Isolation and Characterization of Antibiotic Resistant *Staphylococcus aureus* From Raw Milk Samples of Dairy Farms of Pothohar Region

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Food Poisoning

Antibiotic resistance

Isolation and Characterization of Antibiotic Resistant *Staphylococcus aureus* From Raw Milk Samples of Dairy Farms of Pothohar Region

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FINAL APPROVAL

It is certified that we have read the thesis submitted by Miss. MahamZahoorand it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University , Islamabad for the M.S Degree in Biotechnology.

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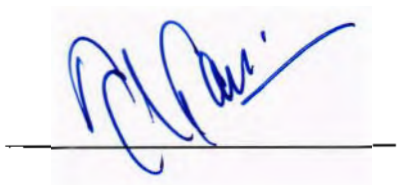

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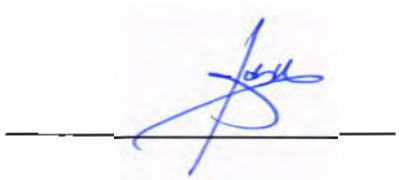

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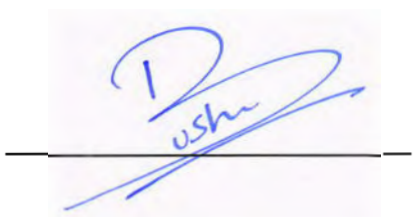

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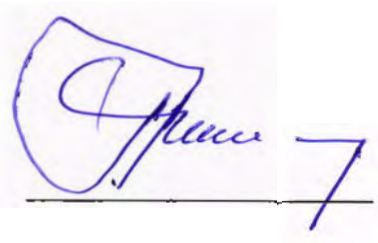

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The degree of MS Biotechnology

DEDICATION

This thesis is dedicated to my beloved parents and my family whose hands always rise in prayers for my success. Their prayers always helped me in coming out from complex situations.

DECLARATION

I hereby declare that the work present in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Date _____

MahamZahoor

CONTENTS

ACKNOWLEDGEMENT

All praise to Allah Almighty, the Omnipotent and the most Merciful and Beneficent, Who is the Creator of Universe, Who bestowed the man with intelligence, knowledge, sight to observe and mind to think and judge. Peace and blessing of Allah be upon the Holy Prophet, Muhammad (Peace Be Upon Him), who is everlasting torch of guidance and knowledge for humanity and who exhorted his followers t seek for knowledge from cradle to grave.

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LIST OF ABBREVIATIONS

Sr.No	Abbreviation	Name
1	MRSA	Methicillin-resistant *Staphylococcus aureus*
2	MSSA	Methicillin-sensitive*Staphylococcus aureus*
3	FOX	Cefoxitin
4	CN	Gentamicin
5	SXT	Sulfamethoxazole
6	DA	Clindamycin
7	C	Chloramphenicol
8	AMP	Ampicillin
9	E	Erythromycin
10	CLSI	Clinical and Laboratory Standards Institute
12	MSA	Mannitol Salt Agar

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ABSTRACT

Staphylococcus aureus (S. aureus) constitutes a grave risk to public health and safety as it can lead to a number different infections in both humans and animals which can develop into life threatening conditions. Appearance of antibiotics resistant *S. aureus*, especially Methicillin-resistant *S.aureus*(MRSA) is fastbecoming a global concern in both healthcare and community related infections. Milk is a vastly valued food source, but raw milk comprises and facilitates the growth of numerous microbes, including *S.aureus*.Such resistance is acquired by the acquisition of*mecA* gene in *S.aureus* .The present study was undertaken to isolate and characterize antibiotic resistant strains of *S.aureus* from raw milk collected from pothohar region , and to investigate whether the resistant strains contained the *mecA gene*.A total of 50 raw milk samples were collected from different local dairy farms and milk venders and subjected to confirmatory biochemical tests and antibiotic resistance profiling. From the collected 50 samples 33 strains of *S.aureus* were selectivelyisolated, including 7 strains from milk samples of cows suffering from mastitis. The isolated strains were subjected to antibiotic sensitivity profiling and then were screened for the presence of *mecA*gene. Results confirmed that 15 % of the isolated strains were positive for *mecA* (i.e 1 out of 3 ,obtained via PCR amplification using XP thermal cycler with the published primer mecA already used for amplification.The detailed analysis revealed that out of total number of 7 drugs that were used in this study ,sulfamethoxazole had the highest resistance of 48% for *S. aureus* which was a test to determine MRSA Methicillin resistant *S. aureus*.Results confirmed that 15 % of the isolated strains were positive for *mecA* (i.e 1 out of 43 samples and 4 out of 7 mastitis samples) ,obtained via PCR amplification using XP thermal cycler with the published primer *mecA* already used for amplification.These results showed the level of contamination of raw milk samples and revealed the poor handling and hygiene involved in handing of milk as well as the alarming level of resistance displayed by most of the isolated strains.

