up of the previously susceptible bacteria take place, either via a mutation or by the introduction of new genetic information. The expression of these genetic changes in the cell result in changes in one or more biological mechanisms of the affected bacteria and ultimately determine the specific type of resistance that the bacteria develops, resulting in a myriad of possible biological forms of resistance (*Sefton, 2002*).

(i) Genetic Mechanisms of Resistance:

For antibiotic resistance to develop, it is necessary that two key elements combine: the presence of an antibiotic capable of inhibiting the

majority of bacteria present in a colony and a heterogeneous colony of bacteria where at least one of this bacterium carries the genetic determinant capable of expressing resistance to the antibiotic (*Levy & Marshall, 2004*).

Once this happens, susceptible bacteria in the colony will die whereas the resistant strains will survive. These surviving bacteria possess the genetic determinants that codify the type and intensity of resistance to be expressed by the bacterial cell. Selection of these bacteria results in the selection of these genes that can now spread and propagate to other bacteria (*Levy & Marshall, 2004*).

Resistance to antibiotics can be natural (intrinsic) or acquired and can be transmitted horizontally or vertically. Whereas the natural form of antibiotic resistance is caused by a spontaneous gene mutation in the lack of selective pressure due to the presence of antibiotics and is far much less common than the acquired one, it can also play a role in the development of resistance (*Alanis, 2005*).

For the most part, however, the micro-ecological pressure exerted by the presence of an antibiotic is a potent stimulus to elicit a bacterial adaptation response and is the most common cause of bacterial resistance to antibiotics. Susceptible bacteria can acquire resistance to antimicrobial agents by either genetic mutation or by accepting antimicrobial resistance genes from other bacteria. The genes that codify this resistance (the “resistant genes”) are normally located in specialized fragments of DNA known as transposons (sections of DNA containing “sticky endings”), which allow the resistance genes to easily move from one plasmid to another (*Sefton, 2002*).

Some transposons may contain a special, more complex DNA fragment called “integron”, a site capable of integrating different antibiotic resistance genes and thus able to confer multiple antibiotic resistance to a bacteria. Integrons have been identified in both gram-negative and gram-positive bacteria, and they seem to confer high-level multiple drug resistance to the bacteria that carry and express them. Once a genetic

mutation occurs and causes a change in the bacterial DNA, genetic material can be transferred among bacteria by several means. The most common mechanisms of genetic transfer are conjugation, transformation and transduction (*Levy & Marshall, 2004*).

(a) Conjugation:

Conjugation is the most important and the most common mechanism of transmission of resistance in bacteria. This mechanism is normally mediated by plasmids (circular fragments of DNA) that are simpler than chromosomal DNA and can replicate independently of the chromosome. The mechanism of transmission of plasmids among bacteria is via the formation of a “pilus” (a hollow tubular structure) that forms between bacteria when they are next to each other, thus connecting them temporarily and allowing the passage of these DNA fragments (*Alfonso, 2005*).

(b) Transformation:

Transformation is another form of transmission of bacterial resistance genes that takes place when there is direct passage of free DNA (also known as “naked DNA”) from one cell to another. The “naked DNA” usually originates from other bacteria that have died and broken apart close to the receiving bacteria. The receiving bacteria then simply introduce the free DNA into their cytoplasm and incorporate it into their own DNA (*Alanis, 2005*).

(c) Transduction:

Transduction is a third mechanism of genetic transfer and occurs via the use of a “vector”, most often viruses capable of infecting bacteria also known as “bacteriophages” (or simply “phages”). The virus containing the bacterial gene that codifies antibiotic resistance (the “resistant DNA”) infects the new bacterial cell and introduces this genetic material into the receiving bacteria. Most times, the infecting bacteriophage also introduces to the receiving bacteria its own viral DNA, which then takes over the bacterial replication system forcing the cell to produce more

copies of the infecting virus until the bacterial cell dies and liberates these new bacteriophages, which then go on to infect other cells (*Alanis, 2005*).

(ii) Biological Mechanisms of Resistance:

Which ever way a gene is transferred to a bacterium, the development of antibiotic resistance occurs when the gene is able to express itself and produce a tangible biological effect resulting in the loss of activity of the antibiotic. These biological mechanisms are many and varied:

(a) Antibiotic destruction:

This destruction or transformation occurs when the bacteria produces one or more enzymes that chemically degrade or modify the antimicrobial making them inactive against the bacteria. This is a common mechanism of resistance and probably one of the oldest ones affecting several antibiotics but especially β -lactam antibiotics via the bacterial production of β -lactamases (*Jacoby & Munoz-Price, 2005*).

(b) Antibiotic active efflux:

Antibiotic active efflux is relevant for antibiotics that act inside the bacteria and takes place when the microorganism is capable of developing an active transport mechanism that pumps the antibiotic molecules that penetrated into the cell to the outside milieu until it reaches a concentration below that necessary for the antibiotic to have antibacterial activity. This means that the efflux transport mechanism must be stronger than the influx mechanism in order to be effective. Efflux was first described for tetracycline and macrolide antibiotics but is now common for many other antibiotics such as fluoroquinolones (*Hooper, 2005*).

(c) Receptor modification:

Receptor modification occurs when the intracellular target or receptor of the antibiotic drug is altered by the bacteria, resulting in the lack of binding and consequently the lack of antibacterial effect. Examples of this mechanism include modifications in the structural conformation of

penicillin-binding proteins (PBPs) observed in certain types of penicillin resistance, ribosomal alterations that can render aminoglycosides, macrolides or tetracyclines inactive, and DNA-gyrase modifications resulting in resistance to fluoroquinolones (*Levy & Marshall, 2004*).

(d) Altered metabolic pathway:

Microorganisms develop an altered metabolic pathway that bypasses the reaction inhibited by the drug. Example: Some sulfonamide-resistant bacteria do not require extracellular PABA but, like mammalian cells, can utilize preformed folic acid (*Brooks et al, 2007*).

(e) Altered enzyme:

Microorganisms develop an altered enzyme that can still perform its metabolic function but is much less affected by the drug. Example: In trimethoprim-resistant bacteria, the dihydrofolic acid reductase is inhibited far less efficiently than in trimethoprim-susceptible bacteria (*Brooks et al, 2007*).

(f) Target overproduction:

In VRSA, alterations in the bacterial cell wall result in reduced autolytic activity and wall thickening. This is thought to result in an impaired ability of vancomycin to reach its binding site and occurs specifically during the cell cycle when the division septum is being formed. These changes are particularly noted after prior exposure to vancomycin (*Holmes et al., 2012*).

Risk factors for development of Antibiotic Resistance?

Different factors play a role in the development of antibiotic resistance but what exactly determines that some bacteria become resistant to a specific drug and not to others and what is the specific role and the “relative weight” of each one of these factors in this process remains to be defined (Table 1).

Practices associated with the development of antibiotic resistance
<ol style="list-style-type: none"> 1. Excessive and irrational over-utilization of antibiotics in outpatient practice and in hospitalized patients, either therapeutically or prophylactically. 2. Use of antibiotics in agricultural industry, particularly in the production of food. 3. Longer survival of severely ill patients. 4. Longer life expectancy with increased use of antibiotics in the elderly. 5. Advances in medical science have resulted in the survival of many patients with severe illness and at risk for infections: Critically ill patients Immunosuppression Congenital diseases (i.e., cystic fibrosis) 6. Lack of use of proven and effective preventive infection control measures: such as hand washing, antibiotic usage restrictions and proper isolation of patients with resistant infections 7. Increased use of invasive procedures 8. Increased use of prosthetic devices and foreign bodies amenable to superinfection with resistant bacteria

Table 1: Risk factors for the development of antibiotic resistance
(Alfonso, 2005)

prevalance of resistance in *S.aureus* and CONS:

(i) *S. aureus*:

Penicillinase-producing hospital strains of *S. aureus* that were also resistant to the commonly available antimicrobials caused considerable clinical problems in the 1950s. These problems were solved initially by the introduction of methicillin and then by the introduction of the related semi-synthetic penicillins, cloxacillin and flucloxacillin, which resulted in a marked decline in these strains. Very shortly after the introduction of methicillin in 1960, three resistant isolates were noted from the same hospital in southern England. Interestingly, despite an early surge in cases in the 1960s, MRSA rates generally fell in Europe through to the early 1980s. The cause of this decline is not clear, but may relate to reductions in the prescribing of tetracyclines and vigorous infection control. In the

1980s, a rise in the frequency of gentamicin resistant MRSA was reported from the USA, Ireland and the UK (*Hawkey, 2008*).

The spread of multidrug-resistant *Staphylococcus aureus* (MRSA) strains in the clinical environment has begun to pose serious limits to treatment options. Yet virtually nothing is known about how resistance traits are acquired in vivo (*Mwangi et al, 2007*).

The spectacular adaptive capacity of this pathogen resulted in the emergence and worldwide spread of lineages that acquired resistance to the majority of available antimicrobial agents. The choice of therapy against such multidrug-resistant *S. aureus* (MRSA) strains has been narrowed to a few antibacterial agents, among them the glycopeptide antibiotic vancomycin, which has become the mainstay of therapy worldwide (*Mwangi et al., 2007*).

A wide variation in the prevalence of MRSA as a proportion of significant *S. aureus* isolates, ranging from ,1% in countries such as the Netherlands to rates of 25%to 50% in much of the Americas, Australia and some countries in southern Europe. MRSA was initially thought to have arisen by a single genetic event in which a large piece of mobile DNA (the staphylococcal cassette chromosome, *SCCmec*) was transferred from a coagulase-negative staphylococcus into the *S. aureus* genome and inserted close to the origin of replication (*oriF*). It is now clear that this event has occurred on a number of occasions as there are multiple types of *SCCmec* cassette arrangements, which represent separate horizontal gene transfers. The *SCCmec* element has a unique mechanism of mobilization by which it excises and integrates into the new host chromosome, the element itself carrying recombinases for the cassette (*ccrAB* and *ccrC*) (*Grundmann, et al., 2006*).

The dramatic increase in the occurrence of infections caused by MRSA led to substantially increased usage of vancomycin, but surprisingly, no resistance was seen prior to 1997. The first resistant strains identified were designated vancomycin intermediate *S. aureus*, and these have now been reported worldwide. There is evidence that these strains have a

thickened cell wall, which results in the elevation of MICs of vancomycin to 8–16 mg/L (*Appelbaum, 2006b*).

The biggest fear with regard to resistance in MRSA has been the possibility of transfer of the *vanA* gene complex from glycopeptide-resistant enterococci into *S. aureus* to produce vancomycin-resistant strains (VRSA) and the rapid dissemination of the clone or clones that have acquired these genes. An early in vitro experiment showed that it was possible to transfer and express *vanA* in *S. aureus* and between 2002 and 2007, six clinical isolates of VRSA—all carrying the *vanA* gene complex—have been reported in the USA (*Sung & Lindsay, 2007*).

(ii) Coagulase negative staphylococci:

CoNS are a major cause of nosocomial bacteremia and septicemia, especially for the patients who have immune deficiency and malignancy, which can lead to morbidity and even mortality. Despite the recent introduction of antimicrobial agents and medical improvements in controlling the frequency and morbidity of staphylococci infections, they are persistent as an important hospital and community pathogen (*Koksal et al., 2009*).

Furthermore, these bacteria have become a major concern to the medical community due to the fact that they have an extraordinary ability to adapt rapidly to antibiotic stress. Because of the widespread use of penicillin in 1950s, penicillin-resistant Staphylococci spread in hospitals. Afterwards methicillin and its derivatives became the drugs of choice for the treatment of infections caused by staphylococci. Soon thereafter, methicillin-resistant staphylococci were reported. All methicillin-resistant CoNS have been displayed to contain a *mecA* gene or its gene product, PBP-2a, and it may easily spread to all methicillin resistant CoNS, probably through transposons (*Koksal et al., 2009*).

Methicillin-resistant staphylococci are resistant to all other penicillins, carbapenems, cepheems and beta-lactam/beta-lactamase inhibitor combinations. Consequently, these antibiotics should not be used for treating of methicillin-resistant staphylococci infections. Recently,

several studies have shown that the methicillin-resistant staphylococci have started to gain resistance to many widely used antibiotics (quinolone, macrolide group antibiotics, aminoglycosides, tetracycline, trimethoprim-sulphamethoxazole, clindamycin, chloramphenicol) (*Koksal et al., 2009*).

Some studies have reported 54–92% resistance rates to gentamicin that has been used along with a beta-lactamase-stable penicillin for empirical treatment of sepsis since early 1970s. The transfer of gentamicin resistance determinants usually residing on conjugative plasmids has been shown between species of coagulase negative staphylococci and between *S. epidermidis* and *S. aureus*. Furthermore in many studies, high resistance ratios against erythromycin, clindamycin, tetracycline and ciprofloxacin were reported. Additionally, prolonged therapy with quinolones may lead to the development of cross-resistance in methicillin-resistant staphylococci. In various reports, the resistance to chloramphenicol was found to be 48–68%. The resistance increase against trimethoprim-sulfamethoxazole, which is an alternative medicine in the treatment of methicillin-resistant staphylococci infections, is recently receiving attention (*Koksal et al., 2009*).

In the United States and Japan, it has been reported that the susceptibility to glycopeptides was reduced. Vancomycin has long been considered as an antibiotic of last resort for multi-drug-resistant staphylococci infection. On the other hand, vancomycin resistance has emerged first in enterococci and, more recently, in *S. aureus* and *coagulase-negative staphylococci*. This condition has led CoNS to become a serious health problem that medical practitioners should be concerned about. The extensive use of glycopeptides in hospitals has been related to decreased susceptibility to these agents. Unfortunately, the therapy chance of multi-resistant staphylococci infections is gradually decreasing (*Koksal et al., 2009*).

The slime production was observed in only one of *S. saprophyticus*, *S. simulans*, and *S. schleiferi* isolates. Slime production was reported in 50% of *S. saprophyticus*. It has been reported that the slime production is

higher in the pathogenic CoNS strains rather than CoNS in normal flora. The slime production by CoNS is accepted by some to be associated with pathogenicity, but the relationship between slime production and antibiotic resistance is a matter of debate. *Koksal et al., 2009*, showed that methicillin resistance was higher in slime producing strains (81%) than in non-slime producing strains (57%) (*Koksal et al., 2009*).

CoNS may adhere to medical devices and surfaces through slime, and the slime allows multi-resistant CoNS to colonize within hospital environment. Thus, they may serve as a reservoir of antimicrobial resistance determinants in hospital. It seems that the therapy of multi-resistant staphylococci infections could become difficult in the near future. For this reason, it is necessary to take preventive measures in order to limit the colonization and spread of multi-resistant staphylococci within hospital environment before a nosocomial infection with these organisms starts (*Koksal et al., 2009*).

The acquisition of methicillin resistance in staphylococci results from the recombinase-mediated insertion of staphylococcal cassette chromosome *mec* (*SCCmec*), the mobile genetic element carrying *mecA*, at the 3' end of a chromosomal open reading frame designated as *orfX*. Eight major *SCCmec* types (I-VIII) are described in MRSA, differing in size and in the allotypic combination of the *mec* (A, B, C) and the recombinase-encoding *ccr* (types 1–4, ie, *ccrAB1* to *ccrAB4*, and type 5, ie, *ccrC*) gene complexes. Major CA-MRSA clones (including USA300, USA400, and ST80) harbor *SCCmecIVa*, a subtype that is also currently diffusing among health care-associated MRSA (HCA-MRSA) strains (*Barbie et al., 2010*).

SCCmec displays more polymorphous structure in methicillin-resistant coagulase-negative staphylococci (MR-CoNS), with frequent *ccr-mec* combinations not described in MRSA, and multiple and/or untypeable *ccr* allotypes. Non-*mecA* *SCC* elements have even been reported in *S. haemolyticus* and *S. epidermidis*, possibly associated with arginine catabolic mobile elements (ACME) in the latter species. Interestingly, recent data from Japan show that *SCCmecIVa* also predominates among

community-acquired *methicillin-resistant S. epidermidis* (CA-MRSE) (*Barbie et al., 2010*).

Several reports involving health care-associated strains suggest that transfer from MR-CoNS to *methicillin-susceptible S. aureus* (MSSA) may occur, although its mechanism remains unclear. MR-CoNS may thus act as a source of *SCCmec* for MRSA. The frequency of methicillin resistance in health care-associated CoNS is currently >160%. On the other hand, little is known about the prevalence of methicillin resistance, the *SCCmec* diversity, and the reservoir of *SCCmecIVa* among carriage strains of CoNS, notably CA-MRSE, in western populations (*Barbie et al., 2010*).

Furthermore, no complete sequence of *SCCmecIVa* from CA-MRSE has been published so far, and homology with that carried by CA-MRSA could not be estimated. *SCCmecIVa* was found to be disseminated in MRSE strains, including from patients not previously exposed to the health care system, and was highly homologous to that sequenced in CA-MRSA (*Barbie et al., 2010*).

Hospitalization in the previous year, long-term hemodialysis, nursing care at home, and living in a rest home increase the risk of MR-CoNS colonization, in agreement with the demonstrated impact of antibiotic pressure and cross-transmission on this carriage in hospitalized patients. The diffusion of MR-CoNS in individuals with no underlying risk factor has been recently reported in non-European population. This spread may elicit additional concerns, given that CoNS are increasingly reported in community-acquired diseases, such as native-valve endocarditis and late-onset infections of prosthetic heart valves, pacemakers, and orthopedic prostheses (*Barbie et al., 2010*).

Response to the increasing burden of antimicrobial resistance “How to combat antibiotic resistance?”:

The first response to high levels of antimicrobial resistance is must be to reduce the selective pressure generated by antibiotic usage. As patients require treatment, it is not always possible to modify substantially or reduce antimicrobial use. However, there have been some clear examples of good antibiotic stewardship leading to reductions in antimicrobial resistance, notably the reduction in penicillin resistance among pneumococci in the UK following a 30% reduction in pharmacy sales of oral β -lactams. However, the correlation between reduced prescribing and resistance is not clearcut (*Livermore et al., 2006*).

The spread of ESBLs within a hospital despite antibiotic restriction, suggesting that infection control may be a better control method for antibiotic-resistant bacteria. Many ESBL-producing strains were introduced from the community following admission of patients colonized with such strains in their bowel. These strains may then have been selected following administration of a range of antimicrobials and caused endogenous infections (*Bisson, et al., 2002*).

Infection control is possibly the single most important control measure that can be applied to the containment of antibiotic-resistant bacteria in a hospital setting, and there are a number of reports of successful control in the literature. One of the problems can be of making a clear case to hospital management for the deployment of scarce resources in infection control. Money spent in this area almost invariably results not just in the control of antibiotic-resistant bacteria but also in reductions in death rates (*Hawkey, 2008*).

Finally, the development and introduction of new agents have often in the past resulted in substantial reductions in the occurrence of resistance to antibiotics already in use due to the elimination of those strains that carry the resistance genes. Following the introduction of penicillin, there

was a rapid rise in resistance to penicillin in *S. aureus* and other drugs such as chloramphenicol, tetracycline and erythromycin through the 1950s. The development and widespread use of penicillinase-stable isoxazolylpenicillins such as methicillin, cloxacillin and flucloxacillin reduced the spread and occurrence of resistant strains of *S. aureus* in the 1960s (**Hawkey, 2008**).

The subsequent rise of MRSA presumably indirectly selected by isoxazolyl penicillins and more recently by cephalosporins and fluoroquinolones has obliterated original reductions in MRSA infections. Recently, carriage of a strain of MRSA (TW) strongly associated with intravascular device-related bacteraemia has been eradicated in ICU patients by treatment with linezolid. It is concluded that pre-emptive treatment of carriers led to the termination of the outbreak (**Edgeworth et al., 2007**).

However, this has not always been the case as sometimes a newly introduced agent will be affected by existing resistance mechanisms (e.g. resistance to cefepime in ESBL-producing *E. coli*). There are a number of novel agents either introduced or on the point of introduction that are active against Gram-positive pathogens (e.g. tigecycline, ceftobiprole, oritavancin and dalbavancin), but in the case of Gram-negative infections, the choice is much smaller with the re-introduction of some older agents, such as temocillin, which has stability to both AmpC and ESBL β -lactamases, but little activity against other pathogens such as *Pseudomonas* or stability to some other mechanisms of resistance, e.g. carbapenemases such as VIM-2 (**Hawkey, 2008**).

There is a pressing need to develop and evaluate novel alternative strategies for combating a worsening clinical situation, to overcome resistance and reduce the morbidity and mortality associated with infections caused by antibiotic-resistant bacteria (**Projan & Shlaes, 2004**).

One strategy would be to use ‘antisense’ or ‘antigene’ agents to inhibit resistance mechanisms at the nucleic acid level. Strictly, ‘antisense’ and ‘antigene’ (hereafter referred to collectively as antisense) oligonucleotides bind mRNA to prevent translation or bind DNA to prevent gene transcription, respectively. Interrupting expression of resistance genes in this manner could restore susceptibility to key antibiotics, which would be co-administered with the antisense compound. This would extend the lifespan of existing antibiotics, which offer clinically proven therapies, and are often cheaper, more effective or less toxic than the alternatives. Antisense molecules that bind complementary mRNA sequences are a well-established means of modifying gene expression in mammalian systems. Indeed, the manipulation of eukaryotic RNA processing pathways with small interfering RNAs (siRNAs) has revolutionized research in mammalian cell biology, with libraries of custom-made molecules spanning entire genomes now commercially available (*Wall and Shi, 2003*).

There is limited proof-of-principle evidence for resistance modulation by antisense agents; the approach has been applied successfully in vitro to reverse, for example, amikacin resistance, chloramphenicol resistance and multidrug efflux in *E. coli*, and glycopeptide resistance in enterococci (*SolerBistue et al., 2007*).

Developing resistance inhibitors is a sound, well-validated strategy, which complements the development of directly antibacterial agents. For example, β -lactamase inhibitors, such as clavulanic acid, tazobactam and sulbactam, are widely used clinically to restore the susceptibility of bacteria to co-administered β -lactam antibiotics. The economic and clinical value of this rationale is demonstrated by efforts to market new combinations (for example, cefixime/clavulanate) or to develop novel β -lactamase inhibitors (for example, NXL104) (*Livermore et al., 2008*).

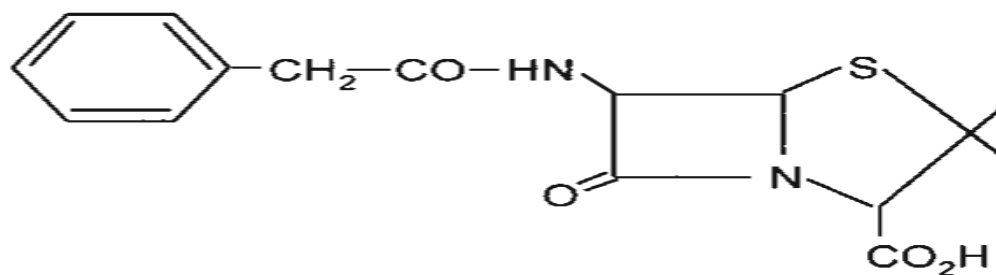
Beyond β -lactamases, efflux pump inhibitors offer a tantalizing and much-explored route whereby bacterial susceptibility could be restored simultaneously to multiple antibiotic classes (*Stavri et al., 2007*).

The principle of using antisense therapeutics as modulators of bacterial resistance is broadly applicable and could be used to overcome resistance, potentially, in any pathogenic species. Furthermore, in contrast to agents targeting essential genes, it may be possible to target only antibiotic-resistant bacteria, limiting disruption of the normal flora, particularly if the antisense allows the co-administration of a narrow-spectrum agent. Toxicity would also be anticipated to be minimal because: (i) antibiotic resistance genes have virtually no homology to human genes, and (ii) humans are continually exposed to bacterial nucleic acids. However, many obstacles must be overcome if these innovative technologies are to be harnessed to reduce the burden of antibiotic resistance for the benefit of patients (*Woodford & Wareham, 2009*).

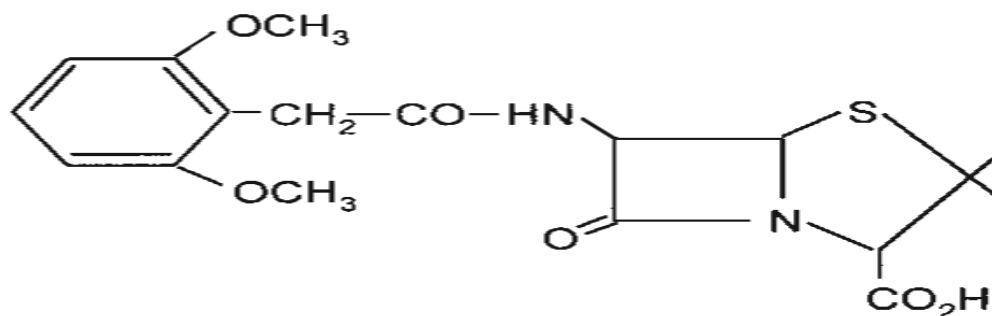
Chapter 3
Mecillin Resistant Staph
aureus

Introduction:

Treatment of *S. aureus* infections before the 1950s involved the administration of benzylpenicillin (penicillin G) (Figure 1), a β -lactam antibiotic, but by the late 1950s *S.aureus* strains resistant to benzylpenicillin were causing increasing concern. Resistant strains typically produced an enzyme, called a β -lactamase, which inactivates the β -lactam. Efforts were made to synthesise penicillin derivatives that were resistant to β -lactamase hydrolysis. This was achieved in 1959 with the synthesis of methicillin, which had the phenol group of benzylpenicillin disubstituted with methoxy groups (Figure 1). The methoxy groups produced steric hindrance around the amide bond reducing its affinity for staphylococcal β -lactamases. Unfortunately, as soon as methicillin was used clinically; methicillin-resistant *S. aureus* (MRSA) strains were isolated. Resistance was not due to β -lactamase production but due to the expression of an additional penicillin-binding protein (PBP2a), acquired from another species, which was resistant to the action of the antibiotic. The use of different types of antibiotics over the years has led to the emergence of multi-resistant MRSA strains, the result of mutations in genes coding for target proteins and through the acquisition and accumulation of antibiotic resistance-conferring genes (*Livermore ., 2000*).



Benzylpenicillin



Methicillin

Fig. 1:

The chemical structures of β -lactam antibiotics benzylpenicillin and methicillin (*Livermore., 2000*).

Penicillin-binding proteins: the targets of β -lactam antibiotics:

The staphylococcal cell is surrounded by a mesh-like structure 20-40 nm thick, called peptidoglycan that is composed of a series of short glycan chains of approximately 20 alternating N-acetylmuramic acid and β -1-4-N-acetylglucosamine residues. Attached to each N-acetylmuramic acid residue is a pentapeptide chain referred to as the stem peptide. The glycan chains in peptidoglycan are linked together via the last glycine residue of a pentaglycine cross-bridge attached to the L-lys residue (position 3) on one stem peptide and the D-Ala residue (position 4) on another (Figure 2) (*Giesbrecht et al., 1998*).