AIMS AND OBJECTIVES

- To selectively isolate *Staphylococcus aureus*strains from raw milk samples.
- To screen isolated strains for antibiotic resistance.
- To investigate presence of *mecA* gene among the isolated*Staphylococcus aureus*strains.

Introduction

INTRODUCTION

1.1 Overview

Bacterial populations are increasingly becoming invulnerable to all commercially accessible agents, with antibiotic resistance being one of the most prevalent dangers to human health in the 21st century (Cohen, 1992).

Staphylococcusaureus is a Gram-positive coccal bacterium that is a member of the Firmicutes.It is frequently found in the, respiratory tract, nose and on the skin(Deresse et al., 2012; Sushma et al., 2012).*Staphylococcusaureus*causes a variety of diseases in human and animals and is known to develop quick resistance to antimicrobial agents. There may be a mild skin infection to severe pneumonia and septicemia (Lowy et al., 1998).*Staphylococcusaureus*usually cause post-operative infections, endocarditis, toxic shock syndrome, food poisoning and osteomyelitis(Benayache et al.,2001).

The bacterium is frequently found associated with subclinical mastitis in dairy cattle (Adesiyun et al., 1998) and may be present in milk and other dairy products (Capurro et al., 2010).Although milk is a highly nutritious valuable commercial food source, however raw milk is often subjected to contamination by many microorganisms (Helena et al., 2010). *Staphylococcus aureus* is the most prevalent and economically significant pathogen causing inflammatory infections in dairy ruminants (Akineden et al., 2001; Cabral et al., 2004; Katsuda et al., 2005). Approximately 30%-40% of all mastitis cases are associated with the bacterium (Asperger et al., 2003).

Staphylococcus aureus can get access to milk either by direct excretion from udders with clinical or subclinical Staphylococcal mastitis or by contamination from the environment during handling of raw milk (Scherrer et al., 2004; Jorgensen et al., 2005). When the udder is infected, *Staphylococcusaureus*may be

excreted through milk in variable numbers up to 108 CFU/mL (Asperger et al., 2003).

The emergence of antibiotic-resistant microorganisms in farm animal environments poses a potential public health concern.It is also a common cause of mastitis in dairy cows and is a primary reason for antibiotic use on farms (Trinidad et al.,1990). *Staphylococcus aureus* is an important opportunistic pathogen both in humans and in dairy cattle.The use of antimicrobial agents on dairy farms as well as in other food animal production systems is a major concern in the emergence of resistant zoonotic bacterial pathogens (Piddock et al.,1996).

1.2 *Staphylococcus aureus*

Staphylococcus aureus was first identified in 1880 in Aberdeen, Scotland, by surgeon Sir Alexander Ogston in pus from a surgical abscess in a knee joint (OgstonA ,1984).*Staphylococcusaureus*can cause a range of illnesses, from minor skin infections, such as pimples, impetigo, boils, cellulitis, folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia, and sepsis(VerbrughH ,1997). It is still one of the five most common causes of hospital-acquired infections and is often the cause of wound infections following surgery. Each year, around 500,000 patients in hospitals of the United States contract a staphylococcal infection, chiefly by *Staphylococcusaureus*Up to 50,000 deaths each year in the USA are linked with S. aureus infections (Bowersoxet al., 1999)

The *Staphylococcusaureus*produces smooth, circular colonies, convex and lustrous; size of the colony may be 0.5-1.5 µm in diameter. Under microscope, it appears like irregular three dimensional bunch of grapes-like cluster of cells. The colony pigmentation may vary from grey, grey-white, with yellowish to orange shades and in blood agar typical β-hemolysis may be produced; depending on the growth condition (Deresse et al., 2012; Sushma et al., 2012).