Pentaglycine cross-bridges are preformed in the cytoplasm by the FemX, FemA, and FemB proteins, which attach the glycine residues to

the L-lysineresidue of the stem peptides. The cross-linking or transpeptidation reactions take place on the external surface of the cytoplasmic membrane in a reaction catalysed by penicillin-binding proteins (PBPs). There are four PBPs in *S. aureus*, PBP1, PBP2, PBP3, and PBP. High molecular weight PBPs have two protein domains, one involved in transpeptidation (crosslinking) the other involved in transglycosylation (extending the glycan chain). The β -lactam antibiotics, which resemble the terminal D-alanyl-D-alanine bond of the stem peptide, inhibit the transpeptidation domain of PBPs (and carboxypeptidase activity of low molecular weight PBPs) thus interfering with the cross-linking reaction. Without cross-linking of the peptidoglycan, the cell wall becomes mechanically weak, some of the cytoplasmic contents are released and the cell dies (*Berger-Büchi and Tschierske., 1998*).

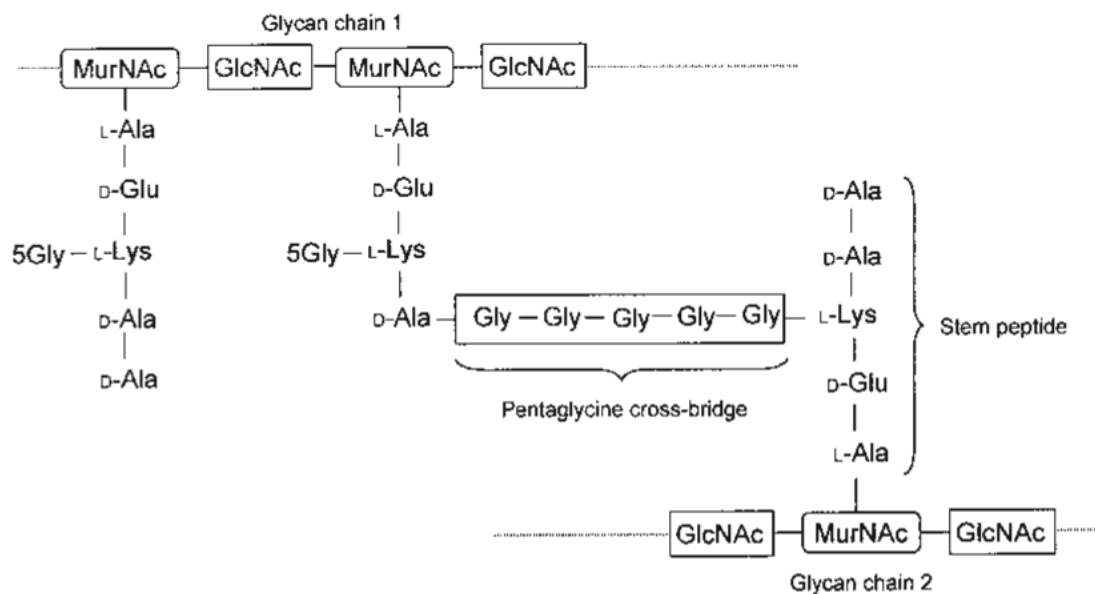


Fig. 2:

A schematic representation of the cross-linking of two glycan chains in peptidoglycan of *S. aureus*. MurNac, N-acetylmuramic acid; GlcNac, N-acetylglucosamine (*Giesbrecht et al., 1998*).

Methicillin resistance:

Methicillin resistance in clinical isolates has been reported to arise from expression of a methicillin-hydrolysing β -lactamase and through the expression of an altered form of PBP2 that has a lower penicillin-binding

affinity and higher rates of release of the bound drug compared to the normal PBP2. However, the main mechanism of methicillin resistance in *S. aureus* is through the expression of a foreign PBP, PBP2a (not to be confused with PBP2), that is resistant to the action of methicillin but which can takeover the transpeptidation (crosslinking) reactions of the host PBPs. Synthesis of PBP2a is regulated and normally kept at low level, but the level of synthesis can be enhanced if mutations occur in the regulatory genes (*Stapelon and Taylor ., 2002*).

PBP2a:

MRSA differ genetically from methicillin-sensitive *S. aureus* isolates by the presence, in the chromosome, of a large stretch of foreign DNA (40-60 Kb), referred to as the *mec* element, and the presence of the *mecA* gene that encodes the 76 KDa penicillin-binding protein, PBP2a (also referred to as PBP2'). The *mecA* gene has been proposed to originate from *Staphylococcus sciuri*. Although the mechanism of gene acquisition from this species is not known, two genes, *ccrA* and *ccrB*, present on the *mec* element from one isolate, have been shown to code for recombinase proteins that are capable of excising and integrating the *mec* element into the chromosome (*Wu et al., 2001*).

Examination of a large number of MRSA isolates has led to the conclusion that the original acquisition of the *mecA* gene has occurred once and that MRSA isolates are descendants of a single clone. Although the arrangement and composition of the *mec* element may vary between isolates, the *mecA* gene itself is highly conserved. In common with other PBPs, PBP2a has the common structural motifs that are associated with penicillin binding yet its affinity for β -lactam antibiotics is greatly reduced (*Katayama et al., 2000*).

Consequently, at therapeutic levels of methicillin that would inhibit the transpeptidational activities of other PBPs, PBP2a remains active ensuring the cross-linking of the glycan chains in peptidoglycan. PBP2a is not able to completely compensate for the other PBPs since cells grown in the presence of methicillin exhibit a marked reduction in the degree of cross-linking. However, the limited degree of crosslinking is enough to ensure survival of the cell (*Oliveira et al., 2000*).

Chapter 4
Vancomycin Resistant
Staph aureus

Glycopeptides, particularly vancomycin, have been considered to be the drugs of choice for treating MRSA bacteremia and sepsis since the prevalence of that organism surged during the 1980s. The high prevalence of MRSA infection has led to increased use of vancomycin in chronic and seriously ill patients and, in turn, to the emergence of multiple phenotypes with reduced susceptibility to glycopeptides. For example, heterogeneous vancomycin-intermediate *S. aureus* (hVISA), defined as organisms with minimal inhibitory concentrations (MICs) of 1–2 ug/mL (but with a subpopulation of daughter cells with the ability to grow at 4 ug/mL), appears to precede the development of vancomycin-intermediate *S. aureus* (VISA), with MICs of 4–8 ug/mL. Finally, VRSA is defined as organisms with MICs 16 ug/mL (**Appelbaum, 2007**).

Since the first documented clinical infection due to hVISA was reported in Japan (in a patient with MRSA pneumonia unresponsive to vancomycin), VISA infections have been reported in patients from the United States, Europe, and Asia. The first documented infection caused by VRSA in the United States was reported by the Michigan Department of Community Health in 2002. Since then, 8 additional cases have been confirmed by the CDC. hVISA and VISA strains probably arose as a result of fundamental changes in the bacterial cell wall and in important metabolic pathways (**Whitener et al., 2004**).

In these *S. aureus* strains, currently unexplained accelerated cell-wall synthesis is correlated with vancomycin trapping in the outer layers, making less vancomycin available for target molecules. On the other hand, VRSA is thought to arise in a different manner, with resistance probably resulting from acquisition of genetic material from enterococci. In vitro transfer of the *vana* resistance determination gene from vancomycin-resistant *E. faecalis* to *S. aureus* has been demonstrated, and conjugative transfer from vancomycin-resistant *E. faecalis* has appeared to be the mechanism of resistance in at least 2 unrelated clinical isolates of VRSA (**Tenover, 2008**).

Most infections with VISA or VRSA have occurred after prior long-term use of glycopeptide antibiotics and in patients with chronic illness, such as preexisting chronic renal failure, diabetes mellitus, or vascular

compromise with devitalized tissue. However, Brazilian investigators reported the presence of 4 coagulase-negative Staphylococcus strains with reduced susceptibilities to vancomycin in healthy carriers inside and outside a health care setting. The isolates were obtained from saliva, indicating the potential for disseminated oral strains to colonize other body sites and other individuals. None of the isolates were found to carry the *vanA*, *vanB* and *vanC* gene according to polymerase chain reaction analysis, and their cell walls became thickened after culture in a medium containing vancomycin (*Appelbaum, 2006*).

Definition of Vancomycin Resistance:

Unfortunately, confusion over the definitions of vancomycin resistance has been generated by recent literature. The source of this confusion seems to be the different breakpoints in vancomycin susceptibilities used in the various countries where vancomycin-resistant staphylococci have been reported. In the United States, the National Committee for Clinical Laboratory Standards (NCCLS) guidelines should be followed. NCCLS guidelines define staphylococci for which the MIC of vancomycin is 4 u/ml to be susceptible, while isolates for which the MIC is 8 to 16 u/ml are intermediate and those for which the MIC is 32 u/ml are resistant. Japan, however, considers some isolates for which the MIC is 8 u/ml to be resistant; as a result, some isolates reported as resistant in Japan have been reclassified as intermediate in the United States (*NCCLS, 2000*).

Confusion with respect to 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, the majority of population is susceptible to vancomycin and a minority population that is resistant. Heteroresistance is likely more common than pure resistance 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 (*Srinivasan, 2002*).

A similar study from the United States also found hetero-resistant populations to be more common than homogenous populations with reduced susceptibilities; however, the overall incidence was much lower, with only 2 of 630 isolates(0.3%) demonstrating hetero-resistance and none showing true reduced susceptibility to vancomycin (*Hubert et al., 1999*).

The clinical significance of heteroresistance is not fully understood. Although one study did show that patients who were infected with heteroresistant strains did have higher mortality rates than patients infected with sensitive isolates, it is difficult to conclusively determine impact based only on one small, retrospective study. Given the uncertain clinical significance and the difficulty and expense in detecting heteroresistance, there does not appear to be any role for screening outside of research studies. If screening is done and heteroresistant isolates are encountered, the MIC for the susceptible, parent strain and not that of the resistant subpopulation should be documented in the patient's record (*Tenover et al., 2001*).

Laboratory Detection of Vancomycin Resistance:

Since vancomycin resistance has not been a homogenous characteristic of the majority of staphylococci that have been examined, agar-based susceptibility test methods, such as agar dilution and the agar diffusion E-test, may be preferred, as they are more sensitive for detecting resistant subpopulations within a strain. However, this detection requires sufficient incubation time for expression of the resistance determinant and subsequent detectable growth. This usually translates into extended incubation times, i.e., a full 24 to 48 h, and precludes the use of the popular, rapid susceptibility methods. Another advantage of the agar methods is the fact that single colonies growing at higher drug concentrations can be visualized on solid media earlier and more readily than in broth-based systems. Whatever method is employed, it should be noted that inconsistencies have been reported even between gold standard testing methods for detecting vancomycin resistance in staphylococci (*Dunne et al., 2001*).

Screening isolates for growth on vancomycin- containing media appears to be a sensitive way to detect even low levels of vancomycin resistance. Commercially prepared agar media appear to be more specific, as susceptible isolates will occasionally grow on media prepared in-house, although in-house media appear to be equally sensitive. Scientists have also described a sensitive system for detecting glycopeptide-intermediate or -resistant subpopulations of staphylococci, which utilizes increased NaCl concentrations (2 to 4%) and the monobactamaztreonam as an inducing agent (*Wonget al., 1999*).

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 u/ml should be sent to the CDC for confirmatory testing (*CDC, 2000*).

Epidemiology of Vancomycin Resistance in *S. aureus*:

To date, there have 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 intermediate *S. aureus*” or “GISA” is synonymous, but because vancomycin is the only glycopeptide used in this country, most American physicians are more familiar with the acronym VISA (*Srinivasan et al., 2002*).

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 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 around the country (Table 3) (*Sieradzki, et al., 1998*).

State and yr (reference)	source	Underlying illness(es)	Vancomycin exposure (wk)
Michigan, 1997	Peritoneal fluid	Renal failure, MRSA peritonitis.	18
New Jersey, 1997	Blood	Acute renal failure, MRSA bacteremia	18
New York, 1998	Blood	Renal failure, MRSA bacteremia	6
Illinois, 1999	Blood	Renal failure, MRSA endocarditis	3.5
Minnesota, 2000	Blood	Renal failure, MRSA osteomyelitis	18
Nevada, 2000	Abscess fluid	Complicated cholecystectomy with polymicrobial intrahepatic abscess (including MRSA)	10
Maryland, 2000	Blood	MRSA endocarditis, psoriasis, sleep apnea	14

Table 2: VISA cases in the United States (*Sieradzki et al., 1998*).

Risk Factors for Vancomycin Resistance:

As is the relative rarity of decreased vancomycin susceptibility in *S. aureus* makes risk factors difficult to ascertain. Exposure to vancomycin (or other glycopeptide antibiotics) again stands out as a strong risk as every patient in this country who developed a VISA isolate had been on vancomycin therapy for a period of time, though the duration varies widely, from just a few weeks to several months. Prior infection caused by MRSA would also appear to be a strong risk, as no known cases of VISA have developed from methicillin-susceptible strains. The relative risk posed individually by vancomycin exposure and MRSA infection is difficult to determine, as they tend to go hand-in-hand in most cases. Renal failure appears to be a significant risk factor, as it was present in five of the seven cases from the United States. Again, whether renal failure itself is a risk or merely serves to increase the risk of MRSA infection and vancomycin exposure is unknown (*Srinivasan et al., 2002*).

Mechanisms of Vancomycin Resistance:

The true mechanism of vancomycin resistance in *S. aureus* is not known. It was initially feared that *S. aureus* would acquire the *van* genes

that code for vancomycin resistance in *Enterococcus* species, especially after this transfer was successfully accomplished in the laboratory. Further, vancomycin-resistant *Enterococcus faecalis* emits a sex pheromone that promotes plasmid transfer, and it has been recently demonstrated that this same pheromone is produced by *S. aureus*. Emission of this pheromone by *S. aureus* organisms that are in proximity to vancomycin-resistant enterococci that contain plasmids encoding van genes could result in transfer of these resistance genes. However, thus far, neither the van genes nor their altered peptidoglycan products have been recovered in vancomycin- intermediate or resistant *S. aureus* isolates. Instead, it appears that vancomycin resistance in *S. aureus* is conferred by other alterations in the bacterial cell wall (*Showshetal., 2001*).

Several years prior to the first clinical VISA isolate being reported, produced laboratory strains of VISA and VRSA that had much thicker cell walls than the sensitive parent strains have been known. Subsequent investigators have demonstrated that cell wall synthesis and turnover are upregulated in VRSA isolates, leading to thicker and more-disorganized cell walls. Further, it appears that resistant isolates have significantly less cross-linking in the peptidoglycan component of the cell wall (*Sieradzki et al., 1998*).

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 (*Sieradzki et al., 1999*).

The role of (PBPs) in vancomycin resistance remains unclear. PBPs are a group of enzymes that catalyze various steps in cell wall synthesis and

are the targets of beta-lactam antibiotics. It is a mutation in one of these enzymes, PBP2a that confers methicillin resistance in MRSA. While some studies have shown an increase in the production of PBPs in VRSA, others have shown that these enzymes are down regulated (*Sieradzki et al., 1999*).

Treatment of Infections Caused by VISA and VRSA:

It is interesting that all isolates have been sensitive to trimethoprim-sulfamethoxazole and tetracycline. Investigators and clinicians have also attempted to exploit the decreased resistance to oxacillin of some of the VISA isolates. In the laboratory, the combination of nafcillin and vancomycin was synergistic in the treatment of VISA endocarditis in rabbits. Beta-lactam antibiotics have been used clinically in the treatment of two of the VISA cases, once in combination with an aminoglycoside and once in combination with an aminoglycoside and vancomycin. In both cases, the infection was cleared, although only one of the patients survived (*Fridkin 2001*).

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 (*Rybak et al., 2000*).

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 (*Fridkin 2001*).

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 (*Tsiodras et al., 2001*).

Chapter 5

Epidemiology of

Staphylococcus Infections

the Epidemiology and the Risk Factors for Invasive Staphylococcus aureus Infections:

Several factors that increase the risk of acquisition of invasive *S. aureus* (ISA) infection have been suggested, such as diabetes, alcohol abuse, immunosuppression, nasal colonization by *S. aureus*, prolonged hospital or intensive care unit (ICU) admission, intravenous drug abuse, hemodialysis, human immunodeficiency virus (HIV) infection, older age, and use of intravenous cannulas. *S. aureus* infections have recently become more severe, because of the appearance of strains with reduced susceptibility to conventional antibiotics, such as vancomycin (*von Eiff et al., 2001*).

Despite their importance, the epidemiology of ISA infections and the risk factors for acquisition have not been defined by use of population-based study design. Other studies have been limited either by the inclusion of only selected patients with ISA or by the failure to include clinical information. As a result, the general-population incidence of and risk factors for acquisition of these infections are not known (*Morin and Hadler., 2001*).

Laupland et al., 2003 in study carried out in The Calgary Health Region, Ireland showed that the annual incidence of ISA infection among residents was 28.4 cases/100,000 populations. One hundred twenty-one (46%) patients had nosocomial ISA infections.

Laupland et al., 2003 detected that the incidence of infection was highest among persons 65 years old. The overall rate of infection was higher in males than in females (35.4 vs. 21.5/100,000 population). Several groups were identified as being at significantly higher risk for acquisition of ISA infection (hemodialysis, peritoneal dialysis, HIV-infection, organ transplantation, heart disease, cancer, IV drug use, alcohol abuse, DM, stroke, chronic obstructive lung disease, systemic lupus, and rheumatoid arthritis).

Laupland et al., 2003 also showed that the mortality rate of ISA infection was 4.9 deaths/100,000 populations, per year. Factors found to be

significant categorical predictors of case fatality were male sex, age \geq 65 years, heart disease, rheumatoid arthritis, history of stroke, catheter-associated infection, soft tissue infection, bone and joint infections, respiratory focus, bacteremia without focus, positive blood culture, empirical antibiotic treatment within 8 h, >4 medications at presentation.

Epidemiology of MRSA:

The epidemiology of *S. aureus*, in particular for MRSA, has changed with the emergence of community-acquired MRSA, as reported by several studies. The epidemiology of infectious diseases relies on typing methods as tools for the characterization and discrimination of isolates based on either their genotypic or phenotypic characteristics. Nowadays, the classification of isolates is mostly based on molecular methods, which usually provide better discriminatory power than phenotypic methods (*Hoet al., 2007*).

Pulsed-field gel electrophoresis (PFGE), after *Sma*I digestion of total bacterial DNA, is still regarded by many authors as the gold standard for benchmarking new typing methods, although it was originally proposed for outbreak investigation. Recently, due to the availability and affordability of DNA sequence technology, several sequenced based typing methods have been developed and are now widely used, such as multilocus sequence typing (MLST) and spa typing, which are the most frequently used for *S. aureus*. DNA sequence-based typing methods generate unambiguous and portable data, amenable to the creation of central databases, which enable the comparison of local data with data from previous studies in different geographical locations (*Faria et al., 2007*).

MRSA clones are named according to their MLST and *SCCmec* types (e.g., clone ST5-MRSA-II). However, the amount of sequencing required for MLST typing and the increasing number of primers need to define *SCCmec* types as new types and variants are found hamper the use of this combination of methods for clonal characterization of large collections, mainly due to cost-related reasons. Other combinations of methods that

provide a similarly fine resolution of the accepted clonal group definition should be explored (*Milheiricoet al., 2007*).

Different laboratories may use different combinations of methods and, over time, implement new typing schemes, the definition of clones is neither universal nor static (*Carric et al., 2006*).

A meta-analysis of studies of *S. aureus* bacteremia that were published from January 1980 through December 2000 demonstrated significantly increased mortality associated with MRSA infection, compared with infection due to MSSA. Data collected from July 2004 through December 2005 by the Active Bacterial Core surveillance network (the laboratory surveillance component of the Emerging Infections Program of the US Centers for Disease Control and Prevention [CDC]) showed an estimated rate of invasive MRSA infection (bloodstream or other sterile sites) of 31.8 cases per 100,000 populations (*Klevenset al., 2007*).

This trend is associated with very high morbidity and mortality. According to one estimate of incidence rates of MRSA infection in 2005, among 5287 patients hospitalized with MRSA infection, there were 988 deaths; on the basis of these data, an estimated 18,650 patients died of invasive MRSA infection in the United States in 2005. If accurate, this projection suggests that MRSA-associated deaths exceeded the total estimated number of deaths (17,011) attributable to HIV infection and AIDS in the United States (*Bancroft, 2007*).

As the prevalence of MRSA strains has steadily increased in health care facilities [HA] MRSA, community-associated (CA) infections have become increasingly endemic in many parts of the world. Primarily associated with skin and soft tissue infections, CA-MRSA can also cause severe pulmonary infections, including pneumonia and empyema, osteomyelitis (or septic arthritis), urinary infections, and bacteremia (*Wang et al., 2008*).

According to The Surveillance Network-USA - an electronic network that collects microbiology data from 300 clinical microbiology laboratories across the United States - rates of MRSA infection have

steadily increased in the United States since 1998 and were still increasing as of March 2005 (*Styers et al., 2006*).

Global epidemiology of MRSA:

The highest rates of HA-MRSA (>50%) are reported in North and South America, Asia and Malta. Intermediate rates (25–50%) are reported in China, Australia, Africa and some European countries [e.g. Portugal (49%), Greece (40%), Italy (37%) and Romania (34%)]. Other European countries have generally low prevalence rates (e.g. The Netherlands and Scandinavia). The prevalence of HA-MRSA has declined in recent years in some European countries, e.g. Austria, France, Ireland, the UK and Greece. In other European countries the prevalence has remained fairly stable. However, very high rates of MRSA (MRSA as proportion of HA *S. aureus* infections) are reported in East Asia, especially in Sri Lanka (86.5%), South Korea (77.6%), Vietnam (74.1%), Taiwan (65.0%), Thailand (57.0%) and Hong Kong (56.8%). In contrast, the values are much lower in India (22.6%) and The Philippines (*Stefani et al., 2012*).

CA-MRSA emerged, and the number of cases escalated, rapidly in the USA in the early 2000s. Compared with the USA, CA-MRSA infections have remained infrequent in Western Europe. In East Asia, the proportion of CA-MRSA as a percentage of total MRSA varied from <5% (Thailand and India) to >30% (Vietnam, The Philippines, Taiwan and Sri Lanka (*Stefani et al., 2012*)).

Recent studies have shown evidence of CA-MRSA infiltrating healthcare settings, most notably in the USA but also in other countries. The proportion of HA-MRSA isolates with *SCCmec* type IV (typical of CA-MRSA) increased from <20% to >50% between 1999 and 2004 in one US hospital. In another US study conducted in an Intensive Care Unit, the proportion of *S. aureus* isolates detected that were resistant to gentamicin, tetracycline and sulfa-trimethoxazole decreased from 1992–2003, although total MRSA incidence as a proportion of total *S. aureus* increased from 35.9% to 64.4% in the same period. Similar results were reported from a French hospital from 1992–2002, during which time the incidence of *SCCmec* type IV HA-MRSA isolates susceptible to

gentamicin, sulfamethoxazole and rifampicin increased markedly. A recent study confirmed the migration of MRSA strains possessing *SCCmec* type IV from the community to the hospital setting in Italy. These strains, despite showing susceptibility to many antibiotics compared with the classical multidrug-resistant nosocomial ones, had acquired some resistance determinants (*Stefani et al., 2012*).

MRSA and burn:

Infection is the leading cause of mortality in burn patients, and MRSA is one of the major nosocomial pathogens affecting this population. The inherent immunosuppression of the burn patient, with reduced T-lymphocyte count and increased suppressor cell activity; uniform exposure to vascular catheters, urinary catheters, & endotracheal tubes; and the open burn wound itself are powerful risk factors for MRSA acquisition (*Safdar et al., 2006*).

Moreover, the risk of acquiring MRSA greatly increases with prolonged hospitalization—the rule in patients with major burns—of which the average length of stay ranges from 1 to 3 months. In a study of 2 MRSA outbreaks in burned patients, scientists reported a 10-fold increased risk of acquiring MRSA with lengths of hospital stay exceeding 21 day. Burn patients colonized with MRSA represent an institutional reservoir for spread of MRSA to the rest of the hospital. The size of the burn has been shown to correlate with likelihood of colonization. Because burn patients typically have prolonged periods of contact with the health care institution, with frequent clinic visits and inpatient admissions, it is plausible that is the mechanism for spread. Nursing and house staff rotate, and that may be another mechanism (*Safdar and Maki, 2002*).

Control of MRSA in burn patients is obviously of high priority. Experiences with MRSA outbreaks in burn patients have found this pathogen very difficult to contain in this uniquely vulnerable patient population. A report of 2 simultaneous MRSA outbreaks, one in a neonatal ICU and the other an adult burn unit, showed that although gowning, enhanced environmental decontamination, and patient cohorting reduced transmission in the NICU, these measures had no

demonstrable effect in containing spread in the burn unit (*Embilet al., 2001*).

MRSA emerged as an important pathogen in burn units in the late 1970s. Since then, there was an increasing report of MRSA outbreaks in those settings. Also, this pathogen reached endemic levels worldwide. The relevance of MRSA colonization in burn patients is a matter of concern. Staphylococcal colonization may progress to infection, with a significant impact on morbidity and mortality. It has been suggested that inpatients colonized with MRSA are more predisposed to infection development than those carrying Methicillin-susceptible *S. aureus* (MSSA). *Rashid et al., 2006* found that 14% of MRSA colonized burned patients developed bacteremia.

Burn units have been recognized not only as wards with increased transmission of MRSA, but also as a reservoir for these bacteria, contributing to their dissemination all through the hospital. However, few studies address specific factors that predispose burned patients to the acquisition of MRSA (*Olivo et al., 2009*).

Some authors made assumptions about risk factors for MRSA acquisition on the basis of analysis of case series, often accompanied by molecular strain typing. The lack of control group makes it hard to validate their inferences. Others, based on univariate analysis of individual or aggregated data, reported some characteristics associated to greater propensity of acquiring MRSA: increasing age, burn extent, longer hospital stay, and previous use of antimicrobial. Those results are somehow similar to findings from studies performed in medical–surgical Intensive Care Units. In these studies, length-of- stay, severity-of-illness, and the use of antimicrobials (especially Cephalosporins and Quinolones) have been identified as predictors of MRSA acquisition (*Thompson, 2004*).

Patients admitted to the burn unit were routinely screened for MRSA through surveillance cultures (nasopharyngeal, oropharyngeal, axillar, perineal and burn wound swabs) at the moment of admission and weekly thereafter (*Olivo et al., 2009*).

Olivo T et al., 2009, showed that out of 175 patients admitted to burn unit, 75 patients acquired MRSA during their stay. The incidence rate was 10.8 per 1000 patient-days. All patients harboring MRSA had positive surveillance cultures. The most frequent isolation sites were burn wound (72.0%), nasopharynx (65.3%), oropharynx (10.7%), axillae (4.0%) and perineum (4.0%). On the other hand, only 23 patients had hospital-acquired MSSA (3.31 per 1000 patients-day). Sites of isolation were burn wound (82.6%), nasopharynx (65.2%), oropharynx (17.4%) and axillae swabs (17.4%). One patient had a previous positive urine culture. Of note, 32 patients had positive cultures for MSSA on admission.

MRSA and hospital acquired Staphylococcal infections in Egypt:

In Egypt, *Abdel Hameed, 2010*, in Ain-Shams university hospital found that MRSA was detected in 11 out of 50 patients (22%), the next most detected organism was methicillin-sensitive *S. aureus* (MSSA) (10 patients, 20%), followed by Gram +ve cocci (8 patients, 16%), the least detected was Gram -ve bacilli (1 patient, 2%), and methicillin-resistant *S. epidermidis* was not detected in any of the studied patients. On the other hand; MRSA was detected in 2 (4%) of the 50 studied health care workers (HCWs), the most detected organism among health care workers was Diphtheroid (18 HCWs, 36%), followed by Gram -ve bacilli (8 HCWs, 16%), then MSSA (4 HCWs, 8%), and the least detected was methicilline sensitive *S. epidermidis* (1 HCW, 2%). Male sex, residence in a rural area, and smoking were identified as significant risk factors for MRSA colonization.