Staphylococcus aureus may be pathogenic or non-pathogenic and the pathogenic strains are usually coagulase-positive and cause disease in their hosts (Smith et al., 2007). *Staphylococcus aureus* strains resistant to methicillin and many other antibiotics ; are major causes of nosocomial infections worldwide (Diekema et al.,2001) . Resistance to methicillin is determined by the mecA gene, which encodes the low-affinity penicillin-binding protein PBP 2A (Beck et al.,1986). The mecA gene is part of a 21- to 60-kb staphylococcal chromosome cassette mec (SCCmec), a mobile genetic element that may also contain genetic structures such as Tn554, pUB110, and pT181 which encode resistance to non-β-lactam antibiotics (Hiramatsu et al., 1998).

Methicillin-resistant Staphylococcus aureus (MRSA) is a major cause of nosocomicalinfections.Emergence of antibiotics resistant *Staphylococcusaureus*especially Methicillin-resistant *Staphylococcusaureus*(MRSA) is a worldwide problem in both healthcare and community settings (Chambers and DeLeo 2009). Outbreak of MRSA includes worldwide diverse population e.g. America India Pakistan and Alaska. MRSA can also be causative of severe and sometimes fatal invasive disease.(lowy,1998;liu et al.,2011).

The frequency of MRSA in Pakistan and India has been shown to be high as compared to northern Europe (Anwar et al. 2004). In Pakistan, the prevalence of methicillin resistance in *Staphylococcusaureus*has been observed with range from 42 to 51 % (Akinkunmi and Lamikanra 2012). With the increase of MRSA associated infections, the use of glycopeptides (vancomycin and teicoplanin) are also rising gradually. The irrational and indiscriminate use of glycopeptides results in the emergence of even low-level vancomycin resistance in S. aureus. Such resistance, though rare, but it is an emerging threat because of its potential to disseminate rapidly (Perwaiz et al. 2007).

1.3 MRSA and Antibiotic Resistance

The different diagnosis of clinical mastitis can be treated with various antibiotics [Morin et al. 1998]. Antibiotics are used to treat diseases of cattle and as well as used as preservatives for milk [Devriese et al. 1997]. The indiscriminate use of antibiotics has led to the development of multiple antibiotic resistances thereby rendering the antibiotic treatment ineffective [Johnston et al. 1983]. Resistant bacteria occur in soil, water, plants and animals. The resistant bacteria present in environments are in contact with human beings and animals. It has been estimated that nearly equal tonnage of antimicrobial agents are used in man and in agriculture worldwide [EFA 1997]. When low doses of antibiotics are used, they inhibit the growth of 146 KalsoomFarzana, Syed NisarHussain Shah and FarzanaJabeensusceptible bacteria while resistance bacteria thrive and grow such as in the presence of tetracycline [Eichner and Gravitz 1999].

MRSA can be transmitted from person to person via skin or the sharing of contaminated objects. In addition, MRSA can evade host immune system and virulence factors disseminated render this bug with limited therapeutic options available (Holcomb et al. 2008). MRSA have capability to breed in the presence of methyl penicillin and its derivatives like methicillin. Resistance to methicillin is mediated by mec-A gene, which encodes the polypeptide PBP2a protein (Oliveira et al. 2006). The mec-A gene have also insertion sites for transposons and plasmids which assist resistance to other antibiotic groups. Accordingly, cross-resistance to non-beta-lactam antibiotic groups including quinolones, sulfamethoxazole, macrolides, aminoglycoside and lincomycin were frequently observed in MRSA isolates (Chambers 2001).

1.4 Virulence by mecA Gene

Resistance to methicillin is determined by the mecA gene, which encodes the low-affinity penicillin-binding protein PBP 2A (Diekema DJ et al.; 2001). The mecA

gene is part of a 21- to 60-kb staphylococcal chromosome cassette mec (SCCmec), a mobile genetic element that may also contain genetic structures such as Tn554, pUB110, and pT181 which encode resistance to non-β-lactam antibiotics (Yonsei, 1998).