In a study conducted on a total of 470 clinical specimens collected from patients attending El-Minia University, EI-Minia General and EI-Minia Chest hospitals; 187 staphylococcal strains were isolated and identified. Out of the 187 isolates, 132 were *S. aureus* and 55 were coagulase negative staphylococci (CoNS) (70.6% and 29.4% respectively). Out of 187 staphylococcal isolates, 80 (57.1 %) were skin infection isolates. The

study revealed that *S. aureus* was the most prevalent isolated strains from patients suffering from skin, respiratory and eye infections, where CoNS were the most frequent species isolated from urinary tract infections. The antibiogram of staphylococcal isolates revealed that *S. aureus* strains showed low rate of resistance to vancomycin (1.5%), and high resistance against ampicillin (87.1 %). Of the isolated *S. aureus*, 24.2% were oxacillin resistant *S. aureus*, while of the isolated CoNS, 23.6% were oxacillin resistant. Vancomycin was the most effective antimicrobial agent against CoNS (*Shawky, 2008*).

The fear from the emergence of *S. aureus* with reduced susceptibility to vancomycin came from reality with the reports of *S. aureus* with reduced susceptibility and even resistance to vancomycin. 957 clinical samples for nosocomial infections were collected and cultured from different departments, units and centers of Mansoura University Hospitals. Cultures yielded 190 *S. aureus* isolates with frequency of 19.8%. for *S. aureus* isolates and 34.7% for MRSA isolates. Vancomycin disk diffusion method failed to detect any *S. aureus* isolates with reduced susceptibility to vancomycin. It was concluded that, neither VISA nor VRSA were detected in this study. Screening using BHI agar containing 4µg/ml of vancomycin was used to detect hVISA isolates. Out of 66 MRSA isolates, 9 (13.6%) isolates were considered as potential hVISA. The E-test method were carried on the 9 potential isolates and showed that 5 isolates were heteroresistant (*El-Sherbini, 2009*).

Chapter 6

Control and Prevention of MRSA in Healthcare Facilities

MRSA remains endemic in many hospitals. Specific guidelines for control and prevention are justified because MRSA causes serious illness and results in significant additional healthcare costs (*Coia et al., 2006*).

Screening for MRSA carriage in selected patients and clinical areas should be performed according to locally agreed criteria based upon assessment of the risks and consequences of transmission and infection. Nasal and skin decolonization should be considered in certain categories of patients. The general principles of infection control should be adopted for patients with MRSA, including patient isolation and the appropriate cleaning and decontamination of clinical areas (*Coia et al., 2006*).

Grades of evidence of infection:

The CDC/ Hospital Infection Control Practices Advisory Committee (HICPAC) system for categorizing recommendations is as follows:

- Category 1a. Strongly recommended for implementation and strongly supported by well-designed experimental, clinical or epidemiological studies.

- Category 1b. Strongly recommended for implementation and strongly supported by certain experimental, clinical or epidemiological studies and a strong theoretical rationale.

- Category 1c. Required for implementation as mandated by federal or state regulation or standard. The UK equivalent is to operate within European Union or UK Health & Safety Legislation.

- Category 2. Suggested for implementation and supported by suggestive clinical or epidemiological studies or a theoretical rationale.

- No recommendation. Unresolved issue Practices for which insufficient evidence exists or for which there is no consensus regarding efficacy.

Recommendations:

1- Suirveillance:

Surveillance must be undertaken routinely as part of the hospital's infection control programme and must be a recognized element of the clinical governance process. As such, there should be clear arrangements identifying those responsible for acting on the results in individual hospital directorates (Category 1b) (*Coia et al., 2006*).

Surveillance data should be fed back to hospital staff routinely, readily intelligible to most hospital staff, considered regularly at hospital senior management committees, and used in local infection control training (*Coia et al., 2006*).

The dataset should include (*Coia et al., 2006*):

- Patient, laboratory, unit/ward and hospital identifiers;
- Patient demographics (address, age, sex);
- Date of admission;
- Date of onset of infection (if appropriate);
- Site of the primary infection, if appropriate (if bacteraemia, source of the bacteraemia);
- Date specimen taken;
- Site of specimen (blood culture, wound, etc.);
- Where the MRSA was acquired (hospital, community, specialty, etc.);
- Antimicrobial susceptibilities.

Other desirable items include the primary diagnosis, an assessment of severity of underlying illnesses, prior antimicrobial therapy and possible risk factors for infection (Category 2) (*Coia et al., 2006*).

2- Antibiotic stewardship:

- Avoidance of inappropriate or excessive antibiotic therapy and prophylaxis in all healthcare settings (Category 1a) (*Enright, et al., 2002*).
- Ensuring that antibiotics are given at the correct dosage and for an appropriate duration (Category 1b) (*Enright, et al.,2002*).
- Limiting the use of glycopeptide antibiotics to situations where their use has been shown to be appropriate. If possible, prolonged courses of glycopeptide therapy should be avoided (Category 1a) (*Onorato et al., 1999*).
- Reducing the use of broad-spectrum antibiotics, particularly third-generation cephalosporins and fluoroquinolones, to what is clinically appropriate (Category 1b) (*Onorato et al., 1999*).
- Instituting antibiotic stewardship programmes in healthcare facilities, key components of which include the identification of key personnel who are responsible for this, surveillance of antibiotic resistance and antibiotic consumption, and prescriber education (Category 1c) (*Onorato, et al.,1999*).

3- Screening:

Active screening of patients for MRSA carriage should be performed and the results should be linked to a targeted approach to the use of isolation and cohorting facilities (Category 2) (*Stanford et al., 1994*).

Certain high-risk patients should be screened routinely, and certain high-risk units should be screened at least intermittently in all hospitals (*Stanford et al., 1994*).

Coia et al., 2006 Reported that Patients at high risk of carriage of MRSA include those who are:

- known to have been infected or colonized with MRSA in the past (Category 1b).
- Frequent re-admissions to any healthcare facility (Category 1b).
- Direct inter-hospital transfers (Category 1b).
- Recent inpatients at hospitals (abroad or local) which are known or likely to have a high prevalence of MRSA (Category 1b)
- Residents of residential care facilities where there is a known or likely high prevalence of MRSA carriage (Category 1b).

Other risk groups may be defined by local experience, based on screening initiatives or outbreak epidemiology. Published examples have included: injecting drug users, patients infected with human immunodeficiency virus and members of professional contact sport teams (Category 2) (*Coia et al., 2006*).

MRSA should be screened at the time of admission unless they are being admitted directly to isolation facilities and it is not planned to attempt to clear them of MRSA carriage (Category 2) (*Farr and Jarvis, 2002*).

Regular (e.g. weekly or monthly, according to local prevalence) screening of all patients on high-risk units should be performed routinely (Category 2) (*Farr and Jarvis, 2002*).

The following sites should be sampled for patients (Category 1b): anterior nares, skin breaks, lesions& wounds, sites of catheters, catheter urine, groin/perineum, tracheostomy, and sputum from patients with a productive cough. The umbilicus should be sampled in all neonates. One should also consider sampling the throat (*Cooper et al., 2004*).

4- Decolonisation:

Nasal decolonization

Patients receiving prophylaxis for an operative procedure and in an outbreak situation under the advice of the infection control team should

undergonasal decolonization. This should be achieved by applying mupirocin 2% in a paraffin base to the inner surface of each nostril (anteriornares) three times daily for five days. The patient should be able to taste mupirocin at the back of the throat after application (Category 1b) (*Wanget al., 2004*).

Mupirocin should not be used for prolonged periods or used repeatedly (i.e. for more than two courses for five days) as resistance may be encouraged (Category 1a) (*Loeb et al., 2003*).

Nasal decolonization using topical nasal mupirocin should be used with other forms of intervention such as skin decolonization with 4% chlorhexidine gluconate aqueous solution (Category 2) (*Loeb et al., 2003*).

Throat decolonization

Systemic treatment should only be prescribed on the advice of the consultant microbiologist in the hospital, with appropriate monitoring [e.g. regular liver function tests (LFTs) to monitor effects of the drugs on the liver]. If treatment is required, this should be restricted to one course of treatment, the course should not be repeated and the possible side-effects should be explained to the patient (Category 1b) (*Maraha et al., 2002*).

Systemic treatment should be given in conjunction with nasal mupirocin and skin decolonization (Category 1b) (*Coia et al., 2006*).

Local treatment for throat carriage such as antiseptic gargles or sprays may be used to reduce the organism load (no recommendation) (*Coia et al., 2006*).

Skin decolonization

Skin decolonization using 4% chlorhexidine body wash/shampoo, 7.5% povidone iodine or 2% triclosan is useful in eradicating or suppressing skin colonization for short times, particularly preoperatively to reduce the risk of surgical site infections (Category 1a) (*Wilcox et al., 2003*).

Patients should bathe daily for five days with the chosen antiseptic detergent. The skin should be moistened and the antiseptic detergent should be applied thoroughly to all areas before rinsing in the bath or shower. Special attention should be paid to known carriage sites such as the axilla, groin and perineal area. The antiseptic should also be used for all other washing procedures and forbed bathing. Hair should be washed with an antiseptic detergent (Category 1a) (*Mody et al., 2003*).

After satisfactory completion of a course of treatment, i.e. each bath and hair wash, clean clothing, bedding and towels should be provided (Category 2) (*Coia et al., 2006*).

Management of MRSA-infected or -colonized patients:

1- Patient isolation:

Patient isolation for those infected or colonized with MRSA will be dependent on the facilities available and the associated level of risk. (*Coia et al., 2006*).

Isolation should be in a designated closed area that should be clearly defined; in most facilities, this will be either single-room accommodation or cohort areas/bays with clinical handwashing facilities (*Coia et al., 2006*).

Hospital staff entering isolation facilities should be required to adopt the prescribed isolation precautions rigorously and these should be audited regularly. Non-staff visitors should be requested to adopt the necessary level of precautions to minimize the risk of spread of MRSA to other areas of the facility (Category 1b) (*Boyce and Pittet, 2002*).

2- Cleaning and decontamination:

Cleaning regimens for isolation facilities should focus on the minimization of dust and the removal of fomites from contact areas. This should be a two-fold approach; firstly, the management of the occupied

facility, and then the terminal clean of the facility after discharge of the patient. Cleaning regimens and products should include the removal of organic material with a general purpose detergent (*French et al., 2004*).

Patient equipment, e.g. wheelchairs, hoists, slings, sphygmomanometer cuffs, etc., should either be capable of being decontaminated and be decontaminated before use with other patients, or should be single-patient use and discarded as clinical waste at the end of a period of usage (Category 1b) (*Duckworth and Jordens, 1990*).

3- Patient movement

The movement of patients with MRSA within a facility should be kept to a minimum to reduce the risk of cross-infection and any potential embarrassment for the patient. Where patients need to attend departments for essential investigations, the receiving area should be notified of the patient's MRSA status in advance of the transfer, and arrangements should be put in place to minimize their contact with other patients, i.e. to be called forward when the department is ready for them and to ensure that they are not held in communal waiting areas. Staff should adopt isolation precautions whilst in contact with the patient (*Coia et al., 2006*).

4- Surgical/invasive procedures

Prior to any planned invasive procedure, efforts should be made to minimize the level of risk of infection through topical and systemic decolonization, and prophylactic antimicrobial therapy, as appropriate (*Coia et al., 2006*).

It may be considered desirable to place the individual at the end of a procedure list. However, in mechanically filtered environments such as operating theatre suites, the number of air exchanges should render this unnecessary. Good infection control practices, which should be in place between all patients, should reduce the risk of cross-infection (Category 1b) (*Ayliffe et al., 2000*).

5- Transportation

The risk of cross-infection from an MRSA-colonized or -infected patient to other patients in an ambulance is minimal. Good infection control practices and routine cleaning should suffice to prevent cross-infection (Category 2) (*Coia et al., 2006*).

6- Discharge

Generally, there is no requirement for patients colonized with MRSA to continue with extended eradication protocols after discharge. Patients and their appropriate contacts should be fully briefed and given relevant information on MRSA, its implications and significance prior to discharge in order to reduce unnecessary anxiety and concern when returning to the home environment (Category 2) (*Harbarth and Pittet, 2005*).

Control of vancomycin-intermediate and -resistant *S. aureus*(VISA and VRSA):

Antibiotic resistance flourishes when antimicrobial drugs are abused, misused and dispensed at levels lower than treatment guidelines dictate. Virtually all strains of *S. aureus* with reduced susceptibility to glycopeptide antibiotics described to date are thought to have arisen from pre-existing reservoirs of MRSA, usually in patients with chronic underlying disease who have received multiple and/or prolonged courses of glycopeptide treatment (*Coia et al., 2006*).

Where the use of such agents is deemed appropriate, clinicians should ensure that adequate dosages are given to ensure that therapeutic levels are obtained at the site of infection and that duration of therapy is not unnecessarily prolonged. These measures will help to reduce the likelihood of resistant strains arising de novo (Category 1b) (*Stelfox et al., 2003*).

Surveillance:

A high level of suspicion must be maintained, particularly in patients who have received multiple and/or prolonged courses of glycopeptide antibiotics or who are known to be colonized/infected with MRSA and VRE (*Coia et al., 2006*).

The laboratory must notify the relevant clinician and infection control personnel as soon as possible after the isolation of presumptive *S.aureus* isolate with reduced glycopeptide sensitivity in order that control measures can be implemented with minimum delay (*Coia et al., 2006*).

Control precautions (all Category 1b):

Coia et al., 2006 reported that Action to be taken on identification of a case of VISA/glycopeptide-intermediate *S. aureus* (GISA) or VRSA:

- The laboratory should immediately notify the relevant clinician and infection control personnel.
- The infection control team should immediately identify where the patient is and where the patient has been during all of the current admission, including transfers from other healthcare facilities.

If the patient is still an inpatient (*Coia et al., 2006*):

- The number of healthcare workers caring for the patient should be reduced. This will cause problems for those who are allocated to care for the patient. These healthcare workers will need support.
- Healthcare workers with chronic skin conditions, e.g. eczema or psoriasis, should not be involved in direct care of the patient.
- All staff caring for the patient should be made aware of how the organism is transmitted and the precautions necessary to prevent this.
- The patient should be cared for in a single room with toilet facilities and a wash hand basin.
- The patient and visitors must understand the need for isolation.
- Fans should not be used to control the patient's temperature.

Appropriate infection control procedures should be implemented:(Coia et al., 2006):

1. Standard precautions should be used. Gowns/disposable aprons and disposable gloves should be worn by all those entering the patient's room. Clean, non-sterile gloves and gowns/aprons are adequate. Consideration should be given to use of theatre- style greens in addition to protective clothing to ensure that healthcare workers do not take uniforms home to launder.
2. Disposable masks and eye protection should be worn by careers for procedures likely to generate aerosols/splashing. Use of closed suction systems will help to reduce aerosols.
3. Hand hygiene should be performed with an antibacterial preparation before and after patient contact. Visibly soiled hands should be washed with soap prior to disinfection.
4. Non-disposable items that cannot be easily cleaned or disinfected (e.g. sphygmomanometer cuffs) should be dedicated for use only by the infected/colonized patient.
5. Patient charts and records should be kept outside the isolation room.
6. Linen should be treated as infected. It must be discarded into alginate bags within the patient's room and a secondary bag outside the room.
7. All waste should be discarded into a clinical waste bag inside the room, and bags should subsequently be disposed of according to hospital policy.
8. Transfers of colonized/infected patients within and between institutions should be avoided unless essential and the receiving institution should be made aware of the patient's colonization/infection status prior to transfer.
9. After discharge, the room in which the patient was cared for should be cleaned according to local disinfection policy, with special attention given to horizontal surfaces and dust-collecting areas. Hot water and detergent are usually satisfactory. Curtains should be changed.
10. Compliance with infection control procedures should be monitored.

Screening (all Category 1b):

Patients:

- Nose, axillae, perineum, skin lesions and manipulated sites of the index case and all other patients in the unit should be screened for carriage of VISA/GISA or VRSA (*Coia et al., 2006*).
- The infection control team should review the admission history of the patient and determine if screening needs to be extended to other areas and other units alerted (*Coia et al., 2006*).

Staff:

- Agreement with staff on the need for screening should be sought (*Coia et al., 2006*).
- Nose, axillae and perineum of health careworkers and others with close physical contact with the case should be screened for carriage of VISA/GISA or VRSA (*Coia et al., 2006*).
- Healthcare workers who maintain contact with the patient will require weekly screening. This may require significant support for these staffm (*Coia et al., 2006*).
- Feedback of results and maintenance of confidentiality should be considered (*Coia et al., 2006*).

Eradication (all Categories 1b):

- Eradication of colonization/carriage patient sand healthcare workers should be attempted (the same like eradication of MRSA carriage)(*Coia et al., 2006*).
- Colonized staff should be excluded from work until eradication of carriage is achieved (*Coia et al., 2006*).

Aim of Work

Aim of Work:

1-Detect prevalence of staphylococcal infection in patients admitted to burn unit at Fayoum hospital.

2-Typing of *staphylococci* isolates by antibiogram.

3-Determine prevalence of infection by different types of *staphylococci* strains isolated from wound specimen collected in the burn unit.

Patients and Methods

I: Patients:

The present study was conducted on 400 patients admitted to burn unit in El-Fayoum general hospital (El-Fayoum-Egypt) in the period from January 2011 to December 2012. Selected Patients are of all age groups, both sexes, and have acute burn injuries.

All patient`s history, including name, age, occupation and medical history were recorded in an individual data sheet.

Inclusion criteria:

- 1-Admission to inpatient due to acute burn and need for rapid treatment.
- 2-Samples taken from patients administered antibiotics for not less than 3 days.

Exclusion criteria:

-Absence of SSTI (Staph soft tissue infection) (e.g: cellulitis, skin abscess, infected surgical incision, infected traumatic wound, diabetic foot ulcer, decubitus ulcer, ischemic ulcer, infected bite).

***Categories of the patients:**

- patients were classified according to:
 - a-size of burn: 10-40%, 40-70%, >70%.
 - b-site of burn: UL and LL, head and neck, chest, trunk.
 - c-age groups: <10 years, 10-20, 20-30, 30-40, 40-50, 50-60, >60 years old.
 - d-infected and non infected patients.
 - e-time of antibiotic administration: 1-3 days, 4-7days,7-14 days.

f-type of antibiotic administered: amoxicillin-clavulanicA, ampicillin-sulbactam, ciprofloxacin, ceftriaxone, cephalexin.

II-methodology:

A-Sampling:

-Swabs were collected from the infected burn wound (400 cases) using sterile disposable plastic swabs (Eipico Co. Egypt).

-samples taken from centre of the wound.

-Then directly inoculated on plates directly.

B- Sample processing:

1-Direct Gram stained film: from the swab.

2-Direct inoculation on routine culture media incubated for 24h at 37c):

a-Blood agar (Oxoid Ltd, Hampshire, UK).

b-MacConkey (Oxoid Ltd, Hampshire, UK).

c- Mannitol salt agar (Oxoid Ltd, Hampshire, UK).

3- Identification of Staphylococci to genus level by (*Gnag R Ket al., 2000*):

a- Gram stain: to detect Gram positive cocci arranged in clusters.

b- Slide catalase test (Artev For Cosmetics, Co. Egypt)

4- Identification of *Staphylococcus aureus*:

- Catalase positive colonies were subcultured on Mannitol salt agar (Oxoid Ltd, Hampshire, UK) to detect mannitol fermentation (yellow color colonies).
- Tube coagulase test (Oxoid Ltd, Hampshire, UK).

****Gram positive cocci, catalase positive, coagulase positive, colonies which yield yellow colonies on MSA (mannitol salt agar) are defined as *S.aureus*.**

5- Identification of ORSA using disk diffusion method with cefoxitin disc and (ORSAB) medium (Oxoid Ltd, Hampshire, UK):

The entire surface of the MHA plate (diameter, 90 mm) was seeded with the required inoculum using the swab soaked with the organism and incubation was performed for 18 h at 37°. Oxacillin resistance was determined with 1-ug disks according to the NCCLS critical diameters. With the low-density inoculums (half McFarland) at 37°C, all MRSA isolates showed cefoxitin inhibition zone diameters of <27 mm. and all MSSA isolates showed larger diameters (*A.Felten et al., 2002*).

ORSAB (oxacillin resistance screening agar base) medium uses aniline blue to detect mannitol fermentation, resulting in intense blue colonies of *S. aureus*. ORSAB was supplemented with lithium chloride, polymyxin B and oxacillin according to the manufacturer's instructions and incubated for 24 h and 48 h at 35–37°C. MRSA grows on this medium yielding blue colonies (*Nsira B S et al., 2006*).

6- Identification of VRSA and VISA using E-test:

Isolated ORSA strains were checked for sensitivity to Vancomycin using E-test strips (Oxoid Ltd. Wade Road, Basingstoke, Hants, RG24

8PW, England). Colonies were made on MHA (muller-hintonagar (Oxoid Ltd, Hampshire, UK). Then the E-test strips were positioned on the agar surface with sterile forceps, and incubated at 37°C for 24 hours. The MICs of Vancomycin from E-Test strips were recorded according to the manufacturer's guidelines (*Hakim S T et al., 2007*): VISA: 8-16 ug/ml. and VRSA: > 32 ug/ml.

7-storage:

Isolated organisms stored on trypticase soya broth eppendorfs in (-20°).

8- Detection of Mec A gene by RT-PCR (real time-PCR):

i-Extraction of bacterial DNA:

Amplification performed using QIAamp DNA Mini and Blood Mini Handbook (Tritan (Rahm and Haas Company, Tween (ICI Americas Inc).

***Kit content:**

- QIAamp Mini Spin Columns.
- Collection Tubes (2 ml).
- Buffer AL, Buffer ATL, Buffer AW1 (concentrate), Buffer AW2 (concentrate), Buffer AE.
- QIAGEN Protease, Protease Solvent, Proteinase K.

***Equipments used:**

- Microcentrifuge.
- microcentrifuge tubes.
- Eppendorfs (1 ml).
- Automatic pippett (200ul, 20ul, 1000ul, 5000ul).
- Tips of automatic pipette (white, yellow, blue).
- Hot water bath.
- Vortex.

***Additional reagents required:**

20 mg/ml lysostaphin, pH 8.0, 2 mM EDTA,* 1.2% Triton.

***Extraction of genomic DNA from MRSA strains:**

1. Bacteria were cultured on broth culture over night. Tubes then were pelleted by centrifugation for 10 min at 5000 x g (7500 rpm).
2. Bacterial pellets were suspended in 180 µl of the appropriate enzyme solution (200 µg/ml lysostaphin; 20 mM Tris·HCl, pH 8.0; 2 mM EDTA; 1.2% Triton).
3. Incubated for at least 30 min at 37°C.
4. 20 µl proteinase K and 200 µl Buffer AL were added and mixed by vortexing.
5. Incubated at 56°C for 30 min and then for a further 15 min at 95°C.
6. Centrifuged for a few seconds.
7. 200 µl ethanol (96–100%) were added to the samples, and mixed by pulse-vortexing for 15 sec. After mixing, drops from inside the lid were removed by brief centrifugation. It is essential that the sample, Buffer AL, and the ethanol are mixed thoroughly to yield a homogeneous solution. A white precipitate may be formed on addition of ethanol. It is essential to apply all of the precipitate to the QIAamp Mini spin column. This precipitate does not interfere with the QIAamp procedure or with any subsequent application.
8. Mixture from step 6 (including the precipitate) was carefully applied to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim, the cap closed, and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube (provided), and the tube containing the filtrate was discarded. Each spin column was closed to avoid aerosol formation during centrifugation. It is essential to apply all of the precipitate to the QIAamp Mini spin column. Centrifugation was performed at 6000 x g (8000 rpm). Centrifugation at full speed will not affect the yield or purity of the DNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all the solution has passed through.
9. The QIAamp Mini spin column was opened carefully and 500 µl Buffer AW1 was added without wetting the rim and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube (provided), and the collection tube containing the filtrate was discarded.

10. Carefully, 500 µl Buffer AW2 was added to the QIAamp Mini spin column without wetting the rim and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min.

11. To eliminate the chance of possible Buffer AW2 carryover, the QIAamp Mini spin column is placed in a new 2 ml collection tube (not provided) and centrifuge at full speed for 1 min (The old collection tube with the filtrate was discarded)

12. The QIAamp Mini spin column was placed in a clean 1.5 ml micro-centrifuge tube (not provided), (discard the collection tube containing the filtrate), 200 µl Buffer AE or distilled water was added, Incubated at room temperature for 1 min, and then centrifuged at 6000 x g (8000 rpm) for 1 min.

13. Step 12 was repeated

N.B

- A 5 min incubation of the QIAamp Mini spin column loaded with Buffer AE or water, before centrifugation, generally increases DNA yield.

- A third elution step with a further 200 µl Buffer AE will increase yields by up to 15%.

- Volumes of more than 200 µl should not be eluted into a 1.5 ml microcentrifuge tube because the spin column will come into contact with the eluate, leading to possible aerosol formation during centrifugation.

- Elution with volumes of less than 200 µl increases the final DNA concentration in the eluate significantly, but slightly reduces the overall DNA yield.

- Eluting with 4 x 100 µl instead of 2 x 200 µl does not increase elution efficiency.

ii-Quantification of Methicillin-resistant Staphylococcus aureus mecA (penicillin binding protein 2) & S. aureus FEMB gene (chromosomal gene) using RT-PCR(PrimerDesign Ltd, Hoffmann-LaRoche AG):

****Device used:**

-Lightcycler platform (La Roche, Deutsch) for RT-PCR.

****Kit Contents:**

- MecA (penicillin binding protein 2) primer/probe mix (150 reactions BROWN) FAM labeled.
- FEMB gene (chromosomal gene) primer/probe mix (150 reactions BROWN) FAM labeled.

- MecA (penicillin binding protein 2) positive control template (for Standard curve RED)(sequence undealed).
- FEMB gene (chromosomal gene) positive control template (for Standard curve RED) (sequence undealed).
- Internal extraction control DNA (150 reactions BLUE).
- Internal extraction control primer/probe mix (150 reactions BROWN) (sequence undealed).
- Endogenous ACTB primer/probe mix (150 reactions BROWN) FAM labeled (sequence undealed).
- RNase/DNase free water.

1-Preparation of reaction mixes:

****Pathogen detection mix:**

- 2 x Precision TM Master Mix: 10ul.
 - Pathogen Primer/Probe mix (BROWN): 1 µl
 - Internal extraction control primer/probe mix (BROWN): 1ul.
 - RNase/DNase free water (WHITE): 3 µl
- Final Volume 15 µl.

****Endogenous ACTB detection mix:**

- 2 x PrecisionTM Master Mix: 10ul.
 - Endogenous ACTB Primer/Probe mix (BROWN): 1 µl.
 - RNase/DNase free water (WHITE): 4 µl.
- Final Volume: 15 µl.

2-15µl of this mix (which one) was pipetted into each well of real-time PCR plate.

3- Sample DNA templates were prepared for each of samples (suggested concentration 5ng/µl) in RNase/DNase free water.then dilute your DNA sample reactions 1:20 (10µl of sample DNA and 190µl of water).

4-5µl of diluted DNA template was pipetted into each well. For negative control wells 5µl of RNase/DNase free water was used. The final volume in each well is 20µl.

5- Preparation of standard curve dilution series.

1) 900µl of RNase/DNase free water was pipetted into 5 tubes and label 2-6.

2) 100µl of Positive Control Template (RED) was pipetted into tube 2 and vortexed thoroughly.

4) 100µl was transferred from tube 2 into tube 3 and Vortexed thoroughly. This step was repeated to complete the dilution series.

6-5µl of standard template was pipetted into each plate well. The final volume in each well is 20µl.

Amplification Protocol:

Amplification conditions using PrimerDesign2XPrecision™

MasterMix (50 cycles):

-UNG treatment: 15 min: 37 oC.

-Enzyme activation: 10 min: 95 oC

-Denaturation: 10s: 95 oC

-Data collection: 60s: 60 oC

Interpretation of Results:

Gene detected	Internal control	Negative control	Positive control	interpretation
+ve	+ve	-ve	+ve	+ve
+ve	-ve	-ve	+ve	+ve
-ve	+ve	-ve	+ve	-ve
-ve	-ve	-ve	-ve	Experiment fail
+ve	+ve	+ve	+ve	Experiment fail

***Statistical methods:**

Collected data were computerized and analyzed using Statistical Package for Social Science (SPSS) version 16. Descriptive statistics were used to describe variables; percent, proportion for qualitative variables. Mean, SD, range for Quantitative variables.

Comparison between groups was done using chi-Square test for qualitative variables fisher Exact test used when expected cell count less than 5, independent t- test for quantitative variables .. p values with significance of less than <0.05% were considered statistically significant.

***sensitivity:**

Sensitivity relates to the test`s ability to identify positive results.

The sensitivity of a test is the proportion of people that are known to have the disease who test positive for. This can also written as:

Sensitivity= $\frac{\text{No of true positives}}{\text{No of true positives} + \text{No of false negatives}}$ = probability of a positive test, given that the patient is ill.

***Specificity:**

Specificity relates to the test`s ability to identify negative results.

Specificity is defined as the proportion of patients that are known not to have the disease who will test negative for it. This can also be written as:

Specificity= $\frac{\text{no of true negatives}}{\text{no of true negatives} + \text{no of false positives}}$ = probability of a negative test given that the patient is well.

***positive predictive value:**

True positive is the event that the test makes a positive prediction, and the subject has a positive result under the gold standard. False positive is the event that the test makes a positive prediction and the subject has a negative result under the gold standard.

PPV = $\frac{\text{No of true positives}}{\text{no of true positive} + \text{no of false positives}}$.

***Negative predictive value:**

True negative is the event that a test makes a negative prediction and the subject has a negative result under the gold standard. And a false negative is the event that the test makes a negative prediction and the subject has a positive result under the gold standard.

NPV = $\frac{\text{no of true negatives}}{\text{no of true negatives} + \text{no of false negatives}}$.

Results

***Summary of Results:**

The present study was conducted on 400 patients admitted to burn unit in El-Fayoum general hospital (El-Fayoum-Egypt) in the period from January 2011 to December 2012 Selected Patients are of all age groups, both sexes, and have acute burn injuries.

Patients were classified according to:

A- infected and non infected patients: and compare between both groups in: age, sex, type of antibiotic used, duration of antibiotic administration, site of burn, size of burn (Fig: 10-15).

B- Size of burn: 10-40%, 40-70%, >70%. And detect its relation with no of patients (fig: 6), and with *S.aureus* (fig: 20), and with MRSA (fig: 32).

C- Site of burn: UL and LL, head and neck, chest, trunk. And detect its relation with no of patient (fig: 5), and with *S.aureus*(fig:19), and with MRSA (fig:31).

D-age groups: <10 years, 10-20, 20-30, 30-40, 40-50, 50-60, >60 years old. And detect its relation with no of patient (fig : 4), and with *S.aureus* (fig:17), and with MRSA (fig:29).

E- Time of antibiotic administration: 1-3 days, 4-7days, 7-14 days. And detect its relation with *S.aureus* (fig: 22), and with MRSA (fig: 34).

🏠 We compare between ORSAB and RT-PCR in (fig: 24, 25) and (table: 19).

🏠 Predictors of *S.aureus* and MRSA were detected in (table: 18, 28).

🏠 Amplification curves of RT-PCR detected in (fig: 26, 27).

Fig (3): Distribution of patients by sex

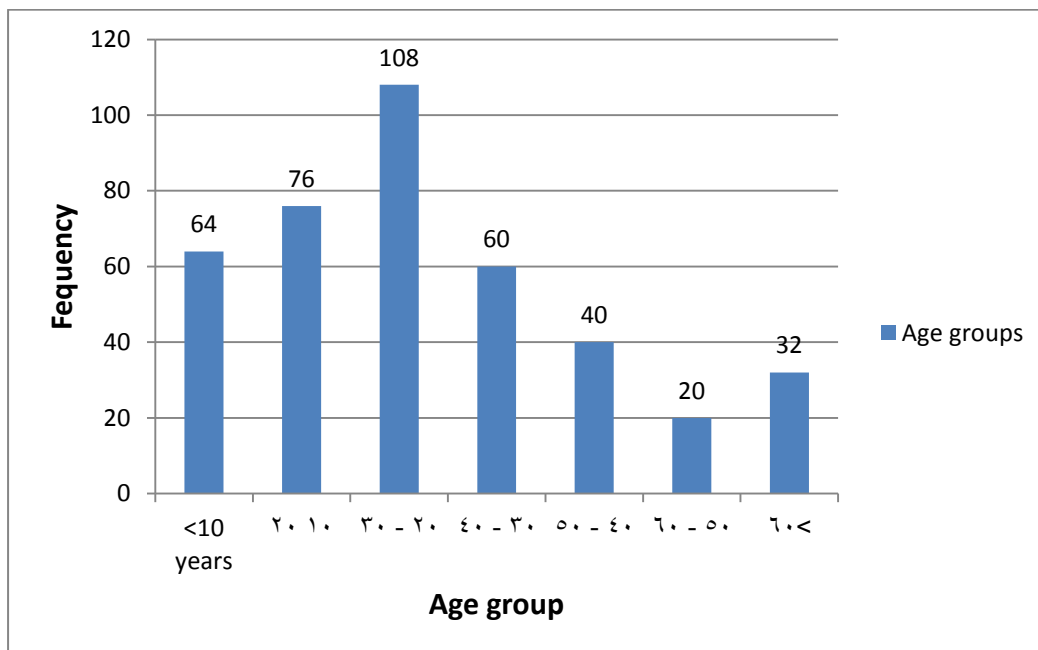
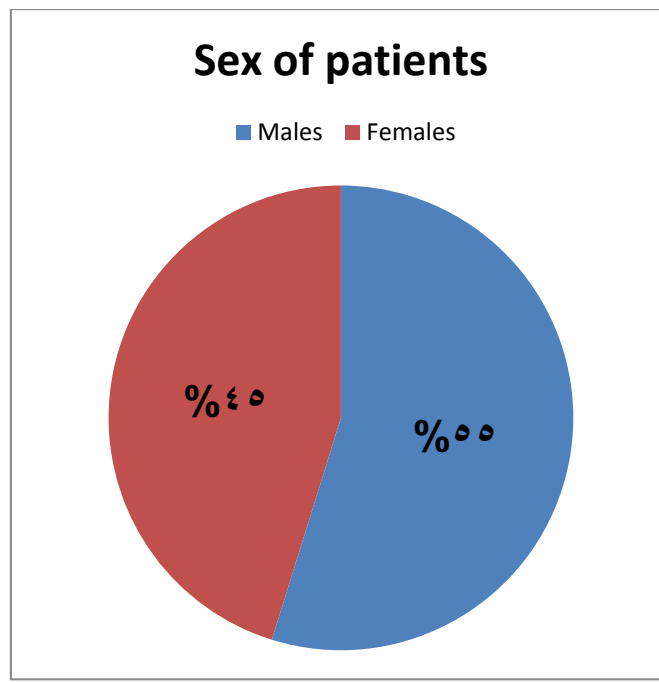
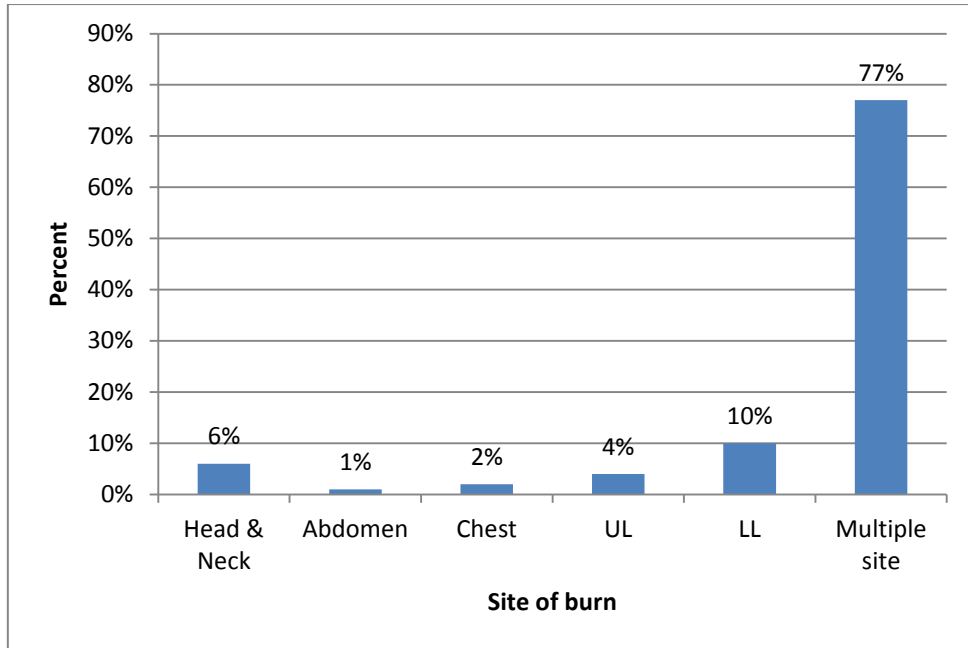


Fig (4): Distribution of patients by age group

Fig -(4) showed that out of the 400 studied patients, the highest percent (27%) of burn patients was found in age group from 20-30 years old.



UL; Upper Limb,

LL; Lower Limb

Fig (5): Distribution of patients by Site of burn

According to site of burn, the highest number of patients having burn wound (308/400 patients “77 %”) is those with multiple sites burns (Fig 5)

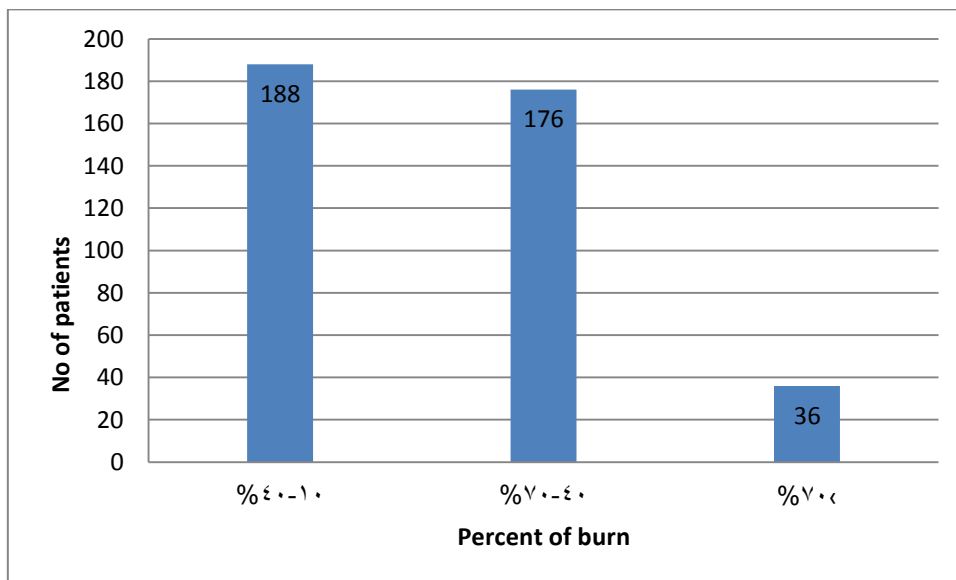


Fig (6): Distribution of patients by percentage of burn.

Fig (6) showed that the highest percent of patients (188 out of 400 “47 %”) have burn percent of 10 – 40 %.

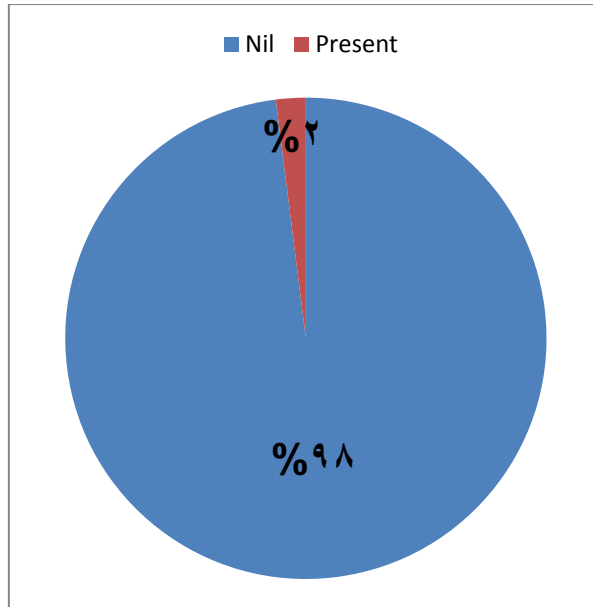


Fig (7): Distribution of patients by DM.

Fig (7): show no statistical difference in presence of DM in patients with burn wound (8 patients have DM "2 %").

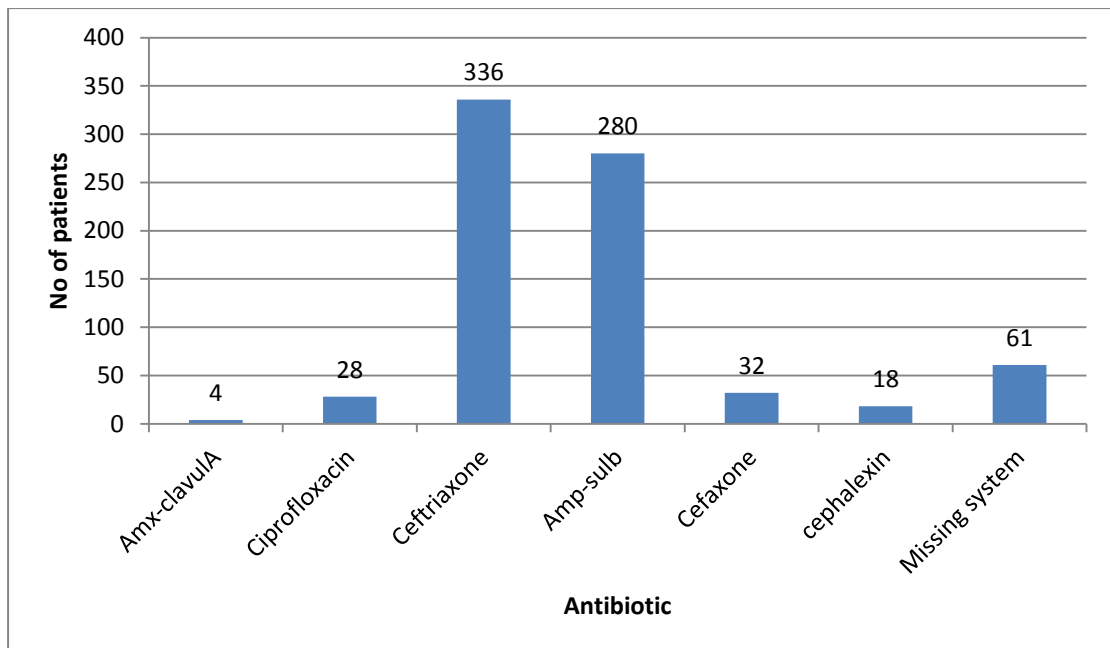


Fig (8): Distribution of patients by antibiotic used in treatment:

Ceftriaxone and ampicillin-sulbactam are the most commonly used antibiotics; Out of 400 patients studied, 336 (84%) and 280 (70%) patients received ceftriaxone and ampicillin-sulbactam respectively “Fig (8)”

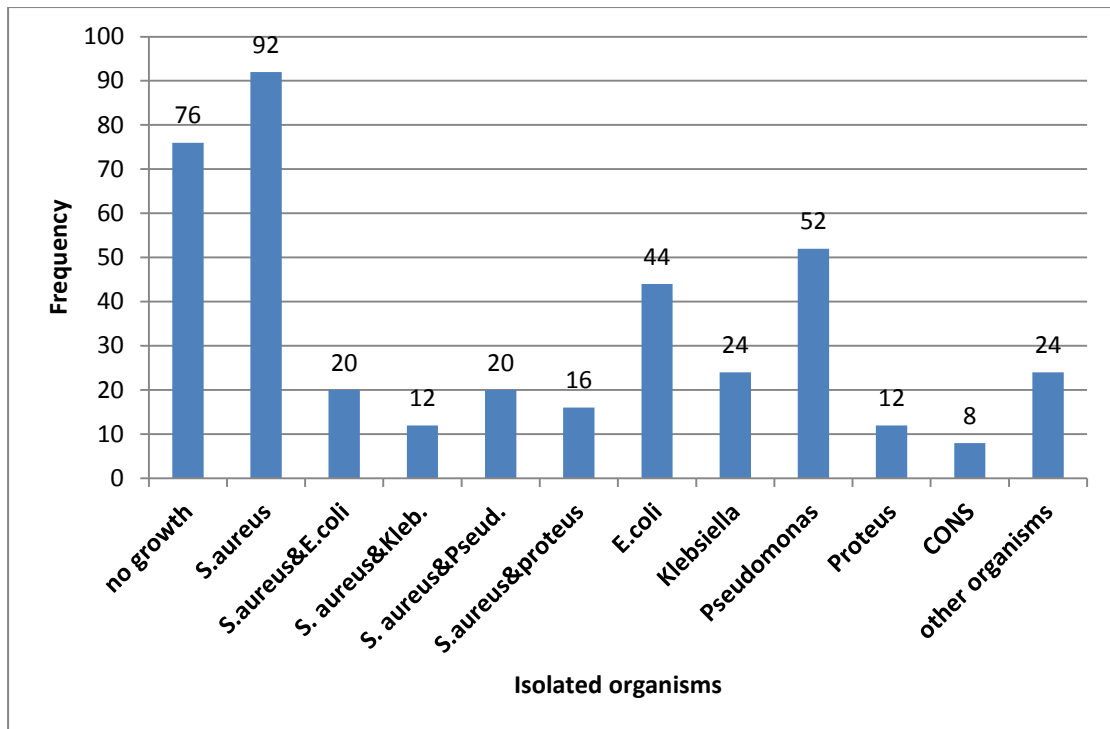


Fig (9): Distribution of isolated organisms

S.aureus represents the most common organisms isolated from burn wound followed by Pseudomonas. As shown by fig (9); within 400 patients, 92 (23%) patients have *S.aureus* only, 20 (5%) patients have *S. aureus&E.coli*, 20 (5%) have *S. aureus& Pseudomonas*, 16 (4%) patients have *S. aureus& Proteus*, and 8 (2%) patients have CONS only.

Table (3): Distribution of sex by infected and non-infected patient's group

		Patient groups			P value	
		Non infected	infected	Total		
sex	male	No (%)	36 (17.0%)	176(83.0%)	212(100%)	0.3
	% within patient group	47.4%	54.3%	53.0%		
females	No (%)	40(21.3%)	148(78.8%)	188(100%)		
	% within patient group	52.6%	45.7%	47.0%		
Total	No (%)	76(19.0%)	324(81.0%)	400(100%)		

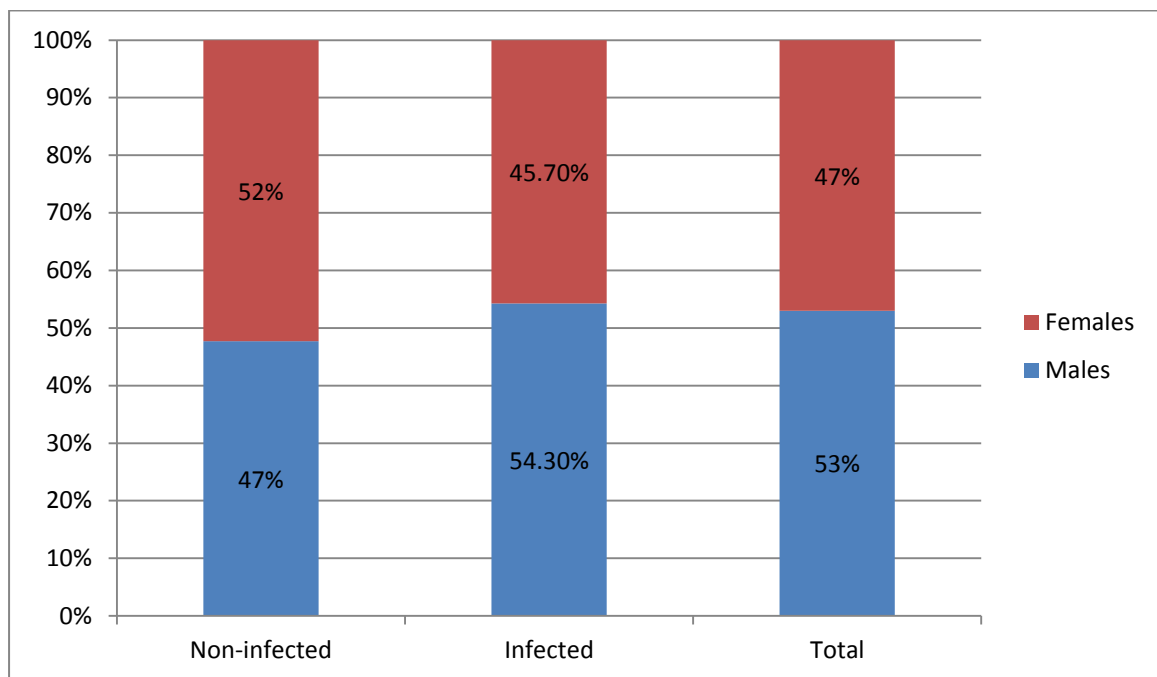


Fig (10): Distribution of sex by infected and non-infected patient's group

Table (3) and fig (10) showed that there is no statistical significant difference in distribution of sex between infected and non-infected patient's groups ($p=0.3$).

Table (4): Distribution of age by infected and non-infected patient's group

Age		Patient groups			P value
		No (%)	Non infected	infected	
<10y	No (%)	16 (25.0%)	48 (75.0%)	64	0.027
	% within patient groups	21.1%	14.8%	16.0%	
10-20	No (%)	12 (15.8%)	64 (84.2%)	76	
	% within patient groups	15.8%	19.8%	19.0%	
20-30	No(%)	16(14.8%)	92(85.2%)	108	
	% within patient groups	21.1%	28.4%	27.0%	
30-40	No (%)	20(33.3%)	40(66.7%)	60	
	% within patient groups	26.3%	12.3%	15.0%	
40-50	No(%)	4(10.0%)	36(90.0%)	40	
	% within patient groups	5.3%	11.1%	10.0%	
50-60	No (%)	4(20.0%)	16(80.0%)	20	
	% within patient groups	5.3%	4.9%	5.0%	
>=60	No (%)	4(12.5%)	28(87.5%)	32	
	% within patient groups	5.3%	8.6%	8.0%	
Total	No (%)	76(19.0%)	324(81.0%)	400	

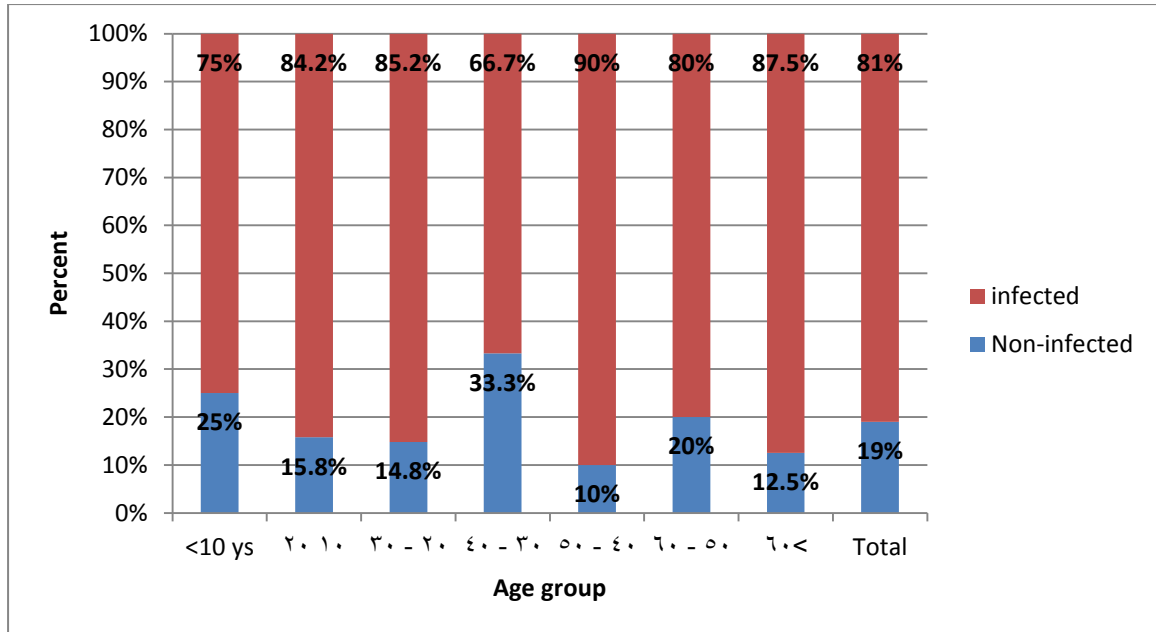


Fig (11): Distribution of age by infected and non-infected patient's group

Table (4) & fig (11) Showed that there is statistical significant difference in distribution of age between infected and non-infected patient's group ($p= 0.027$) with the highest percent of infection occurred in age group (40 – 50 years).