Two hypotheses have been raised to explain the evolutionary origin of methicillin-resistant *Staphylococcusaureus*(MRSA) strains. The single clone hypothesis, based on early analyses of the restriction fragment length polymorphisms obtained for MRSA isolates collected worldwide by using probes for mecA and Tn554, suggests that mecA entered the *Staphylococcusaureus*population on one occasion and resulted in the formation of a single MRSA clone that has since spread around the world (Yonsei, 1998)(Kreiswirth B et al.;1993). The second hypothesis, based on the detection of mecA in diverse *Staphylococcusaureus*multilocus enzyme electrophoresis types, proposes that MRSA strains evolved a number of times by means of the horizontal transfer of mecA into phylogenetically distinct methicillin-susceptible *Staphylococcusaureus*(MSSA) precursor strains (J Clin Microbiol,1992). By using DNA microarray technology, mecA has been detected in at least five divergent lineages, implying that horizontal mecA transfer has played a fundamental role in the evolution of MRSA (Fitzgerald JR et al.; 2001). The transfer of mecA from *S. epidermidis* to *S. aureus*was recently witnessed in vivo, suggesting that mecA may transfer more frequently to MSSA (Wielders CL et al.; 2001).

Material and methods

MATERIAL AND METHODS

2.1 Sample Collection

A total of 50 milk samples were collected from different dairy farms and local milk venders around the vicinity of the twin cities. Samples were collected in sterile glass containers and transported to the microbiology lab where culturing and sensitivity profiling was done. The apparatus was sterilized before use. The details of the areas from where the samples were collected are presented in the table 2.1

2.2 Media preparation

Staphylococcus aureus was enumerated using mannitol salt agar and the plates were incubated at 37°C for 24 hours. The *Staphylococcus aureus* appears as yellow colonies with yellow zones in the media. Mannitol salt agar's composition is presented in the table 2.3

111 grams of dehydrated medium was dissolved in 1 liter of distilled water. Then it was mixed properly and heated it so that the powder is dissolve properly. The media was then sterilized by autoclaving at 121°C for 15 min. After autoclave the media was allowed to cool. The media was then dispensed into plates and left for some time to solidify. After solidification, covered with the lids of plates and stored in refrigerator.

2.3 Identification of Samples:

Staphylococcus aureus was identified on the basis of colony morphology via culturing on MRSA selective medium (Mannitol Salt Agar – MSA), nutrient agar and by Gram staining.

2.4 Biochemical tests for identification of *Staphylococcusaureus:*

After morphologically identifying various strains of staph aureus, different biochemical tests including catalase test and coagulase were performed according to Bergy's Manual of determinative Bacteriology for confirmation of *Staph ylococcus aureus.*

2.5 Antibiotic Sensitivity Profiling:

Once the bacterial strains were confirmed as *Staphylococcus aureus,* the strains were again cultured on nutrient agar plates. Different antibiotic discs were used in order to determine sensitivity by placing the discs on the surface of the medium inoculated with *Staphylococcus aureus.* After 18-24 hours the diameter of zone of inhibition were measured according to reference tables(Hammods,1995). Antibiotic susceptibility testing was done for staph aureus under standard CLSI guidelines 2013 for the following antibiotics:

Table 2.2 CLSI 2013 guidelines for antimicrobial agents used

Antibiotics	**Abbreviations**	**Disc Potency**	**Zone of inhibition**
Cefoxitin	FOX	30ug	≥21mm
Chloramphenicol	C	30ug	≥12mm
Ampicillin	AMP	10ug	≥9mm
Gentamicin	CN	10ug	≥12mm
Erythromycin	E	15ug	≥15mm
Clindamicin	DA	5ug	≥14mm
Sulfamethoxazole	SXT	5ug	≥10mm

2.6 Bacterial Genomic DNA Extraction :

Resistant *Staphylococcusaureus* strains previously profiled were further cultured in 5ml LB broth and incubated over night at 37 C . After that 1ml cell suspension was taken and centrifuged at 8000 rpm for 5 minutes. The supernatant was discarded and pellet was re suspended in 400ul STE buffer and centrifuged again for same speed and time . Cell pallets were re-suspended in 200ul TE buffer. For cell lyses, 100ul Tris saturated phenol was added and vortexed for 60 seconds.afterthis the reaction mixture was again centrifuged for 13000 for 5 minutes at 4C to separate the aqueous phase from the organic phase. 160ul upper aqueous was transferred to a clean 1.5ml tube. To make it 200ul, 40ul TE was added. Then chloroform 100ul was added and centrifuged for 5 minutes at 13000rpm at 4C until white interface completely disappeared. In the end 150ul upper layer was transferred to clean 1.5ml tubes to be used for PCR. The isolated DNA was stored at -20 C for further use.