Table (5): Distribution of site of burn among infected and non-infected group:

site			Patient group			P value
			Non-infected	Infected	Total	
Head&neck	No (%)	0 (0%)	24 (100%)	24 (100%)	<0.001	
	% within patient's group	.0%	7.4%	6.0%		
Chest	No (%)	0 (0%)	4 (100%)	4 (100%)		
	% within patient's group	.0%	1.2%	1.0%		
Abdomen	No (%)	4 (50%)	4 (50%)	8 (100%)		
	% within patient's group	5.3%	1.2%	2.0%		
UL	No (%)	8 (50%)	8 (50%)	16 (100%)		
	% within patient's group	10.5%	2.5%	4.0%		
LL	No (%)	12 (30%)	28 (70%)	40 (100%)		
	% within patient's group	15.8%	8.6%	10.0%		
Head&neck + UL	No(%)	20(22.7%)	68 (77.3%)	88 (100%)		
	% within patient's group	26.3%	21.0%	22.0%		
Head&neck + LL	No (%)	4 (9.1%)	40 (90.9%)	44 (100%)		
	% within patient's group	5.3%	12.3%	11.0%		
Multiple sites	No (%)	28(15.9%)	148(84.1%)	176		
	% within patient's group	36.8%	45.7%	(100%)44.0%		
Total	No (%)	76(19.0%)	324(81.0%)	400		
				(100%)		

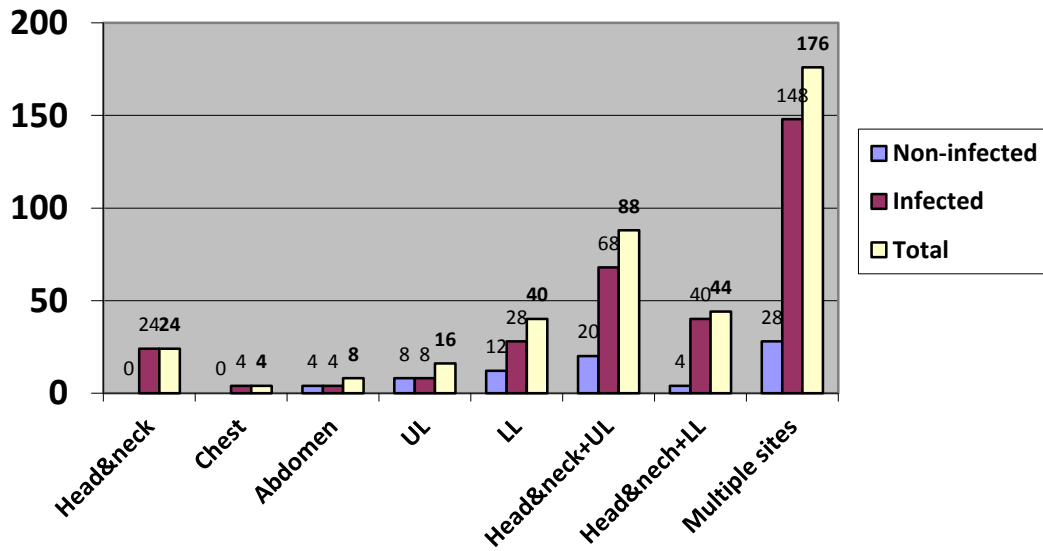


Fig (12): Distribution of site of burn among infected and non-infected group

Table (5) & fig (12) showed that there is statistical significant difference in distribution of site of burn between infected and non-infected patient's group ($p < 0.001$) with the highest infection rates occurs in burns of multi-sites (45.7%), followed by head & neck and UL (21%).

Table (6): Distribution of burn percent by infected and non-infected patient's group:

burn_percent		Patient groups			P value
		No (%)	Non infected	infected	
10-40	No (%)	52(27.7%)	136(72.3%)	188	<0.001
	% within patient group	68.4%	42.0%	47.0%	
40-70	No (%)	20(11.4%)	156(88.6%)	176	
	% within patient group	26.3%	48.1%	44.0%	
>70	No (%)	4(11.1%)	32(88.9%)	36	
	% within patient group	5.3%	9.9%	9.0%	
Total	No (%)	76(19.0%)	324(81.0%)	400	

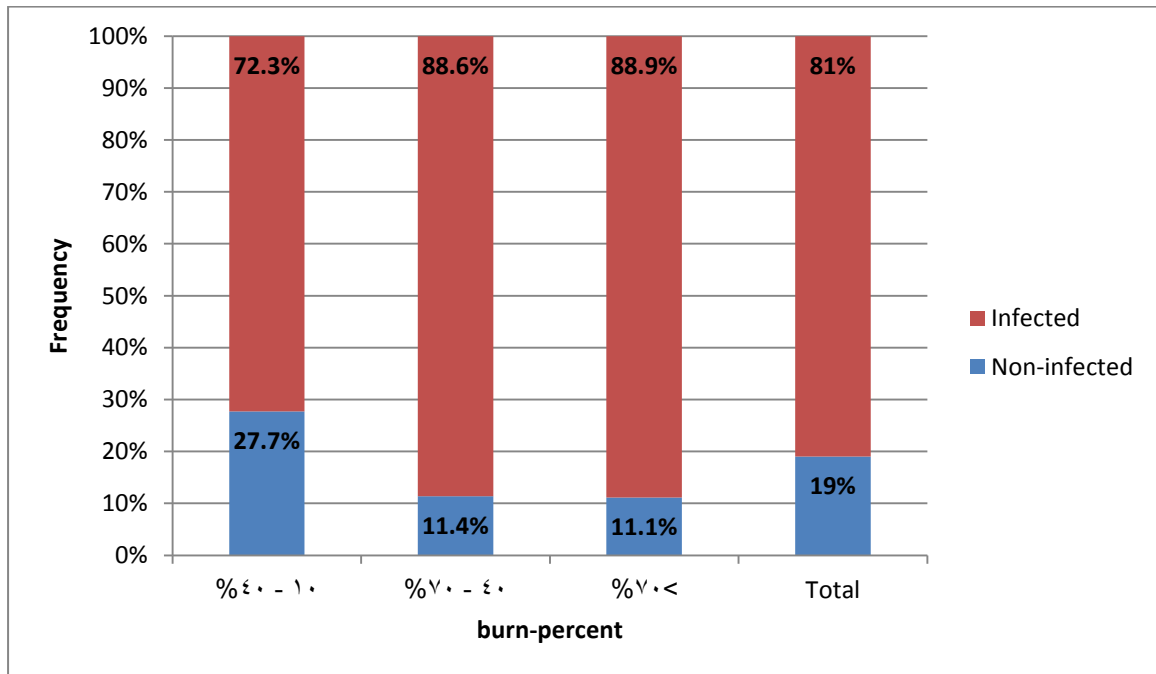


Fig (13): Distribution of burn percent by infected and non-infected patient's group

Table (6) & fig (13) showed that there is statistical significant difference in distribution of burn percent between infected and non-infected patient's groups ($p < 0.001$). Burn infections increased with increase of burn-percent.

Table (7): Relation of antibiotics by infected and non-infected patient's groups:

		Patient's group			P-value
		Non infected	infected	total	
cephalexin	No (%)	3(16.7%)	15(83.3%)	18	1.000
	% within patient's group	5.0%	5.4%	5.3%	
Cefaxone	No (%)	0(0%)	32(100%)	32	0.004
	% within patient's group	0%	9.9%	8.0%	
Amp-sulbactam	No (%)	64(22.9%)	216(77.1%)	280	0.003
	% within patient's group	84.2%	66.7%	70.0%	
Ceftriaxone	No (%)	76(22.6%)	260(77.4%)	336	<0.001
	% within patient's group	100.0%	80.2%	84.0%	
Amx-clavA	No (%)	4(12.5%)	28(87.5%)	32	0.328
	% within patient's group	5.3%	8.6%	8.0%	
Ciprofloxacin	No (%)	0(0%)	28(100.0%)	28	0.008
	% within patient's group	0(0%)	8.6%	7.0%	
Amx-clavA	No (%)	0(0%)	4(100.0%)	4	0.99
	% within patient's group	0%	1.2%	1.0%	
Total	No (%)	76	324	400	

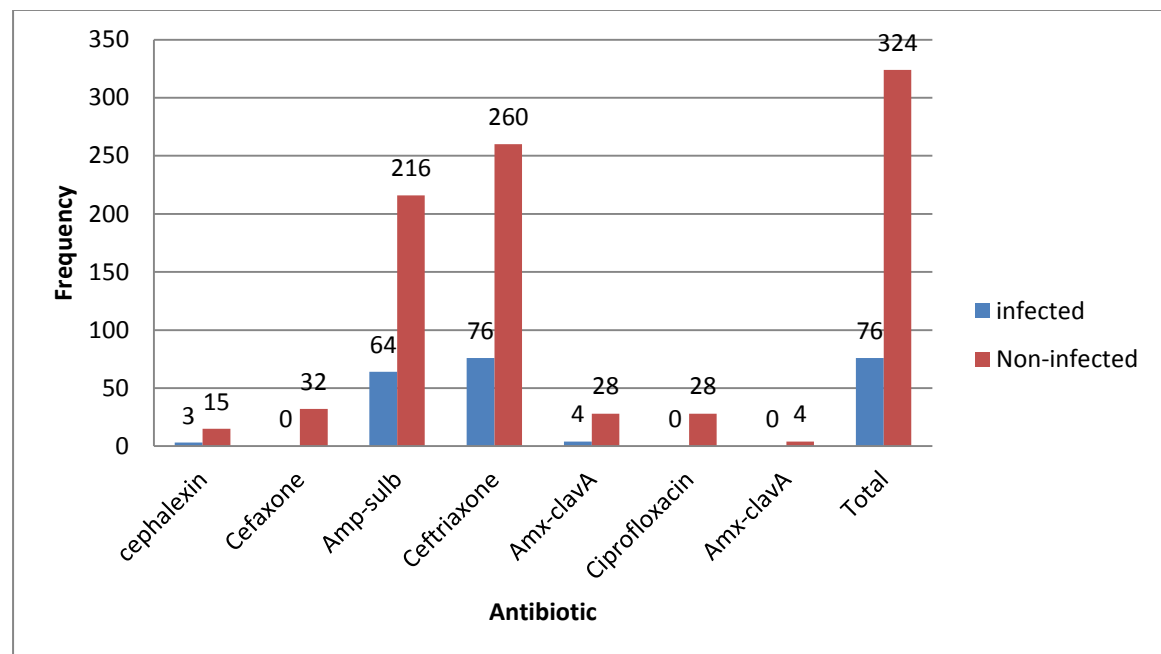


Fig (14): Relation of Antibiotic by infected and non-infected patient's groups

Table (7) & fig (14) showed that there is statistical significant difference in occurrence of infection in burn patients with the use of antibiotics cefaxone, Amp-sulbactam, ceftriaxone, ciprofloxacin (p values = 0.004, 0.003, < 0.001, and 0.008 respectively), while use of ceporex, Amx-clav A does not make any difference. 100% of patients using ciprofloxacin or cefaxone have infected burns.

Table (8): Relation between duration of antibiotic intake by infected and non-infected patient's groups

duration		No (%)	Patient groups		Total	P value
			Non-infected	infected		
1-3			28 (17.5%)	132(82.5%)	160	0.17
	% within patient's group		36.8%	40.7%	40.0%	
4-7			4 (10.0%)	36 (90%)	40	
	% within patient's group		5.3%	11.1%	10.0%	
8-14			44 (22.0%)	156(78.0%)	200	
	% within patient's group		57.9%	48.1%	50.0%	
Total		Not%	76 (19%)	324 (81%)	400	

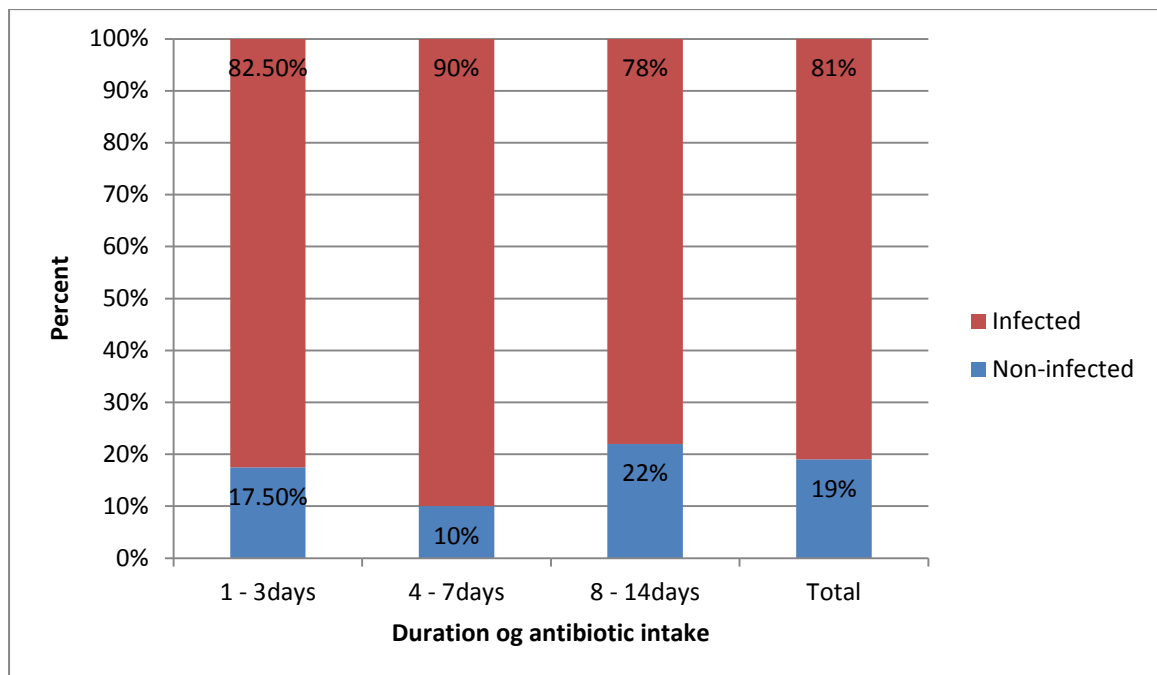


Fig (15): Relation between duration of antibiotic intake among infected and non-infected patient's group

Table (8) & figure (15) showed that there is no statistical significant difference in relation of duration of antibiotic intake between infected and non-infected patient's groups (p= 0.17)

Table (9): Distribution of antibiotic combinations among non-infected patients:

		antibiotic			
		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Ceftriaxone	12	15.8	15.8	21.1
	Ceftriaxone, Amp-sulbactam	56	73.7	73.7	94.7
	Amx-clavA,Ceftriaxone, Amp-sulbactam	4	5.3	5.3	5.3
	Ceftriaxone,Amp-sulb,Ceprex	4	5.3	5.3	100.0
	Total	76	100.0	100.0	

Table (9) showed that among non-infected patient's group the most commonly used antibiotic combination is ceftriaxone and Amp-sulabactam (73.3%).

Table (10): Distribution of *S. aureus* by sex

Sex		<i>S.aureus</i>		Total	P-value
		+ve	-ve		
males	No (%)	104(49.1%)	108 (50.9%)	212 (53%)	<.001
	% within <i>S.aureus</i>	65.0%	45.0%		
females	No(%)	56 (29.8%)	132 (70.2%)	188 (47%)	
	% within <i>S.aureus</i>	35.0%	55.0%		
Total	No(%)	160 (40%)	240 (60%)	400 (100%)	

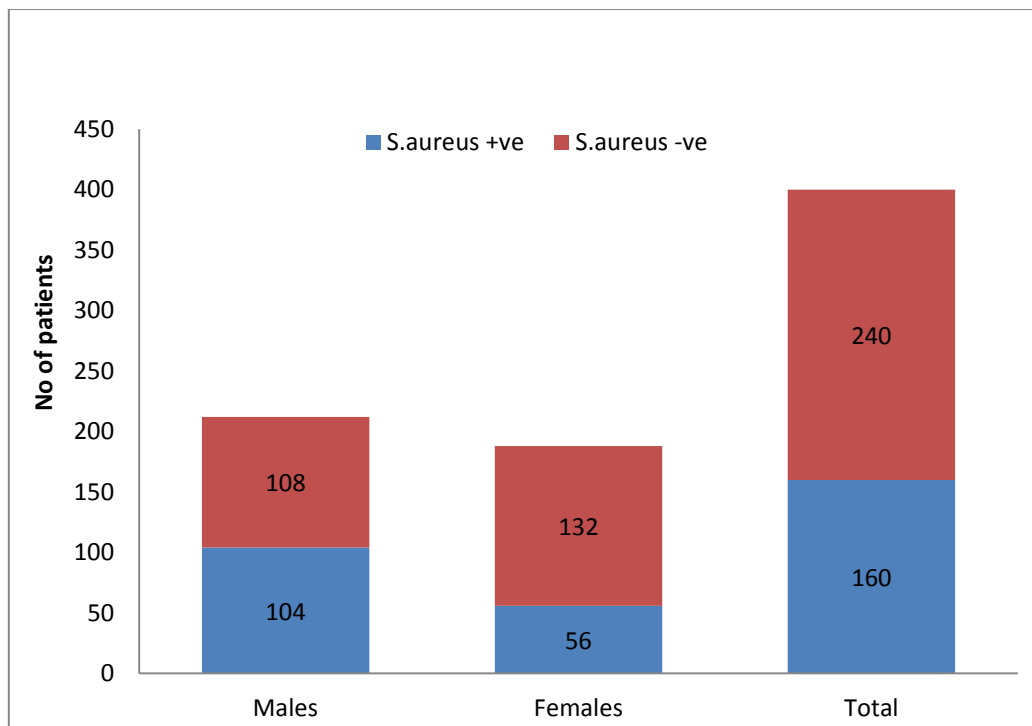


Fig (16): Distribution of *S. aureus* by sex

Table (10) and fig (16) showed that there is statistical significant difference in distribution of *S. Aureus* between males and females ($p < 0.001$). *S. aureus* infections are more common in males (65%) than females (35%).

Table (11): Distribution of *S. aureus* by age group

Age	No(%)	<i>S. aureus</i>		Total	p-value
		+ve	-ve		
<10y	No(%)	24 (37.5%)	40 (62.5%)	64 (16%)	.003
	% within <i>S. aureus</i>	15%	16.7%		
10-20	No(%)	20 (26.3%)	56 (73.7%)	76 (19%)	
	% within <i>S. aureus</i>	12.5%	23.3%		
20-30	No(%)	52 (48.1%)	56 (51.9%)	108 (27%)	
	% within <i>S. aureus</i>	32.5%	23.3%		
30-40	No(%)	16 (26.7%)	44 (73.3%)	60 (15%)	
	% within <i>S. aureus</i>	10%	18.3%		
40-50	No(%)	20 (50%)	20 (50%)	40 (10%)	
	% within <i>S. aureus</i>	12.5%	8.3%		
50-60	No (%)	12 (60%)	8 (40%)	20 (5%)	
	% within <i>S. aureus</i>	7.5%	3.4%		
>=60	No (%)	16 (50%)	16 (50%)	32 (8%)	
	% within <i>S. aureus</i>	10%	6.7%		
Total	No	160 (40%)	240 (60%)	400 (100%)	

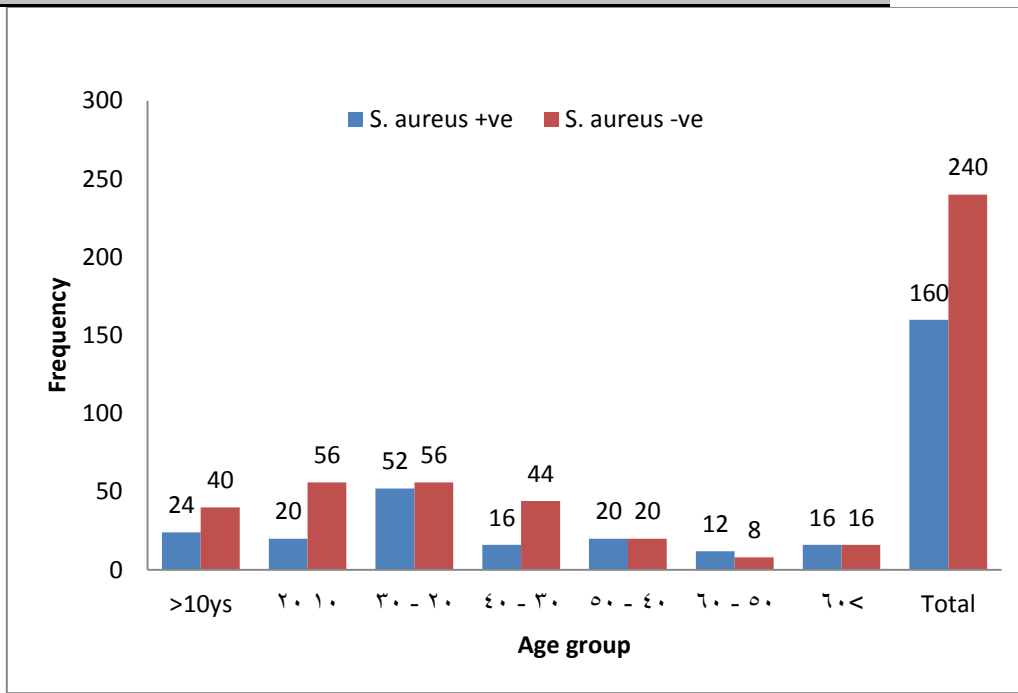
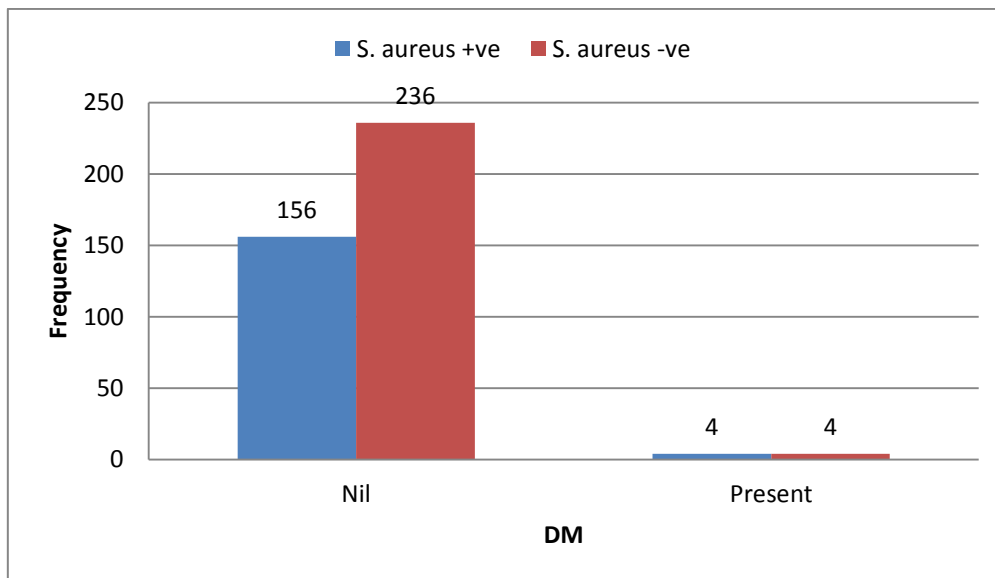


Fig (17): Distribution of *S. aureus* by age group

Table (11) and fig (17) show that there is a statistical significant difference in distribution of *S. aureus* isolates among different age groups of patients (P=0.003), being maximum in 50 - 60 years age group

Table (12): Relation of *S. aureus* by DM:

DM		<i>S. aureus</i>		Total	p-value
		+ve	-ve		
nil	No (%)	156 (60.2%)	236 (39.8%)	392 (98%)	0.72
	% within <i>S. aureus</i>	97.5%	98.3%		
present	No(%)	4 (50%)	4 (50%)	8 (2%)	
	% within <i>S. aureus</i>	2.5%	1.7%		
Total	No(%)	160 (40%)	240 (60%)	400 (100%)	



DM; Diabetes Mellitus

Fig (18): Relation of *S. aureus* by DM

Table (12) and Fig (18) show that there is no statistical significant difference in relation of *S. Aureus* between diabetic and non-diabetic patients (P= 0.72).

Table (13): Relation of *S. aureus* with site of burn

Site	No (%)	<i>S. aureus</i>		Total	p-value
		+ve	-ve		
Head& neck	No (%)	16 (66.6)	8 (33.4)	24 (6%)	0.004
	% within <i>S.aureus</i>	10%	3.3%		
chest& abdomen	No (%)	0	12 (100%)	12 (3%)	
	% within <i>S.aureus</i>	0%	5%		
UL or LL	No (%)	20 (35.7%)	36 (64.3%)	56 (14%)	
	% within <i>S.aureus</i>	12.5%	15%		
Head & limbs	No (%)	52 (39.4%)	80 (60.6%)	132 (33%)	
	% within <i>S.aureus</i>	32.5%	33.3%		
Multiple sites	No (%)	72 (40.9%)	104 (59.1%)	176 (44%)	
	% within <i>S.aureus</i>	45%	43.3%		
Total	No (%)	160 (40%)	240 (60%)	400 (100)	

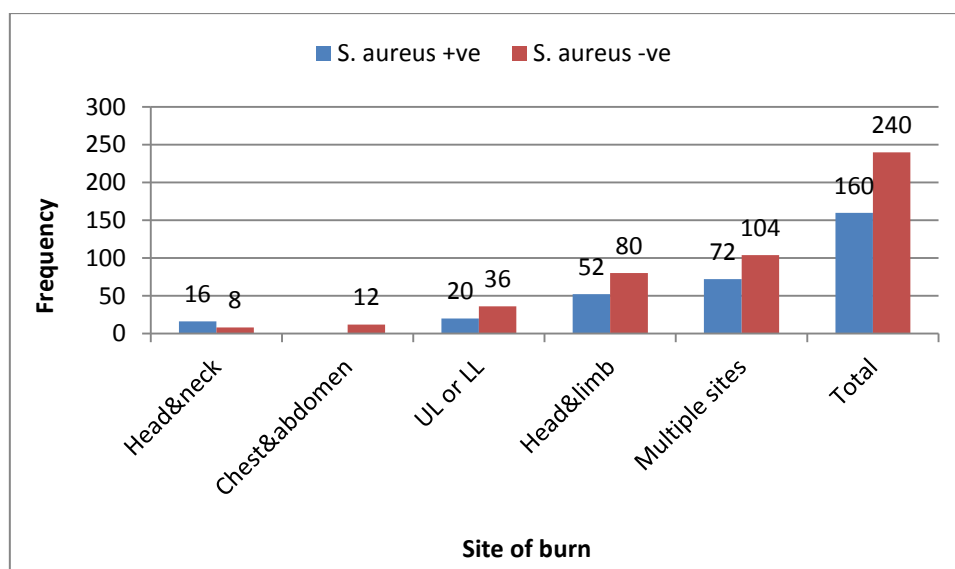


Fig (19): Relation of *S. aureus* with site of burn

Table (13) and fig (19) showed that there is statistical significant difference in *S. aureus* relation among patients with different sites of burn (P=0.004). The highest percent (45%) of *S. aureus* was isolated from patients with multiple-sites burn followed by burn of head & limb (32.5%), while no *S. aureus* was found in chest and abdomen burns.