2.7 PCR Protocol for mecA Gene Detection :

Out of 33 positive samples , 16 resistant MRSA isolates were selected and screened for detection of mecA gene using PCR at institute of Biomedical and Genetic Engineering IB&GE at KRL Hospital Islamabad , Pakistan. Three step PCR method was carried out using XP thermal cycler (Oliveira *et al.,)* with the published primer mecA already used for amplification from (Manisha et al.,2000).For amplification of 163 bp region the forward primer sequence (CTGGTGAAGTTGTAATCTGG) and reverse primer sequence (ACTGCTATCCACCCTCAAAC) were used.

In order to perform PCR a reaction volume of 20ul was prepared. Conditions for PCR were set at initial denaturation at 94C for 5 minutes followed by 35 cycles of denaturation at 94C for 2 minutes. Annealing temperature was set at 57C for 2 minutes then for extension , temperature of 72C for 1 minute was set. Final extension was carried out at 72C for 7 minutes.

Agarose gel of concentration 1.5 % was used to separate the amplification product by electrophoresis. It was visualized by ethidium bromide staining. DNA ladders of 100 bp and 1000 bp were used as DNA molecular weight standards.

Table 2.3 Primer for mecA gene

Gene Name	Primer Name	Oligonucleotide Sequence	Location	Amplified Gene Size
mecA	GMECAR-1	ACTGCTATCCACCCTCAAAC	1325-1344	163
mecA	GMECAR-2	CTGGTGAAGTTGTAATCTGG	1325-1344	163

Table: 2.1 List of the milk samples and dairy farm

Dairy Farms	Sample Code
1.Gujjar Dairy farm- Rawat	SA001
2.Wahab Cattle and Dairy Farm- Adyala	SA002
3.Burhani Dairy Farm- KalarSyedan	SA003
4.Crown Dairy Farm - Shahdara ,Islamabad	SA004
5.Fazal Dairy Farm- ChakShehzad	SA005
6.Islamabad Cattle Farm- AlipurFarash, Islamabad	SA006
7.Malik Cattle Farm- Shah Allah Dittah, Islamabad	SA007
8.Arco Cattle Farm – Rawat	SA008
9.Horse & Cattles Farm- Misrail Road,	SA009

Rawalpindi	
10.Janjuas Cattle Farm- KallarSyedan	SA0010
11.Milker Dairy Farm- Chautara , Rawalpindi	SA0011
12. DeraChGulsiraj- Dhamyal Road, Rawalpindi	SA0012
13.Rich Farms- DHA Phase 2	SA0013
14.Sethi Farm House- Tumair, Islamabad	SA0014
15.Bhatti Farms-Bharakahu, Islamabad	SA0015
16.Aqua Farms- PindBigwal, Islamabad	SA0016
17.Abbasi Farm House- Islamabad	SA0017
18.Master Cattle Farm- Gujjar Khan	SA0018
19Darkala Farm-Nelore Factory , Islamabad	SA0019
20.Malik Dairy Farm - Rawalpindi	SA0020
21.Raja Cattle Farm- Dhamyal, Rawalpindi	SA0021
22.Gahi Syedan Farmhouse - Chakri	SA0022
23.Hamza Dairy Farm	SA0023
24.Kachhi Dairy Farm	SA0024
25.Malik Nasar Dairy Farms	SA0025
26.Green Land Farms	SA0026
27.Ibrahim Dairy and Livestock Farms	SA0027
28.Gujrat Dairy Farm	SA0028
29.AL-Kausar Farms	SA0029
30.Shah Ji Dairy Farm	SA0030
31.Al-Makkah Dairy Farm	SA0031
32.Munna Dairy Farm	SA0032
33.Data Dairy Farm	SA0033
34.Bismillah Dairy Farm	SA0034
35.Meekon Dairy Farm	SA0035

36.Chauhdry Dairy Farm	SA0036
37.Ismabad Farm House	SA0037
38.SKRG Farms	SA0038
39.Rehman Dairy Farm	SA0039
40.Dairy Farm in ISB Farms	SA0040
41.Milker Dairy Farm	SA0041
42.Gujjar Dairy Farm	SA0042
43.Riaz Raja Dairy Farm	SA0043
44.Pathar garh Dairy Farm	SA0044
45.Taj Bibi Dairy Farm	SA0045
46.Village jhang Dairy farm	SA0046
47.Village NaikaMehmood Dairy Farm	SA0047
48.Village Shahpursheerbhadur Farm	SA0048
49.Arshad Khan Dairy Farm	SA0049
50.Village shahpurFahad Khan Dairy farm	SA0050