Table (14): Distribution of *S. aureus* by burn percent:

Burnpercent		<i>S. aureus</i>		Total	p-value
		+ve	-ve		
10-40	No (%)	68 (63.8%)	120 (36.2%)	188 (47%)	0.335
	% within <i>S. aureus</i>	42.5%	50%		
40-70	No (%)	76 (43.2%)	100 (56.8%)	176 (44%)	
	% within <i>S. aureus</i>	47.5%	41.7%		
>70	No (%)	16 (44.4%)	20 (55.6%)	36 (9%)	
	% within burn_percent	10%	8.3%		
Total	No (%)	160 (40%)	240 (60%)	400 (100)	

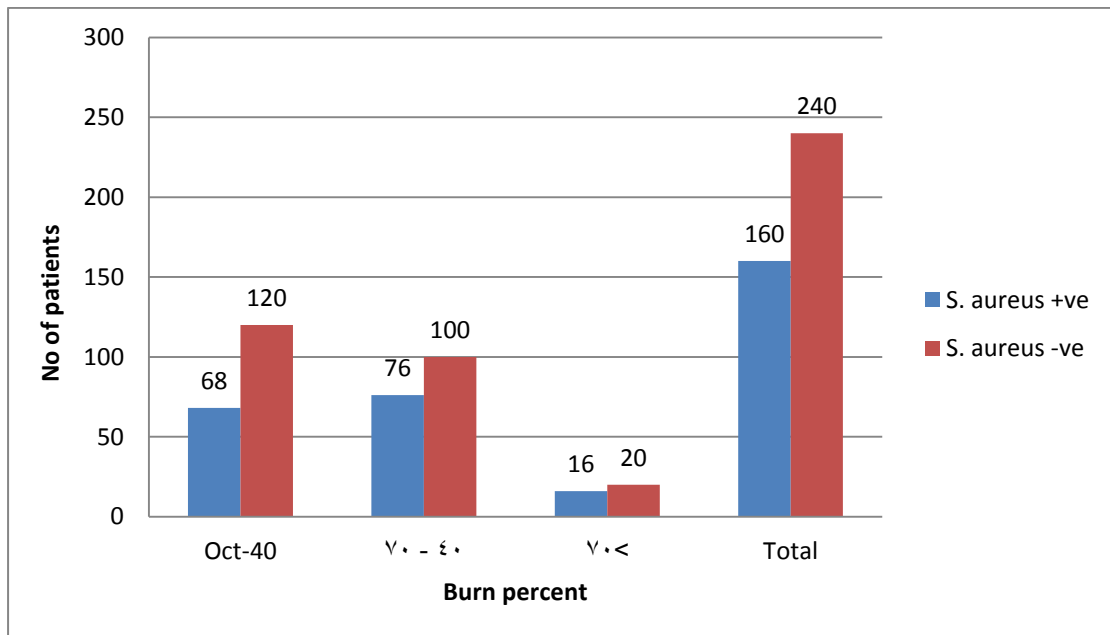


Fig (20): Distribution of *S. aureus* by Burn percent

Table (14) and fig (20) show that there is no statistical significant difference in distribution of *S. aureus* between patients with different burn percent ($p= 0.335$).

Antibiotic	<i>S. aureus</i>		Total	% within <i>S. aureus</i>	p-value
	+ve	-ve			
Amx-clavA	0(0%)	4(100%)	4(1%)	0%	0.101
Ciprofloxacin	16 (57.1%)	12 (42.9%)	28 (7%)	10%	0.055
Amx-clavA	8(25%)	24(75%)	32(8%)	5%	0.071
Ceftriaxone	128(38.1%)	208(61.9%)	336(84.0)	80%	0.075
Amp-sulbact	100(35.7%)	180(64.3%)	280(70%)	62.5%	0.008
Cefaxone	28(87.5%)	4(12.5%)	32(8.0%)	17.5%	<0.001
cephalexin	9(50.0%)	9(50.0%)	18(5.3%)	6.5%	0.410

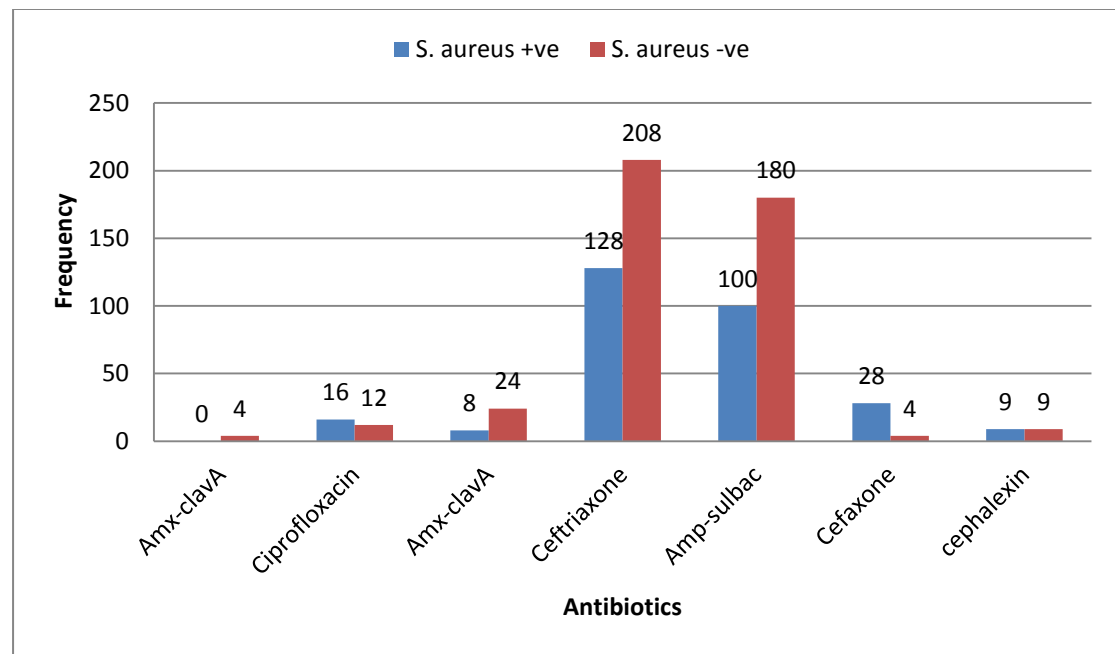


Fig (21): Distribution of *S. aureus* by type antibiotic used:

Table (15) and fig (21) show that *S. aureus* distribution among studied patients is not affected by type of antibiotics used in their treatment (p values ranged from 0.05 to 0.4) except for cefaxone and Amp-sulbactam (P<0.001 & 0.008 respectively) which seems to affect *S. aureus* distribution, 87.5% of patients receiving cefaxone have *S. aureus* infection.

Table (16): Distribution of *S. aureus* by duration of antibiotic administration

Duration	No	<i>S.aures</i>		Total	P value
		-ve	+ve		
1-3 D	88(55%)	72(45%)	160	0.16	
% within <i>S.aureus</i>	36.7%	45.0%	40.0%		
4-7 D	28(70%)	12(30%)	40		
% within <i>S.aureus</i>	11.7%	7.5%	10.0%		
8-14D	124(62%)	76(38%)	200		
% within <i>S.aureus</i>	51.6%	47.5%	50.0%		
Total	Count	240(60%)	160(40%)	400	

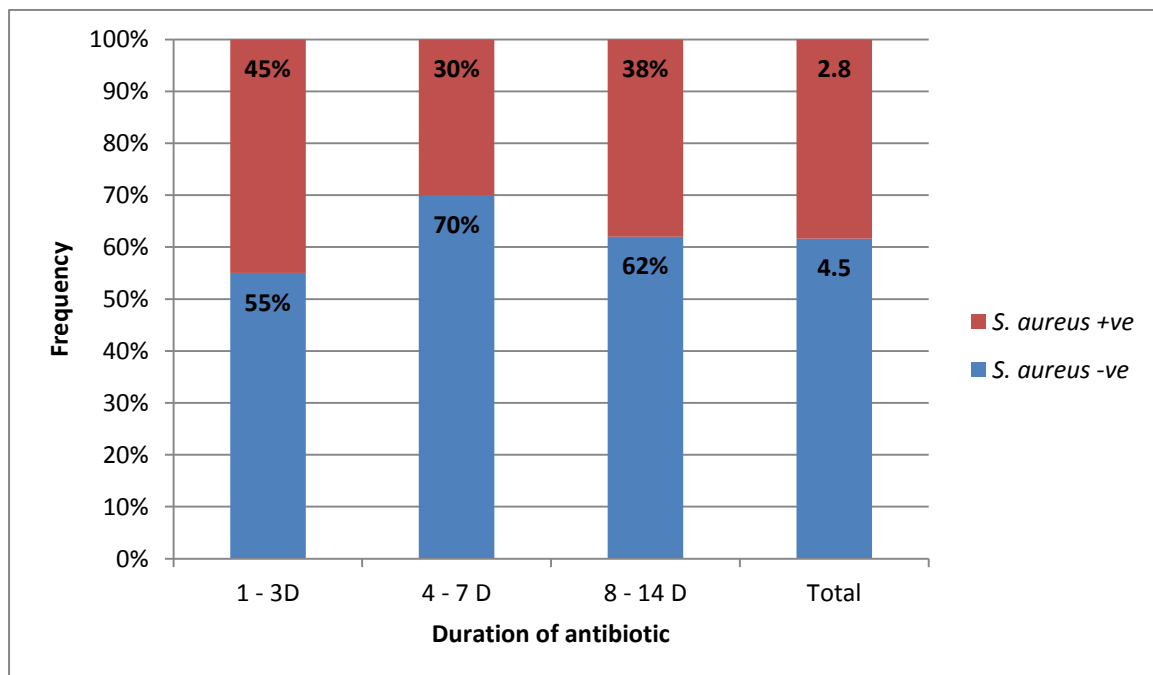


Fig. (22): Distribution of *S. aureus* by duration of antibiotic administration

Table (16) & Figure (22) showed that there is no statistical significant difference in distribution of *S. aureus* among patients receiving antibiotics for different durations (P= 0.16).

Table (17): Distribution of *S. aureus* by antibiotic combinations

Antibiotic		<i>S. aureus</i>		Total	P value
		-ve	+ve		
Ceftriaxone only	No (%)	44 (55%)	36 (45%)	80	<0.001
	% within <i>S. aureus</i>	18.3%	22.5%	20%	
Amp-sulbct only	No (%)	12 (75%)	4 (25%)	16	
	% within <i>S. aureus</i>	5.0%	2.5%	4.0%	
Ceftriaxone+Amp-sulb	No (%)	144 (66.7%)	72 (33.3%)	216	
	% within <i>S. aureus</i>	60%	45.0%	54%	
DitheraPy	No(%)	20 (35.7%)	36 (64.3%)	56	
	% within <i>S. aureus</i>	8.3%	22.5%	14.0%	
Tritherapy	No (%)	20 (62.5%)	12 (37.5%)	32	
	% within <i>S. aureus</i>	8.3%	7.5%	8.0%	
Total	No (%)	240 (60%)	160 (40%)	400	

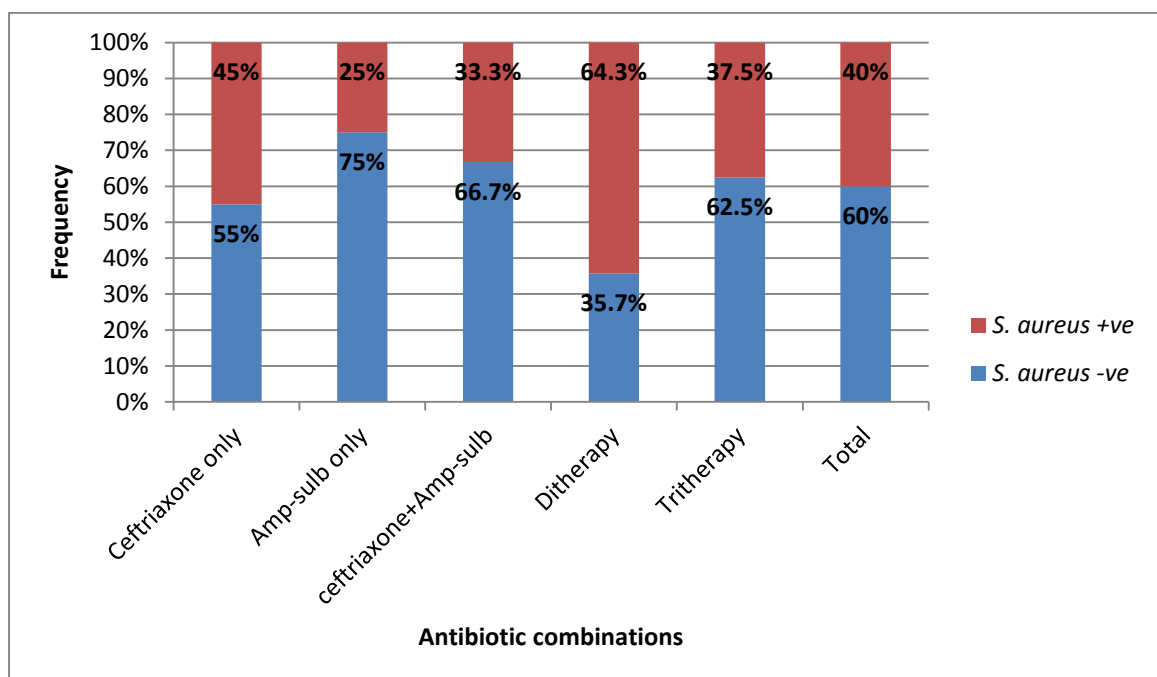


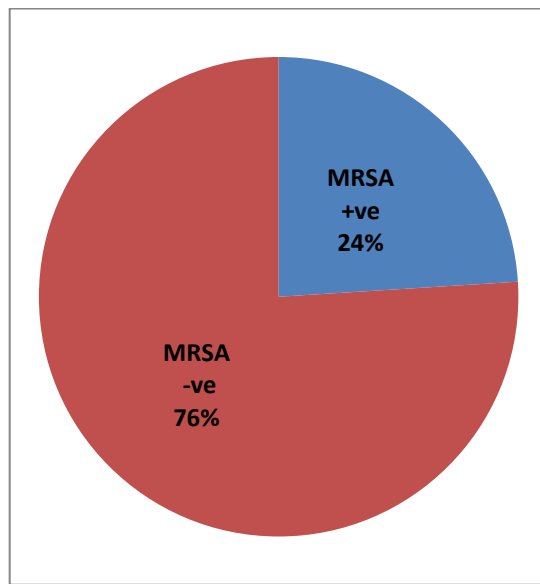
Fig. (23): Distribution of *S. aureus* by antibiotic combinations

Table (17) & figure (23) showed that there is statistical significant difference in distribution of *S. aureus* among patients receiving different combinations of antibiotics ($p < 0.001$). Amp-sulbactam alone (mono) showed lower rates of *S. aureus* infections than ceftriaxone + Amp-sulbactam combination, which has the lowest rate of infection among antibiotic combinations.

Table(18): Predictors of *S. aureus*:

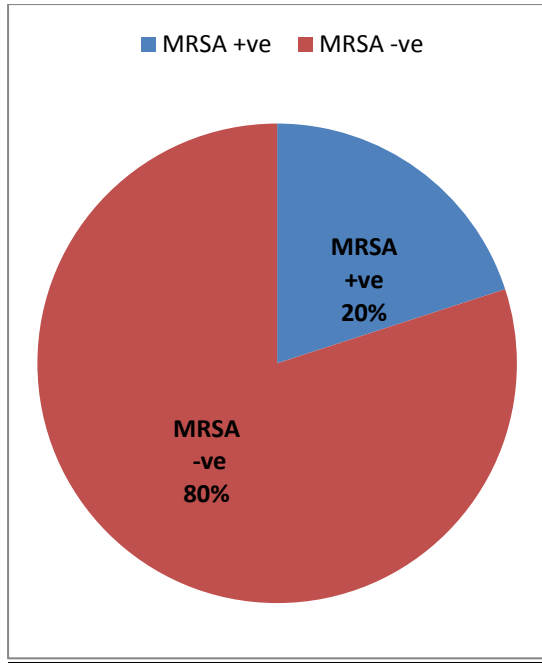
	P	Odd ratio	95.0% C.I.for Odd ratio	
			Lower	upper
Sex	<.001	.334	.197	.567
age	.419	.994	.979	1.009
DM	.159	2.959	.654	13.387
Percent	.226	1.008	.995	1.021
Amx-clavA	.999	.000	.000	.
Ciprofloxacin	.140	2.456	.744	8.102
Emoxiclave	.018	.280	.098	.801
Ceftriaxone	.477	.687	.244	1.935
Cefaxone	<.001	14.598		72.456
Amp-sulbactam	.052	.576	.330	1.005
Cephalexin	.079	.265	.060	1.166
Constant	.581	1.795		

Table (18) showed that sex and intake of cefaxone antibiotic are predictor of *S. aureus* infection among studied burn patients ($p < 0.001$ and 0.001 respectively)



Within 400 cases of burn wound infection studied, 96 cases (24%) of MRSA infection have been detected by ORSAB media(Fig24)

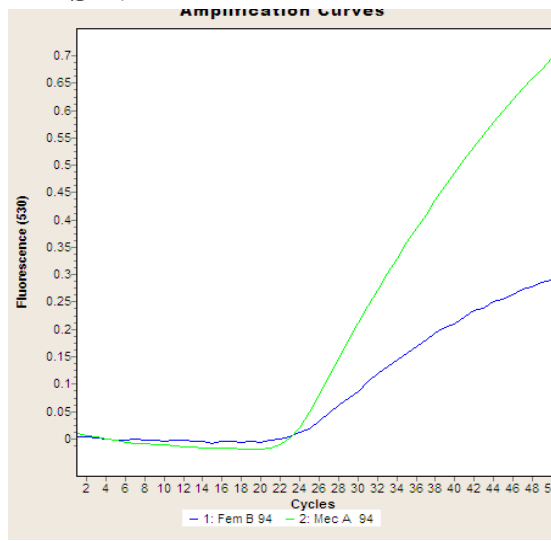
Fig(24): Distribution of MRSA among burn wound patients detected by ORSAB



Fig(25): Distribution of MRSA among burn wound cases detected by RT-PCR:

There are 80 (20%) MRSA isolates detected by RT-PCR among 400 studied cases of burn wound (Fig 25)

Fig(26): Amplification curves for a staphylococcus isolate positive for Fem B gene of *S. aureus*, and Mec A gene of MRSA.



X for no of cycles Y for amount of fluorescence

Fig(27): Amplification curves for a staphylococcus isolate positive for Fem B gene of *S. aureus* but negative for Mec A gene of MRSA.

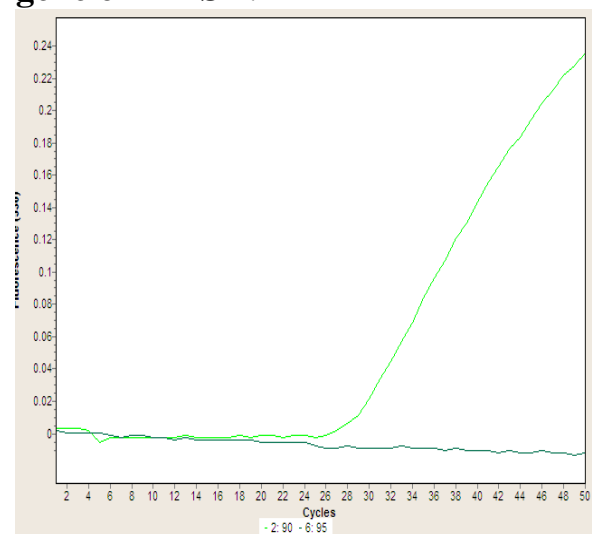


Table (19) :Comparison between ORSAB and RT-PCR for MRSA detection :

		ORSAB		Total	K
		+ve	-ve		
RT-PCR	+ve	80	0	80	0.81
	-ve	16	64	80	
Total		96	64	160	

Sensitivity= 83.3%
Specificity= 100%

Positive predictive value (PPV) = 100%
Negative predictive value (NPV) = 80%

Out of 400 isolates studied, ORSAB detect 96 cases of MRSA and RT-PCR detect 80 cases. Considering ORSAB media the gold standard for detection of MRSA; Sensitivity, specificity, positive and negative prediction values of RT-PCR are 83.3%, 100%, 100%, and 80%, respectively “table (19)”

Table (20): Distribution of MRSA by sex

Sex			MRSA		Total	P value
			+ve	-ve		
Males	No (%)	68 (32.1%)	144 (67.9%)	212	.100	
	% within MRSA	70.8%	47.4%	53.0%		
Female	No (%)	28 (14.9%)	160 (85.1%)	188		
	% within MRSA	29.2%	52.6%	47.0%		
Total	No (%)	96 (24%)	304 (76%)	400(100 %)		

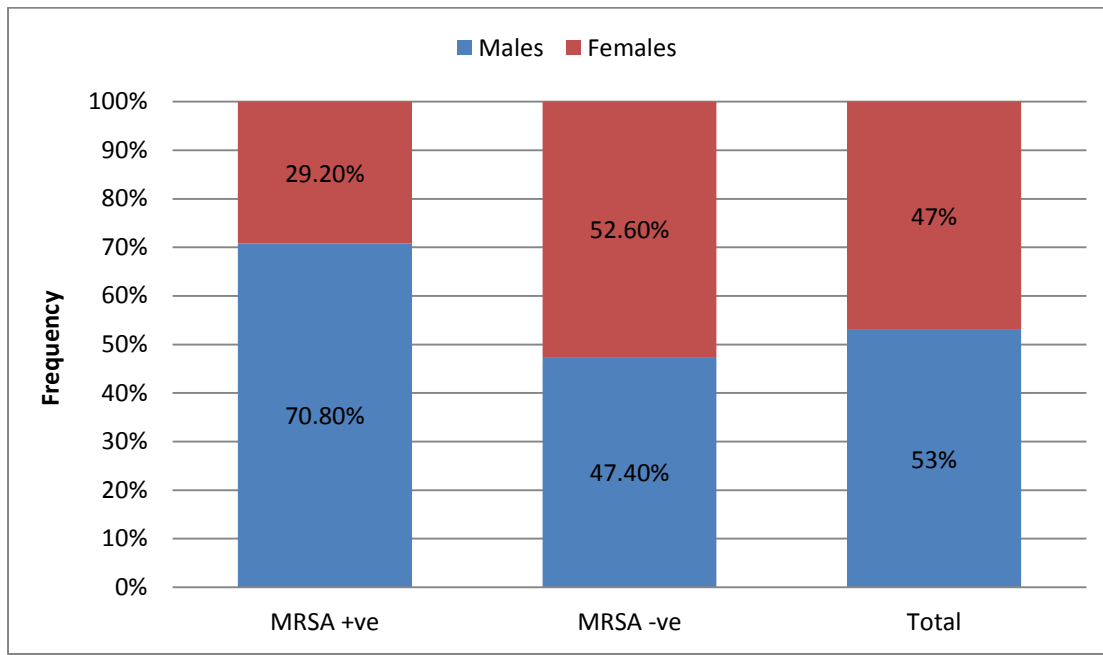


Fig (28): Distribution of MRSA by sex

Table (20) and fig (28) showed that there is statistical significant difference in distribution of MRSA between males and females. MRSA infections are more prevalent in males than females.

Table (21): Distribution of MRSA by age:

Age		MRSA		Total	p-value
		+ve	-ve		
<10y	No (%)	4 (6.2%)	60 (93.8%)	64	<.001
	% within MRSA	4.2%	19.7%	16%	
10-20	No (%)	12 (15.8%)	64 (84.2%)	76	
	% within MRSA	12.5%	21.1%	19%	
20-30	No (%)	40 (37.0%)	68 (63.0%)	108	
	% within MRSA	41.6%	22.4%	27%	
30-40	No (%)	12 (20%)	48 (80%)	60	
	% within MRSA	12.5%	15.8%	15%	
40-50	No (%)	12 (30%)	28 (70%)	40	
	% within MRSA	12.5%	9.2%	10%	
50-60	No (%)	12 (60%)	8 (40%)	20	
	% within MRSA	12.5%	2.6%	5%	
≥60	No (%)	4 (12.5%)	28 (87.5%)	32	
	% within MRSA	4.2%	9.2%	8%	
Total	No(%)	96 (24%)	304 (76%)	400 (100%)	

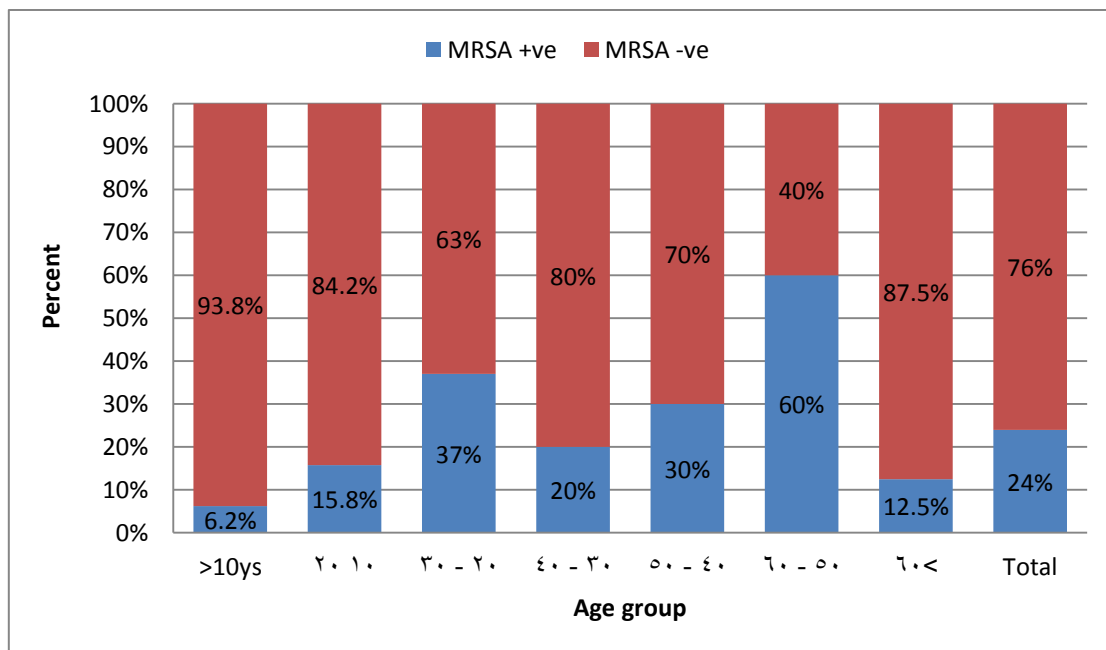


Fig (29): Distribution of MRSA by age:

Table (21) and fig (29) show that there is a statistical significant difference in distribution of MRSA between patients with different age group ($P < 0.001$). MRSA infections occur in (60%) of patients of age group (50-60ys) and only in 6.2% of patients of age group (<10ys).

Table (22): Distribution of MRSA by DM:

DM		MRSA		Total	p-value	
		+ve	-ve			
Nil	No (%)	96 (24.5%)	296 (75.5%)	392	0.207	
	% within MRSA	100.0%	97.4%	98.0%		
	present	No (%)	0 (0%)	8 (100%)		8
	% within MRSA	0%	2.6%	2.0%		
Total	No(%)	96 (24.0%)	304 (76.0%)	400		

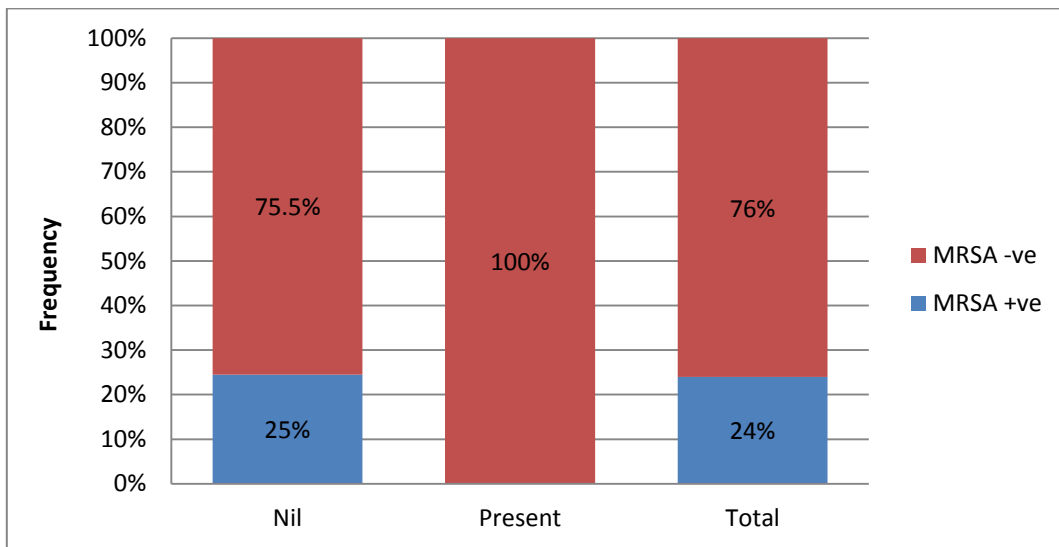


Fig (30): Distribution of MRSA by DM

Table (22) & fig (30) showed that there is no statistical significant difference in MRSA distribution between Diabetic and non-diabetic patients.

Table (23): Distribution of MRSA by site of burn

Site		MRSA		Total	P value
		+ve	-ve		
head& neck	No (%)	4 (16.7%)	20 (83.3%)	24 (6.0%)	0.22
	% within MRSA	4.2%	6.6%		
chest& abdomen	No (%)	0 (0%)	12 (100%)	12 (3.0%)	
	% within MRSA	0%	3.9%		
UL or LL	No (%)	12 (21.4%)	44 (78.6%)	56 (14%)	
	% within MRSA	12.5%	14.5%		
head & limbs	No (%)	32 (24.2%)	100 (75.8%)	132 (33%)	
	% within Mrs	33.3%	32.9%		
multiple sites	No(%)	48 (27.3%)	128 (72.7%)	176 (44%)	
	% within Mrs	50.0%	42.1%		
Total		No (%)	96 (24%)	304 (76%)	400

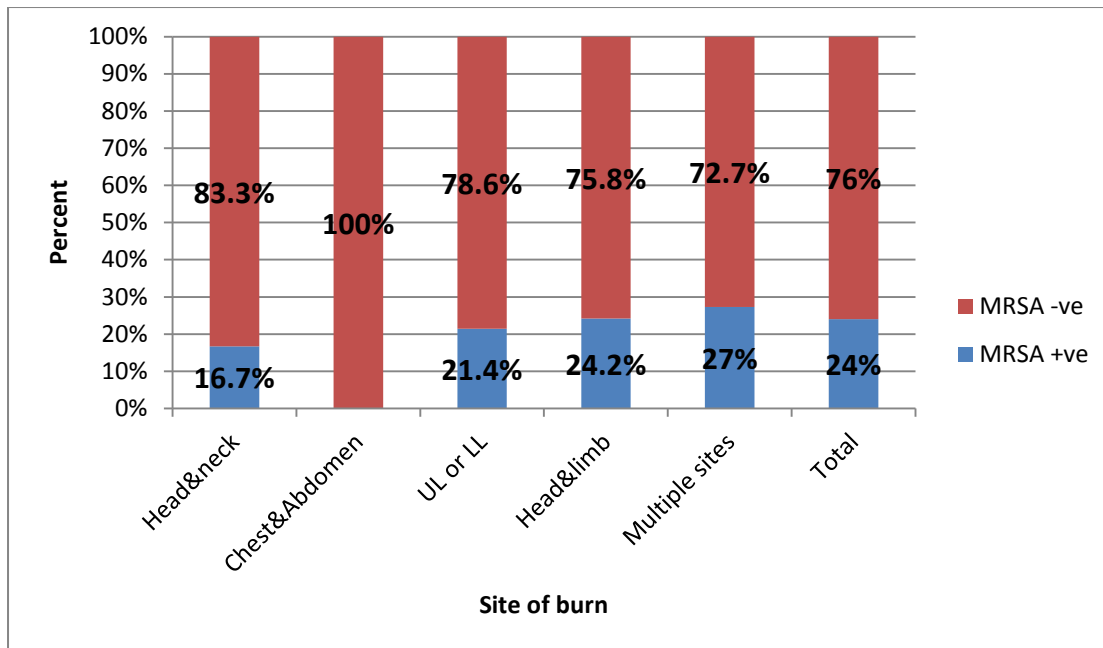


Fig (31): Distribution of MRSA by site of burn

Table (23) & fig (31) showed that there is no statistical significant difference in distribution of MRSA between patients of different burn sites (p=0.22).

Table (24): Distribution of MRSA by percent of burn:

burn_percent		MRSA		Total	p-value
		+ve	-ve		
10-40	No (%)	44 (23.4%)	144 (76.6%)	188	0.009
	% within MRSA	45.8%	47.4%	47%	
40-70	No (%)	36 (20.5%)	140 (79.5%)	176	
	% within MRSA	37.5%	46%	44%	
>70	No (%)	16 (44.4%)	20 (55.6%)	36	
	% within MRSA	16.7%	6.6%	9%	
Total	No (%)	96 (24%)	304 (76%)	400(100%)	

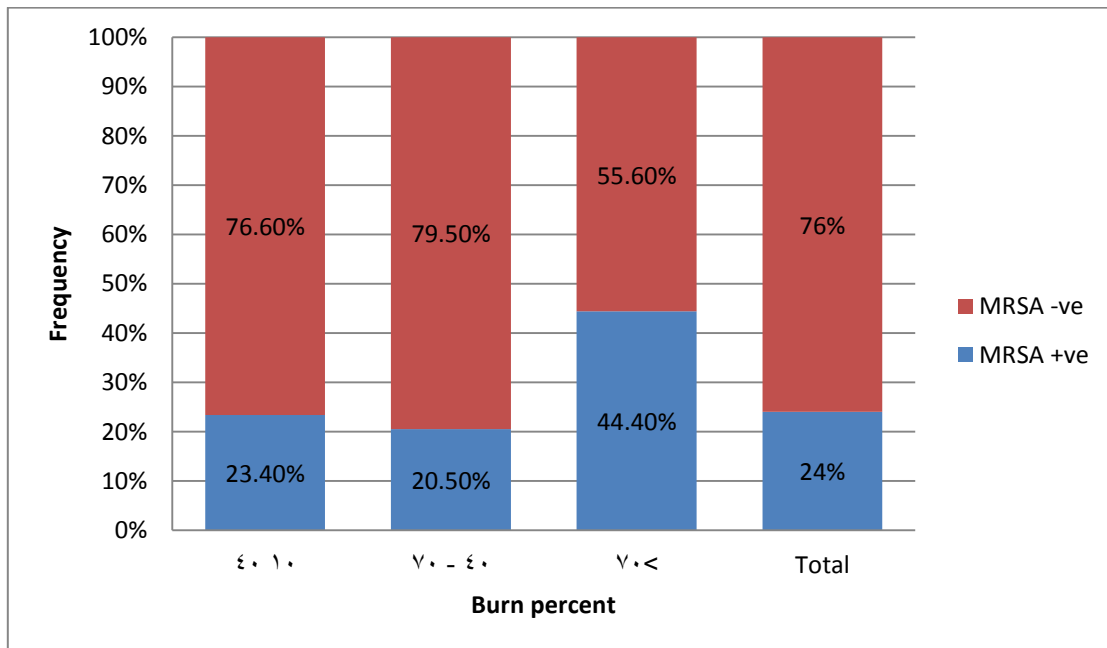
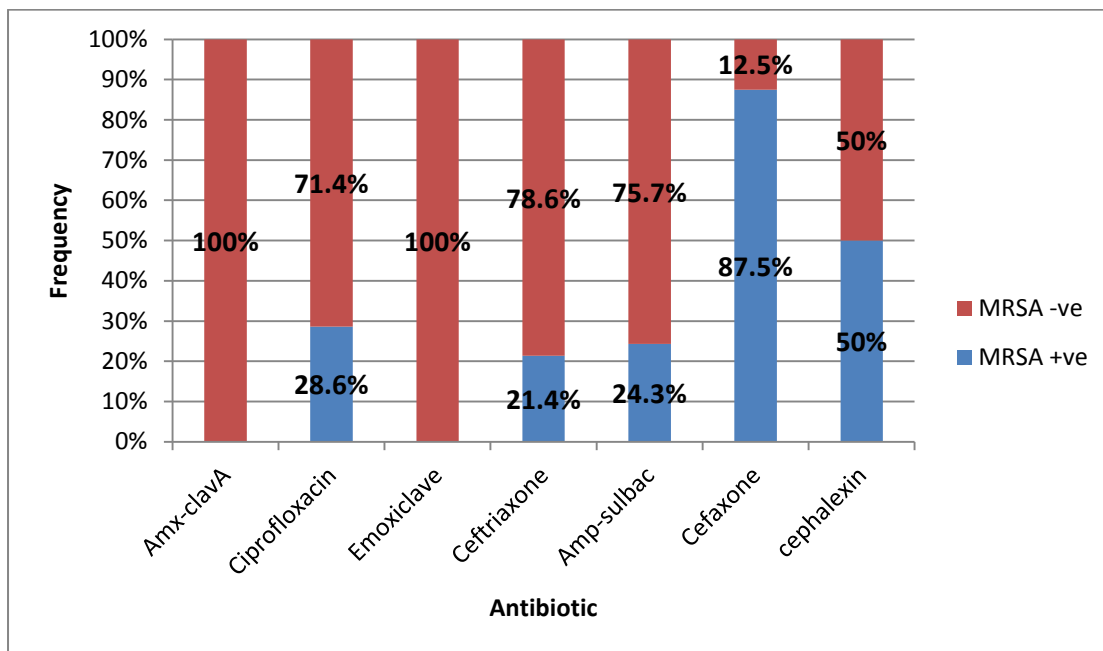


Fig (32): Distribution of MRSA by percent of burn:

Table (24) and fig (32) show that there is a statistical significant difference in distribution of MRSA between patients with different percent of burn (P= 0.009). MRSA infections increase with the increase in percent of burn.

Table (25): Distribution of MRSA by antibiotic used:

Antibiotic	MRSA		Total	%within MRSA	p-value
	+ve	-ve			
Amx-clavA	0(0%)	4(100%)	4(1%)	0%	0.57
Ciprofloxacin	8 (28.6%)	20 (71.4%)	28 (7%)	8.3%	0.55
Emoxiclav	0(0%)	32(100%)	32(8%)	0%	<.001
Ceftriaxone	72(21.4%)	264(78.6%)	336(84.0%)	75.0%	.006
Amp-sulbactam	68(24.3%)	212(75.7%)	280(70%)	70.8%	.838
Cefaxone	28(87.5%)	4(12.5%)	32(8.0%)	29.2%	<.001
Cephalexin	9(50.0%)	9(50.0%)	18(5.3%)	11.8%	.004



Fig(33): Distribution of MRSA according to type of antibiotic use

Table (25) and fig (33) showed that there is a statistical significant difference in MRSA distribution according to antibiotics used. MRSA infections increase with intake of ceftriaxone, cefaxone and cephalixin (p value=0.006, <0.001, 0.004 respectively) and decrease with intake of Emoxclave (p< 0.001).

Table (26): Distribution of MRSA by duration of antibiotic administration

Duration		MRSA			P value
		-ve	+ve	Total	
1-3 D	No (%)	124 (77.5%)	36 (22.5%)	160	0.611
	% within MRSA	40.8%	37.5%	40.0%	
4-7D	No (%)	28 (70%)	12 (30%)	40	
	% within MRSA	9.2%	12.5%	10.0%	
8-14D	No (%)	152 (76%)	48 (24%)	200	
	% within MRSA	50.0%	50.0%	50.0%	
Total	No (%)	304(76%)	96(24%)	400	

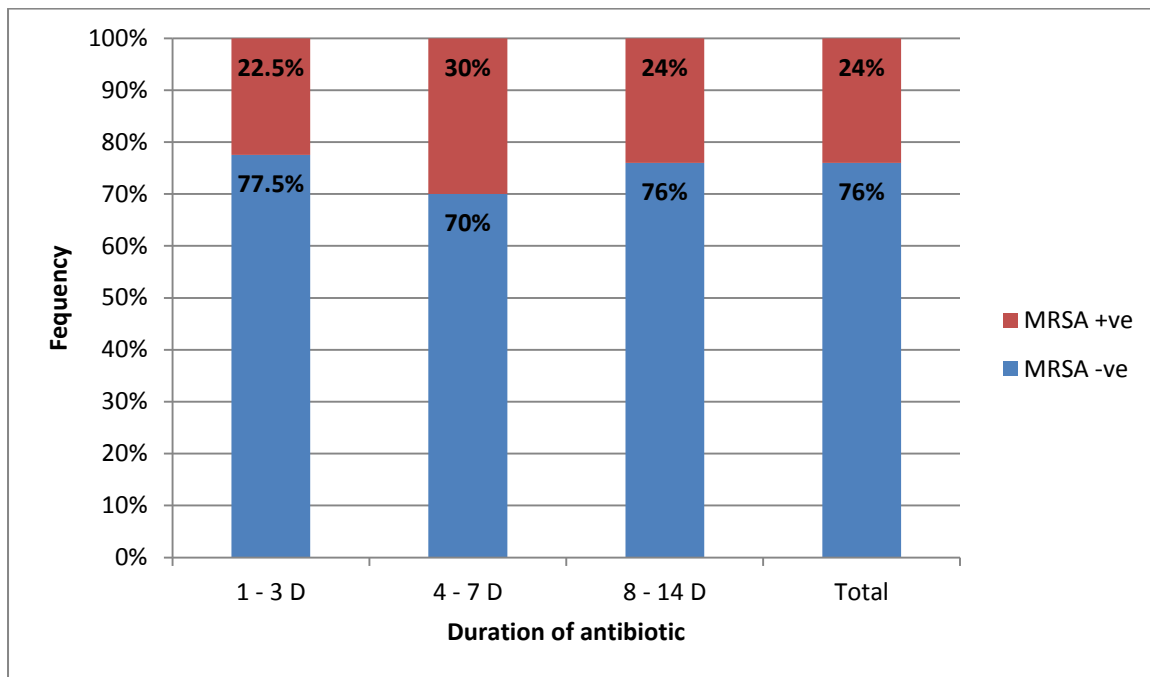


Fig. (34): Distribution of MRSA by duration of antibiotic administration

Table (26) & Fig. (34) showed that there is no statistical significant difference in distribution of MRSA among patients receiving antibiotics for different durations (P =0.611)

Table (27): Distribution of MRSA by antibiotic combinations

Antibiotic		MRSA		Total	P value
		-ve	+ve		
Ceftriaxone	No (%)	68 (85%)	12 (15%)	80	0.001
	% within MRSA	22.3%	12.5%	20%	
Amp-sulbactam	No (%)	16(100%)	0 (0%)	16	4.0%
	% within MRSA	5.2%	0%	4.0%	
Ceftriaxone + Amp-sulbac	No(%)	168(77.7%)	48(22.3%)	216	54%
	% within MRSA	55.2%	50.0%	54%	
Ditherapy	No (%)	28 (50%)	28 (50%)	56	14.0%
	% within MRSA	9.2%	29.1%	14.0%	
Tritherapy	No(%)	24 (75%)	8 (25%)	32	8.0%
	% within MRSA	7.8%	8.3%	8.0%	
Total	No (%)	304 (60%)	96 (40%)	400	

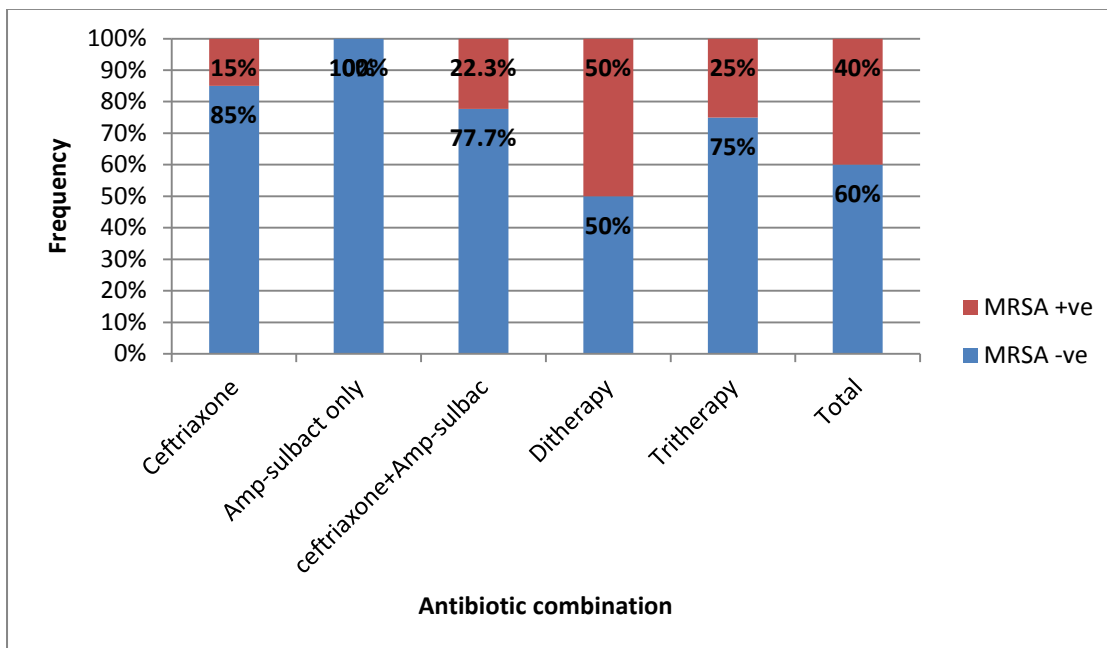


Fig. (35): Distribution of MRSA by antibiotic combinations

Table (27) & fig. (35) showed that there is statistical significant difference in distribution of MRSA among patients receiving different combinations of antibiotics ($p < 0.001$). Ceftriaxone or Amp-sulbactam alone (mono) showed lower rates of MRSA infections than cefotriaxone + Amp-sulbactam combination which showed lowest rate of MRSA infection among antibiotic combinations.

Table (28): Predictors of MRSA:

	P	Odd ratio	95.0% C.I. for Odd ratio	
			Lower	Upper
Sex	.002	.329	.166	.655
age	.301	1.010	.991	1.028
Percent	.004	1.024	1.008	1.040
DM	.999	.000	.000	.
Cefaxone	<.001	45.009	8.093	250.311
Amx-clavA	.999	.000	.000	
Ciprofloxacin	.059	3.664	.953	14.081
Emoxiclav	.998	.000	.000	.
Ceftriaxone	1.000	1.000	.231	4.331
Amp-sulbac	.129	1.802	.843	3.855
cephalexin	.742	.771	.164	3.629
Constant	.999	5.487E7		

Table (28) showed that the predictors of MRSA infection in burn patients are sex, percent of burn and intake of cefaxone antibiotic (p= 0.002, <0.001 and 0.004 respectively).

****E-test: 96 cases show no presence of VISA or VRSA in any case (MIC<2ug\m.**

Discussion

Thermal injury is one of the most common traumas in daily life, and the causes are in great diversity. In China millions of burns happened every year, while in the USA 1.2 million burns occurred because of fires every year (*Chen et al., 2012*).

Skin is regarded as the first barrier protecting people from invading microorganism, so it is easier to get infections after burns. Burn wound infections have been reported to be one of the most common and vital complications in burn centers which greatly influence mortality. *S. aureus* or MRSA is one of the leading causes of infections among burn centers (*Chen et al., 2012*).

S. aureus is a major human pathogen causing a great number of illnesses, ranging from skin & soft tissue infections and toxin-mediated disease to invasive infections. The emergence and spread of MRSA among burn centers results in number of poor outcomes such as prolonged hospitalization, economic burden, bacteremia or sepsis and even death, which prompts great urgency in the development of and advocacy for prevention and treatment efforts (*Chen et al., 2012*).

In the present study we examined 400 patients in burn unit in El-Fayoum general hospital aiming at: Detecting prevalence of staphylococcal infection in patients admitted to burn unit, typing of staphylococci isolates by anti-biogram, and determining prevalence of infection by different types of staphylococci strains isolated from burn wounds. Swabs taken from burn sites were examined for contaminating organisms. The presence of *S. aureus* was detected by conventional culture and biochemical tests, while MRSA was detected by culture on ORSAB media and RT-PCR. The presence of VIRSA or VISA was examined by E-test.

In the present study; out of 400 studied cases, 92 cases (23%) showed multiple isolates, 232 cases (58%) showed single isolates, while no isolates were obtained in 76 cases (19%).

On comparing infected and non-infected cases we found that; older age (40 – 50 years old), the higher burn percent (40-70%), burns in multiple sites (more than 2 sites) were associated with infected patient's group.

Singh et al., 2003 showed that multiple isolates were found in (40%) of cases, single isolates were found in (45%) of cases and (12.5%) of samples showed absence of bacterial pathogens. **Begum et al., 2011** found bacterial isolates in (92.9%) of cases and only (7.1%) were sterile.

Appelgren et al., 2002, found that 83 patients were infected, whereas 147 patients were not infected, infected patients were older in age than non-infected (50ys vs 38ys), TBSAB% larger in infected than non-infected (10% vs 5%).

Infection rate in burn unit have been reported to be high due to: poor quality of nursing care - nursing overload and patient crowding are the most important factors - presence of patient relatives in the same room with burned patients, and indiscriminate use of broad spectrum antibiotics without antibiotic policy (**Singh et al., 2003**).

The present study showed that , the most frequently isolated organism causing wound infection was *S. aureus* (23%), followed by *E.coli* (13%), *pseudomonas* (11%), *Klebsiella* (6%), *Proteus* (3%) and *CONS* (2%).

In a study similar to ours, whereas all patients started antibiotic at the day of admission **Appelgren et al., 2002** found that the most frequent organisms causing burn wound infection was MSSA (57%), followed by *P. aeruginosa* (25%), β -haemolytic *streptococcus* spp (24%), coagulase-negative *staphylococci* (15%), *Enterococcus* (12%) and *Enterobacter* (9%).

Singh et al., 2003 found that *Pseudomonas* spp. was the commonest isolated pathogen (31%) followed by *S. aureus* (22%), *Klebsiella* species (19%), *.E. fecalis* (10%) and *Acinetobacter* spp. (9%).

In Egypt, *Nasser et al., 2003*, showed that *Pseudomonas* spp. was the most common isolated pathogen in Ain Shams Hospital Burn Unit (21.6%) followed by *Klebsiella* (15.2%), *E. coli* (13.6%), *S. aureus* (13.2), *CONS* (11.6%), *S. pyogenes* (8.3%), *Enterobacter* (6.6%), *E. feacalis* (5.9%), *Candida albicans* (3.6%) and No MRSA have been detected.

In the present study *S. aureus* and MRSA represent 40% and 26% of isolated organisms respectively. *Theodorou et al., 2013* detect a lower percent of *S. aureus* (4%), and MRSA (2%), on the other hand *Chen et al., 2012* and *Maina et al., 2013* detected higher percent of MRSA (55%).

Rashid et al., 2006 screened for MRSA outbreak in Ireland Regional Burn Unit and detected that MRSA represent (31%) (n=16\52 cases).

Buchanan et al., 2012, examined 942 culture-positive infections over six-year period, of these 82 (8.7%) were MRSA infections with 66 (7.0%) of these being CA-MRSA infections. Of the remaining 860 (non-MRSA cases), staphylococcal and streptococcal species made up to 71.3% of these cases.

Aragon et al., 2011, revealed that out of the 219 patients with *S. aureus* isolates, 89 (40.64%) patients had MSSA, 33 patients (15.07%) had CA-MRSA and 97 (44.3%) had HA-MRSA isolates.

S. aureus, may be able to proliferate rapidly in and invade through the nonviable burned tissue, and can multiply also in the wet dressing, and the risks of infection are greater especially in burns unit and when burned patients were nursed in general units (*Alsaimary., 2009*).

In the present study, out of studied infected burn patients 54.3% were males 45.7% were females (P=0.3). *S. aureus* infections are more common in males (65%) than females (35%). Also MRSA infections are more prevalent in males (70.8%) than females (29.2%).

Similar results were obtained by *Nasser et al., 2003* who examined 70 patients admitted to burn unit, faculty of medicine, Ain Shams University Hospital, 39 females and 31 males (55.7% and 44.3%) respectively. *Chen et al., 2012* detected 80% of burn infection in males, and *Schweizer et al., 2012* found that 66% of the infected burn patients were male. On the other hand in Bangladesh, *Mashreky et al., 2011*, detected more burn infection in females (90%) -especially in rural areas- where burns occurred in the kitchen associated with flame.

Macedo and Santos, 2005, found that 28.4% of all isolates were *S. aureus* of which (59.1%) were males and (40.9%) were females. *Ekrami and Kalantar, 2007*, found that infection with *S. aureus* occurred in ratio of 1.45:1 in males and females.

Rashid et al., (2006) detect 16 cases of MRSA in his study. These cases divided into (12 males and 4 females) which represent 75% and 25% respectively.

In our study the highest percent of burn infection (27%) was found in age group 50 - 60 years old ($P= 0.02$), with both *S. aureus* and MRSA infections being maximum (60%) in age group 50 – 60 years ($P= 0.003$, and 0.000 respectively)

This finding is in accordance with results of *Hendrix et al., 2011* study which described risk factors for burns infection in the elderly in USA, and *Schweizer et al., 2012* study which found that the median age of infected burn patients was 54 years. Also *Appelgren et al., 2002*, detect that median age of infected patients 50 years old. On the other hand *Begum et al., 2011* detected more than 50% of infected burn patients in age from 11 to 30 years.

Macedo and Santos, 2005, found that highest percent of *S. aureus* infection occur in age group (20-30 years), while *Vostrugina et al., 2006* found that highest percent of *S. aureus* infection occur in age group (40-50 years).

Ekrami and Kalantar., 2007, found that 12% of cases were MRSA which occurred mainly in age group (10-20 years).

Our study showed that, out of infected burn patients, we have 8 diabetic patients (2%), 4 of them (50%) have *S. aureus* infection (P=0.27), and none has MRSA. None of our patients have hypertension. DM may be a cause of decreasing immunity so half of DM cases have *S. aureus* infection.

Olivo et al., 2009, showed that (4%) of cases of MRSA have DM which is not significant (P=1.0)

Memmel et al., 2004 detected that diabetic patients represented 10.4% of burned patients and they had multiple infectious organisms (*S. aureus*, MRSA, proteus and pseudomonas).

In the present study higher percent of infections occur in patients with burned total body surface area (BTBSA) >70% (P= 0.009). MRSA infections increase with the increase in percent of burn, with the highest rate of MRSA infections (44.4%) being in burn percent >70%. The highest rate of *S. aureus* infections (47.5%) occurred in burn percent [40-70%] which was insignificant (p= 0.335).

The large area of burns suppresses the immune functions allowing the organism to flourish in the wound (**Theodorou et al., 2013**).

Keen et al., 2010, revealed that Analysis of isolates by percentage TBSA burns showed that the percentage of MDR isolates was higher in patients with 30–60% TBSA. In contrast **Appelgren et al., 2002** and **Rashid et al., 2006**, revealed that higher percent of infection occur in patients with BTBSA (0-10%), [83% (n=193\230)] of patients had (0-10%). **Begum et al., 2011**, detect that 28.5% (n=32\104) of infected burn patients have burn percent (20-30%).

Macedo and Santos., 2005, found that *S. aureus* infection occurred more in patients with burn percent (10-40%). Also **Vostrugina et al., 2006**, found that MRSA infection was higher in patients with burn percent (10-40%).

Bagdonas et al., 2003, showed that in MRSA group major burns (TBSAB>70%) were predominated, but no significant difference was found (p=0.9), In *S. aureus* group the distribution of patients according to the severity of the burn injury was even and no significant difference was found (p=1).