Table 2.5 list of mastitis affected cows samples

Sample number	**Sample code**
1	SA002
2	SA0019
3	SA0025
4	SA0028
5	SA0030
6	SA0031
7	SA0049

Table 2.4 Composition of Mannitol salt agar media

COMPONENTS	Quantity used for 1000ml
Sodium Chloride	75g
Proteose Peptone	10g
Mannitol	10g
Beef Extract	1g
Phenol red	0.025g
Agar	15g

Results

RESULTS

3.1 Sample information

A total number of 50 milk samples were collected from various dairy farms and local milk venders distributed across pothohar region (table 2.1). From the 50 milk samples at least 7 samples were from cows suffering from mastitis (table2.5).

From the 50 milk samples , 33 tested positive for *staphylococcus aureus* and 17 samples tested negative when cultured on selective media MSA. All 7 mastitis samples tested positive for *staphylococcus aureus.*

For the distribution of *Staphylococcus aureus*, MSSA and MRSA isolates from the collected 50 samples revealed a percentage of MRSA 66% and MSSA 34% as shown in figure 3.1.

3.2 Morphological characterization of strains

Strain identification was carried out on the basis of colony morphology. From the collected 50 samples, 33 that were deemed positive for *Staphylococcus aureus*, including the 7 mastitis samples, because they produced bacterial colonies that were Gram-positive cocci, forming clumps. On MSA growth medium,*Staphylococcus aureus*colonies were golden yellow to golden brown, appearing circular, pinheaded and convex with complete margins as shown in figures 3.6 ,3.7, 3.8 and 3.9.Gram staining confirmed that all strains of *Staphylococcus aureus* were gram positive which appeared as grape-like clusters in purple color under microscopic slide (Figure 3.10)

Gram positive / negative	Shape	Color	MSA Media Positive Result	MSA Media Negative Result
Gram positive cocci	Circular, pinheaded and convex with complete margins	Colonies were golden yellow to golden brown	Media turned yellow with staph positive colonies	Media turned pink or remained red with staph negative colonies

3.3 Biochemcal tests for identification of Staphylococcus aureus

a. Catalase test

In catalase test the results were the gas formation of (O2) in the form of bubbles in all staph samples which indicated staph had a catalase positive reaction.(Figure 3.11)

b. Coagulase test

In Coagulase test the plasma coagulation was observed for all samples that were suspected staph and the coagulate was stable which confirmed coagulate positive *Staphylococcus aureus*.(Figure 3.12)

3.4 Antibiotic resistanceprofiling

Data analysis of 33 staph strains isolated from 50 milk samples showed pattern and percentage results of 7 drugs Genyamicin (CN), Chloramphenicol (C), Ampicillin(AMP), Cefoxitin(FOX),Erythromycin(E), Sulfamethoxazole(SXT), Clindamycin (DA).(Table 3.2) (Figure 3.15) (Figure 3.16).

Staphylococcus aureus showed resistance when abitiotic drug discs produced zones of inhibition of specific diameter according to CLSI standards .

Data revealed that resistance to Gentamicin producing zone of inhibition(>12mm) was 15%. Staphylococcus showed resistance when chloramphenicol disc produced zone of inhibition of (>12mm) in diameter. Data revealed that resistance to chloramphenicol was 24%. Clindamycin resistance was demonstrated when zone of inhibition was (>14mm) resulting in 36% resistance. Drug Erythromycin showed resistance of 39% producing zones of inhibition (<15mm). Resistance to Ampicillin was 33% with zone of inhibition of (< 9mm) in diameter. 30% of strains showed resistance to Cefoxitin (FOX) showing zone of clearance of (>21mm).the last drug was sulfamethoxazole and 48% strains developed resistance against this drug , producing zone of inhibition of (>10mm) in diameter.

The detailed analysis revealed that out of total number of 7 drugs that were used in this study ,sulfamethoxazole had the highest resistance of 48% for staph aureus which was a test to determine MRSA Methicillin resistant staph. Next in number was Eeythromycin showing

that 39% of strains testing positive for staphylococcus aureus were resistant. The third drug that staph strains showed most resistance to was clindamycin with a resistance of 36%. Drugs to which staph aureus were most susceptible in this study were Gentamycin and chloramphenicol with 15% and 24% resistance respectively.