In our study, we calculate percent of infection in different burned sites in body, the highest infection rates occurred in burns of multi-sites (45.7%) (p= 0.000), followed by head & neck and UL (21%), with *S. aureus* (P=0.004) and MRSA (p=0.22) infections being maximum (45% and 50% respectively) in patients with multiple-sites burn followed by burn of head & limb (32.5% and 33.3% respectively).

Fraze et al., 2005, found that *S. aureus* infection more in LL (48%), UL (27.7%), followed by head and neck or trunk (23.3%).

Lee et al., 2005 found that *S. aureus* infection more in LL (28.6%), UL (17.1%), followed by trunk (14.3%), and followed by head and neck (11.4%).

Olivo et al., 2009, found that MRSA infections was higher in burn in UL (89.3%), followed by trunk (85.3%).

Patients in our study received the following antibiotics: penicillins (Unasyn, Augmentin and Emoxclav), ciprofloxacin, cephalixin (1st generation cephalosporin) and ceftriaxone (cefaxone). Infection increased with the use of antibiotics cefaxone, unasyn, ceftriaxone, ciprofloxacin (p = 0.004, 0.003, 0.000, and 0.008 respectively), 100% of patients using ciprofloxacin or cefaxone have infected burns. *S. aureus* distribution among studied patients is not affected by type of antibiotics used in their treatment (p values ranged from 0.05 to 0.4) except for cefaxone and unasyn (P=0.000

& 0.008 respectively) which seems to affect *S. aureus* distribution, 87.5% of patients receiving cefaxone have *S.aureus* infection. MRSA infections increase with intake of ceftriaxone, cefaxone and ceporex (p value=0.006, 0.000, 0.004 respectively) and decrease with intake of Emoxclave (p=0.001).

Resistance rates of *S. aureus* and MRSA were as follows respectively: (62.8%) & (54.5%) for ceftriaxone, (57.1%) & (28.6%) for ciprofloxacin, (50%) for each for cephalexin, (25%) & (0%) for amoxicillin-clavulanate, and (35.7%) & (24%) for ampicillin-sulbactam. None of *S. aureus* or MRSA isolates showed resistance to vancomycin when tested by E-test.

In our study, the duration of antibiotic intake as well as use of antibiotic combination has no effect on rates of *S. aureus* or MRSA or other organisms infections (p=0.611, 0.160, 0,17 respectively). The use of unasyn alone (for *S. aureus*) or either unasyn or ceftriaxone alone (for MRSA) was more effective than (ceftriaxone + unasyn) combination (P=.000) which showed the lowest rates of burn infections among antibiotics combinations.

Our hospital mainly deals with patients residing in urban slums and cross-infection is frequent because of overcrowding of the in-patient unit, these strains establish themselves in hospital environment in areas like sinks, taps, railing, mattress, toilets and spread from one patient to another.

Macedo and Santos, 2005, found that *S. aureus* isolates were sensitive to: amoxicillin\clavulanic A (active ingredient of Unasyn), vancomycin, gentamicin, amikacin and ciprofloxacin.

Ekrami and Kalantar., 2007 performed antibiotic sensitivity of *S. aureus* and found that organism was sensitive to: ampicillin and vancomycin.

Bagdonas et al., 2003 showed that Systemic antibiotics were given to 19 patients (19%) for the treatment or prevention of *S. aureus* infection. For the treatment of MSSA infection, Oxacillin was given in 8 cases (20.5%), and

1st generation cephalosporins in 3 cases (7.7%). For the treatment of MRSA infection, Vancomycin was given in 8 cases (13%). The rest of the patients were managed without anti-*S. aureus* antibiotic therapy.

Al-Haddad et al., 2001 isolated 128 MRSA from burn unit and examined their antibiotic sensitivity. They found that: isolates were resistant to tetracycline, kanamycin and ciprofloxacin, but sensitive to minocycline, vancomycin and teicoplanin.

Chalise et al., 2008, detected antibiotic sensitivity to *S. aureus* and found that they are sensitive to: ciprofloxacin, ofloxacin, amikacin, tobramycin, nitrofurantoin, ceftazidime, but resistant to: azithromycin, cloxacillin, ceftriaxone, gentamycin, ampicillin, amoxicillin, cotrimoxazole.

Keen et al., 2010, showed that culture isolates recovered within the first 5 days of admission are more susceptible to antibiotics compared to isolates recovered after 15 days of hospitalization. Analysis of total cultures obtained from admission through hospital day 5 versus hospital days 15–30 revealed that: resistance of *S.aureus* increase 40% for oxacillin ($p < 0.05$).

Singh et al., 2003 performed antibiotic sensitivity of *S. aureus* by disk method and agar dilution method and detected resistance rates as follows: vancomycin (0%), co-trimoxazole (89%), ciprofloxacin (90%), netilmycin (10%), erythromycin (91%) and cloxacillin (94%). Such high resistances were due to non-adherence to the hospital antibiotic policy and empirical use of broad spectrum antibiotics which exert selective pressure on bacteria promoting isolation of multidrug resistant strains.

Olivo et al., 2009, detected resistance rates of: oxacillin (58.7%), amoxicillin-clavulanate (20%), piperacillin-tazobactam (4%), ciprofloxacin (25.3%), gentamycin (8%), imipenem (5.3%), vancomycin (9.3%) and ceftazidime (1.3%) in patients with MRSA after 48h of administration to hospital.

Mania et al., 2013 examined 176 samples and detected 82 *S. aureus* isolates, of which 69 were MRSA. Antibiotic sensitivity of both organisms showed that most isolates were susceptible to: ciprofloxacin and vancomycin, and decreased sensitivity to: gentamycin, erythromycin and cotrimoxazole.

Bagdonas et al., 2003, showed that the mean duration of antibiotic intake in cases of major burns in MRSA and MSSA groups was, respectively, 34.7 and 27.2 days, but no significant difference was found ($p=0.11$).

In the present study we use conventional disc diffusion method using cefoxitin as well as chromogenic ORSAB medium to detect MRSA. Out of 400 studied cases 160 *S. aureus* isolates were detected, of which 96 (24%) were proved to be MRSA by both disc diffusion and ORSAB.

Becker et al., 2002, use ORSAB to detect MRSA, 102 out of 104 MRSA-positive clinical specimens (98%) were correctly identified.

Cherkaoui et al., 2007, compare 4 chromogenic media (ORSAB, MRSA ID, Chromogen oxacillin *S. aureus* and MRSASelect), he detected relative sensitivities of 87%, 90%, 53% and 91% and specificities of 68%, 95%, 80% and 79% respectively.

In our study, we have developed a rapid and reproducible real-time PCR using the LightCycler platform that enables the detection of MRSA within 2 h. An advantage of the RT-PCR assay besides rapidity includes amplification and detection in a closed system of a capillary minimizing hands-on time and potential amplicon contamination. The RT-PCR has disadvantage that amplification mix must be made immediately before each use and cannot be frozen in the capillary. These capillaries are fragile and need to be handled with care during the aliquoting of reagents (**Costa et al., 2005**).

In the present study we compared results of ORSAB (chromogenic media) and RT-PCR in detection of MRSA strains. Out of 160 *S. aureus* isolates ORSAB media detected 96 MRSA isolates, and RT-PCR detected 80 isolates. The sensitivity, specificity, positive predictive value, negative predictive values of RT-PCR were 83.3%, 100%, 100%, and 80% respectively.

Our results are in accordance with **Rajn et al., 2007**, who revealed that Real time PCR initially identified only three of five (60%) new MRSA patients; one was negative and one was unresolved using PCR but both patients were identified as being MRSA positive by one of the other culture methods.

Titécata et al., 2012, showed that among 9 discordant results, 4 false-negative results were obtained with no detection of *mecA* gene, whereas MRSA strains were found in culture for 2 of them (sampled of the same patient). On the other hand **Gilpina et al., 2007** detected 12 PCR(+) /culture (-) samples. It is possible that RT-PCR has detected MRSA in samples which are below the limit detectable by culture, perhaps due to patient factors, e.g. current antibiotic therapy or de-colonization regimen.

According to CLSI, MRSA are those strains of *S. aureus* that express *mecA* or another mechanism of methicillin resistance, such as changes in affinity of penicillin binding proteins for oxacillin (modified *S. aureus* [MOD-SA] strains). The mechanisms of MRSA lacking *mecA* may be associated to hyper-production of β -lactamase, production of normal PBP with altered binding capacity, or other factors yet unidentified (**Chen et al., 2012**).

Paule et al., 2009, revealed that out of 500 samples a total of 171 samples grew confirmed MRSA (on any media), and 186 samples proved to

be MRSA by RT-PCR, there were 15 PCR-positive / culture-negative samples from patients with a history of MRSA.

Pasanen et al., 2009, compared ORSAB and RT-PCR in their study and found that: 29 samples were culture (+) / PCR (+), 2 samples were culture (+) / PCR (-), 138 samples were culture (-) / PCR (+), with PCR sensitivity of 93.5%, specificity of 88.6%, PPV of 17.3%, and NPV of 99.8%.

We use E-test to detect presence of VISA or VIRSA. None of *S. aureus* isolates in our study were proved to be VIRSA or VISA.

Dhanalakshmi et al., 2012 detect MIC of MRSA strains by using the agar dilution method and it was rechecked by the E-test and he found that No VISA or VIRSA were found among the 250 *S.aureus* isolates.

Kaleem et al., 2012, used E-test to detect VISA and VIRSA from 267 MRSA isolates indicated that there is emergence of increased vancomycin resistance among MRSA strains. Tough there was no VISA or VIRSA strain detected but a large number of isolates turned out to be having vancomycin MIC > 1 µg/ m.

Conclusion & Recommendation

The present study showed that the most common organism contaminate burn wounds in El-Fayoum general hospital was *S.aureus* (40%), and MRSA was detected in (26%) of studied cases.

Risk factors for acquisition of *staphylococcal* burn wound infections in the present study include: male, sex, old age (50-60 years old), large burn surface area (BTBSA >70%), burns in head and neck, and intake of cefaxone antibiotics.

ORSAB media is intended as a medium for the screening for MRSA containing peptones for growth, a high salt concentration and lithium chloride to suppress non-*staphylococcal* growth with mannitol and aniline blue for the detection of mannitol fermentation. In this study it considered the gold standard for MRSA detection; it detected 96 cases of MRSA while RT-PCR detected only 80 cases.

In our study, we use a rapid and reproducible real-time PCR using the LightCycler platform that enables the detection of MRSA within 2h. an advantage of the RT-PCR assay besides rapidity includes: amplification and detection in a closed system of a capillary minimizing hands-on time and potential amplicon contamination. Sensitivity, specificity, PPV, NPV was 83.3%, 100%, 100%, 80% respectively.

Careful surveillance of infection, good isolation techniques, procedure routines and a restrictive antimicrobial policy can keep antimicrobial resistance rates as well as infection rates low in infection-prone burn patients.

Summary

Thermal injury is one of the most common traumas in daily life, and the causes are in great diversity. In China millions of burns happened every year, while in the USA 1.2 million burns occurred because of fires every year.

S. aureus is a major human pathogen causing a great number of illnesses, ranging from skin & soft tissue infections and toxin-mediated disease to invasive infections. The emergence and spread of MRSA among burn centers results in number of poor outcomes such as prolonged hospitalization, economic burden, bacteremia or sepsis and even death, which prompts great urgency in the development of and advocacy for prevention and treatment efforts.

The present study was conducted to: Detect prevalence of staphylococcal infection in patients admitted to burn unit at Fayoum hospital. Typing of *staphylococci* isolates by antibiogram and Determine prevalence of infection by different types of *staphylococci* strains isolated from wound specimen collected in the burn unit.

Burn sites of 400 patients were swabbed and cultured on conventional culture media. Isolates were identified using conventional biochemical tests. *S.aureus* and MRSA isolates were identified using conventional methods and culture on chromogenic agar (ORSAB) and were confirmed by molecular method real-time polymerase chain reaction (RT-PCR).

The present study detected *s.aureus* in (40%), MRSA in (26%) and CONS in (8%) of studied cases and no VRSA or VISA were detected. Out of 400 cases ORSAB media detect 96 cases of MRSA while RT-PCR detect only 80 cases. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of RT-PCR were 83.3%, 100%, 100% and 80% respectively.

Risk factors for acquisition of *staphylococcal* burn wound infections in the present study include: male sex, old age (50-60 years old), large burn surface area (BTBSA >70%), burns in head and neck, and intake of cefaxone antibiotics.

Careful surveillance of infection, good isolation techniques, procedure routines and a restrictive antimicrobial policy can keep antimicrobial resistance rates as well as infection rates low in infection-prone burn patients.

المخلص العربي

تعتبر البكتريا العنقودية الذهبية المكوره من اهم الميكروبات التى تصيب الانسان بالعديد من الامراض. وهذه الامراض قد تتراوح من امراض جلديه الى امراض تسببها السموم او امراض تغزو الانسجه والاعضاء. وقد بدأ ظهور البكتريا العنقودية المقاومه للميسيثيلين وانتشرت فى وحدات الحروق. مما ادى الى ظهور العديد من الاثار السلبيه مثل: المكوث فى المستشفى لفترات اطول، زياده التكلفه الماديه، انتشار الميكروب بالدم، تسمم الدم وقد ينتهى الامر بالوفاه. وهذا يحتاج لتطوير وابتكار وسائل جديده لمنع انتشار وعلاج هذا الميكروب.

وقد هدفنا فى هذه الدراسه الى: تحديد مدى انتشار البكتريا العنقودية فى وحده الحروق بمستشفى الفيوم العام، تقسيم البكتريا العنقودية باستخدام طريقه الحساسيه للمضادات الحيويه، ثم تحديد نسبه تواجد كل نوع من هذه الانواع فى هذه الحروق.

لقد قمنا بتجميع ٤٠٠ عينه من هذه الحروق باستخدام المسحه ثم قمنا بزراعتها على مستنبتات الزرع التقليديه، ثم حددنا نوع البكتريا الموجوده بالجرح باستخدام التحاليل البيوكيميائيه. ثم قمنا بالبحث عن البكتريا العنقودية المقاومه للميسيثيلين باستخدام الميديا المحدده للمقاومه للاوكساسيلين والتأكيد على هذه النتيجة باستخدام الطرق البيولوجيا الجزيئية (الزمن الحقيقى لتفاعل البوليميراز السلسلى -RT-PCR).

وباستخدام هذه التحاليل وجدت البكتريا العنقودية بنسبه ٤٠% والبكتريا العنقودية المقاومه للميسيثيلين ٢٦% والبكتريا العنقودية السالبه للتخثر ٨% ولم تظهر اى حاله للبكتريا العنقودية المقاومه للفانكوميسين. وباستخدام الميديا المحدده للمقاومه للاوكساسيلين وجد ٩٦ حاله من البكتريا العنقودية المقاومه للميسيثيلين باستخدام الوسائل الجزيئية (الزمن الحقيقى لتفاعل البوليميراز السلسلى -RT-PCR) وجد ٨٠ حاله فقط من البكتريا العنقودية المقاومه للميسيثيلين ووجد ان حساسيه هذه الطريقه تمثل ٨٣,٣%، ودقة هذه الطريقه تمثل ١٠٠%، وقيمتها لتحديد العينات الموجبه ١٠٠%، اما قيمتها لتحديد العينات السالبه ٨٠%.

وقد وجدنا فى هذه الدراسه ان عوامل الخطر التى ادت لزياده الاصابه بالبكتريا العنقودية فى جروح الحروق هى: الاصابه اكثر فى الرجال والسن الكبير (من ٥٠-٦٠ سنه)، مساحه الحرق الكبيره (اكثر من ٧٠%)، الحروق فى الرأس والرقبه، تناول مضاد حيوى سيفاكسون.

انه من الضرورى لكل وحدات الحروق بكل المستشفيات ان تحدد نوعيه البكتريا التى تسبب عدوى للجروح بها، وان تحدد حساسيه هذه الانواع من البكتريا للمضادات الحيويه المختلفه، وهذا مما

يساعد هذه الوحدات على مواجهة وعلاج اى عدوى تظهر مبكرا باستخدام المضاد الحيوى المناسب دون انتظار نتيجة المزارع بالمعمل، مما يقلل نسبة الاصابه بالعدوى ونسبه الوفيات بسبب هذا النوع من العدوى.

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Appendix

Muller-Hinton agar:

An antimicrobial susceptibility testing medium which may be used in internationally recognised standard procedures.

Typical Formula*	gm/litre
Beef, dehydrated infusion from	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0
pH 7.3 ± 0.1 @ 25°C	

* Adjusted as required to meet performance standards

Directions

Add 38g to 1 litre of distilled water. Bring to the boil to dissolve the medium completely. Sterilise by autoclaving at 121°C for 15 minutes.

Description

Mueller-Hinton Agar was designed to be a reproducible culture medium for the isolation of pathogenic *Neisseria* species (Mueller & Hinton¹). The inclusion of starch ensures that toxic factors found during growth will be absorbed and its presence is often essential to establish growth from very small inocula².

The major use of Mueller-Hinton Agar is for Antimicrobial Susceptibility Testing (AST). It has become the standard medium for the Bauer-Kirby method 3, 4 and its performance is specified by the NCCLS.

Blood agar base:

A non-selective general purpose medium which may be enriched with blood or serum.

Typical Formula*	gm/litre
`Lab-Lemco' powder	10.0
Peptone Neutralised	10.0
Sodium chloride	5.0
Agar	15.0
pH 7.3 ± 0.2	

* Adjusted as required to meet performance standards

Directions

Suspend 40g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes.

For blood agar, cool the Base to 50°C and add 7% of Defibrinated Horse Blood SR0050. Mix with gentle rotation and pour into petri dishes or other containers.

Description

Oxoid Blood Agar Base is a non-selective general purpose medium widely employed for the growth of pathogenic and non-pathogenic bacteria:

(i) Without additions, the medium may be employed as a nutrient agar (a richer medium than Nutrient Agar CM0003), or as a medium for the short-term maintenance of stock cultures.

(ii) With added serum or other enrichments, the medium becomes suitable for the cultivation of many fastidious organisms. Serum and other thermolabile enrichments should be added to the sterilised medium cooled to 45-50°C.

(iii) With added blood, the medium is not only enriched, but becomes suitable for the determination of the typical haemolytic reactions which are important diagnostic criteria for *streptococci*, *staphylococci*, and other organisms. For blood agar, 7% of sterile blood should be added to the sterilised medium cooled to 45-50°C.

Blood Agar Base was used during investigations on irradiated *Escherichia coli* and other bacteria. It was the most suitable medium for investigating the phages of *Clostridium perfringens*³ and as the basis of a selective medium for *Clostridium perfringens*⁴. It was used with added phenolphthalein phosphate for the detection of phosphatase-producing staphylococci⁵ and with added salt and agar for the assessment of surface contamination on equipment and pig carcasses⁶. It was used for determining the salinity range of growth of marine flavobacteria⁷.

MACCONKEY Agar:

A differential medium for the isolation of coliforms and intestinal pathogens in water, dairy products and biological specimens.

Typical Formula*	gm/litre
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral red	0.075
Agar	12.0
pH 7.4 ± 0.2	

* Adjusted as required to meet performance standards

Directions

Suspend 52g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes. Dry the surface of the gel before inoculation.

Description

A differential medium for the detection, isolation and enumeration of coliforms and intestinal pathogens in water, dairy products and biological specimens. MacConkey Agar corresponds to the medium recommended by the World Health Organization, the Dept. of Health and by Windle Taylor for the bacteriological examination of water.

Although principally used for coliforms, this medium may also be employed for the differentiation of other enteric bacteria (including pathogens) and is suitable for the differentiation of *Pasteurella* species⁴.

Technique

Pathological specimens

Due to its ability to support the growth of pathogenic Gram-positive cocci (e.g. *staphylococci* and *enterococci*) as well as Enterobacteriaceae, MacConkey Agar is particularly recommended for the cultivation of pathogens which may be present in a variety of specimens such as urine,

faeces and wound swabs. Whilst it is selective it does not suppress a mixed bacterial flora to the same extent as other inhibitory media (including other MacConkey agars). It provides a number of other diagnostic indications in addition to bile tolerance, such as colony morphology and chromogenesis. MacConkey Agar should be used in parallel with other selective indicator media such as Desoxycholate Citrate Agar, Bismuth Sulphite Agar, Brilliant Green Agar and Brilliant Green Bile (2%) Broth, and a non-selective medium such as Blood Agar..

Colonial characteristics

After 24 hours at 35-37°C typical colonies are as follows:

Organism	Colour	Remarks
<i>Escherichia coli</i>	red	non-mucoid
<i>Aerobacter aerogenes</i>	pink	Mucoid
<i>Enterococcus</i> species	red	minute, round
<i>Staphylococcus</i> species	pale pink	Opaque
<i>Pseudomonas aeruginosa</i>	green-brown	fluorescent growth

Mannitol salt agar:

A selective medium for the isolation of presumptive pathogenic staphylococci. Most other bacteria are inhibited, with the exception of a few halophilic species.

Typical Formula*	gm/litre
`Lab-Lemco' powder	1.0
Peptone	10.0
Mannitol	10.0
Sodium chloride	75.0
Phenol red	0.025
Agar	15.0
pH 7.5 ± 0.2 @ 25°C	

* Adjusted as required to meet performance standards

Directions

Suspend 111g in 1 litre of distilled water and bring to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes.

Description

A selective medium prepared according to the recommendations of Chapman¹ for the isolation of presumptive pathogenic *staphylococci*. Most other bacteria are inhibited by the high salt concentration with the exception of some halophilic marine organisms. Presumptive coagulase-positive *staphylococci* produce colonies surrounded by bright yellow zones whilst non- pathogenic staphylococci produce colonies with reddish purple zones.

Mannitol Salt Agar is recommended for the detection and enumeration of coagulase-positive staphylococci in milk², in food³ and other specimens⁴.

Technique

Heavily inoculate the Mannitol Salt Agar plate and incubate for 36 hours at 35°C or for 3 days at 32°C - the latter is recommended by the APHA³.

Presumptive coagulase-positive *staphylococci* produce colonies with bright yellow zones whilst coagulase-negative staphylococci are surrounded by a red or purple zone. Pick off suspect colonies and subculture in a medium not containing an excess of salt (e.g. Nutrient Broth No.2 CM0067) to avoid interference with coagulase or other diagnostic tests.

ORSAB(OXACILLIN RESISTANCE SCREENING AGAR BASE):

Vial contents (each vial is sufficient for 500ml of medium)	per vial	per litre
Polymyxin B	25,000IU	50,000IU
Oxacillin	1.0mg	2.0mg

Directions

Suspend 51.75g of Oxacillin Resistance Screening Agar Base in 500ml of distilled water and bring gently to the boil to dissolve. Sterilise by autoclaving at 121°C for 15 minutes. Cool to 50°C and aseptically add the contents of one vial of ORSAB Selective Supplement SR0195, reconstituted as directed below. Mix well and pour into sterile Petri dishes.

Description

ORSAB is intended as a medium for the screening for methicillin resistant *Staphylococcus aureus*(MRSA) directly from routine swab samples. The screening of patients and staff for the early detection of MRSA colonisation is essential if epidemics are to be prevented. ORSAB is based on traditional Mannitol Salt Agar CM0085 with a reduction in salt concentration from 75g/l (7.5%) to 55g/l (5.5%). This lower level of salt is still sufficient to inhibit most bacteria other than *staphylococci* whilst optimising growth of low numbers of MRSA.

Oxacillin Resistance Screening Agar Base is a nutritious and selective medium containing peptones for growth, a high salt concentration and lithium chloride to suppress non-*staphylococcal* growth with mannitol and aniline blue for the detection of mannitol fermentation.

The antibiotics contained in ORSAB Selective Supplement SR0195 are oxacillin at 2 mg/litre to inhibit methicillin sensitive *Staphylococcus aureus* (MSSA) and polymyxin B for the suppression of other bacteria able to grow at such a high salt concentration, e.g. *Proteus* spp.

Typical colonies of MRSA are intense blue in colour on a colourless background enabling the organism to be more easily identified in mixed culture than the pale yellow colonies seen on Mannitol Salt Agar.

Culture media	Colony Colour	
	Positive	Negative
ORSAB CM1008 & SR0195	Intense blue on colourless media	Straw / No Growth
Mannitol Salt Agar CM0085	Pale yellow on red media	Pink-Red / No Growth

Technique

Take a routine swab sample from the patient or person to be screened. Rub the swab onto an ORSAB plate in one set of streaks near the plate perimeter. The sample material should then be streaked across the plate using the diminishing sweep technique. Incubate at 37°C for 24 hours. Examine after 24 hours for blue colonies. Confirm suspected MRSA with coagulase test Staphytest Plus DR0850 or Dryspot Staphytest Plus DR0100 and PBP2' DR0900. Re-incubate negative plates for a further 24 hours if necessary. Do not re-incubate positive plates.

M.I.C.Evaluator Strips:

Description

The M.I.C.Evaluator™ (M.I.C.E.™) strips are a range of devices for the accurate determination of the minimum inhibitory concentration (MIC) of a test organism to an antimicrobial. The M.I.C.E. strips consist of a gradient of stabilised antimicrobial covering 15 doubling dilutions. The innovative new design makes the clinical interpretation of the MIC even easier by removing the half step values. The accuracy of the test is still maintained if required using the black sections on the strip.

On application of a M.I.C.E. strip to a pre-inoculated agar plate, the antimicrobial releases from the polymer strip, forming a defined concentration gradient in the area around it. After appropriate incubation, growth develops with a zone of inhibition around the strip. The large print font makes MIC is easy to read, as the value in the section where the growth first touches the strip.

Each M.I.C.E. strip is individually sealed in an easily peelable sachet with a desiccant to maintain the long term stability of the product. When the sachet is opened, the handle of the M.I.C.E. strip is conveniently presented, allowing easy placement of the strip on the agar plate. The sachets are presented in an easily stackable, durable box, which protects the strips from physical damage during transportation. M.I.C.E. strips are available in packs of 10 and 50 to allow maximum flexibility to meet your requirements.

M.I.C.E. strips allow for the rapid and accurate determination of the MIC of an organism to an antibiotic which can significantly improve patient management.

M.I.C.EVALUATOR READING GUIDE:

strip design makes the clinical interpretation of the MIC even easier by removing the half-step values – less really is more! The new design allows for the use of a larger font which will enable the MIC to be more easily read.

The MIC is read where the growth of the organism touches the strip. If the growth intersects on the line between the sections then the MIC is read as the value in the lower section.

If there is growth along the entire length of the strip, the MIC should be read as greater than the highest value shown on the M.I.C.E. scale. e.g. >256

If growth is inhibited all around the strip, the MIC should be read as less than the lowest value on the M.I.C.E. scale. e.g. <0.015

For clinical interpretation, the white sections on the strip are used as indicated in the diagram below in blue. If a clinical interpretation is required and the growth of the organism intersects the strip on a black section, then the MIC is read as the value in the white section above.

If an actual MIC is required then the black sections on the strip are also used as indicated in the diagram below in red.

دراسه رصد وتصنيف للبكتريا المكوره العنقوديه بوحدده الحروق

رسالة توطئة
للحصول على درجة الدكتوراه
في الميكروبيولوجيا الطبية والمناعة

مقدمة من

الطبيبة/ ايناس جمعه ابراهيم
بكالوريوس الطب والجراحة – جامعة القاهرة
ماجستير الميكروبيولوجيا الطبية والمناعة (جامعة القاهرة)

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كلية الطب
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