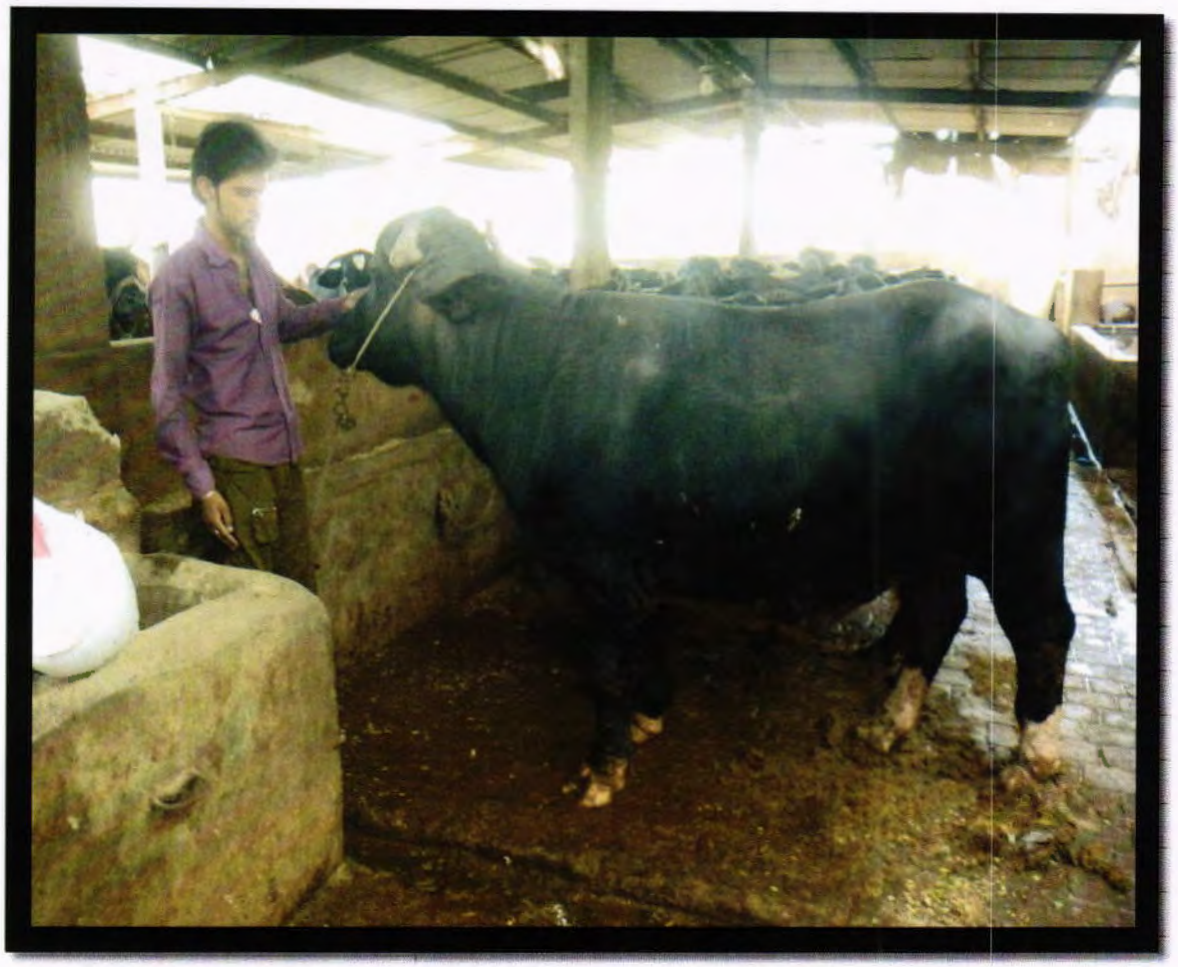
3.5 Detection of mecA Gene of MRSA by PCR

Fromthe 50 milk samples that were collected , 33 had tested positive for staph aureus . Staph positive samples that showed resistance to 4 or more of the 7 drugs used in the study to generate the antibiotic resistance profile were selected as potential confirmed MRSA and analyzed for mecA gene detection by PCR. Amplified product of 163bp was observed in 5 samples when checked on 1.5% agrose gel. From the 5 confirmed MRSA strains with mecA gene (figures 3.10 and 3.11), 4 were from the samples taken from cows suffering from mastitis (total number of mastitis samples taken was 5), and 1 was from the general data collected.

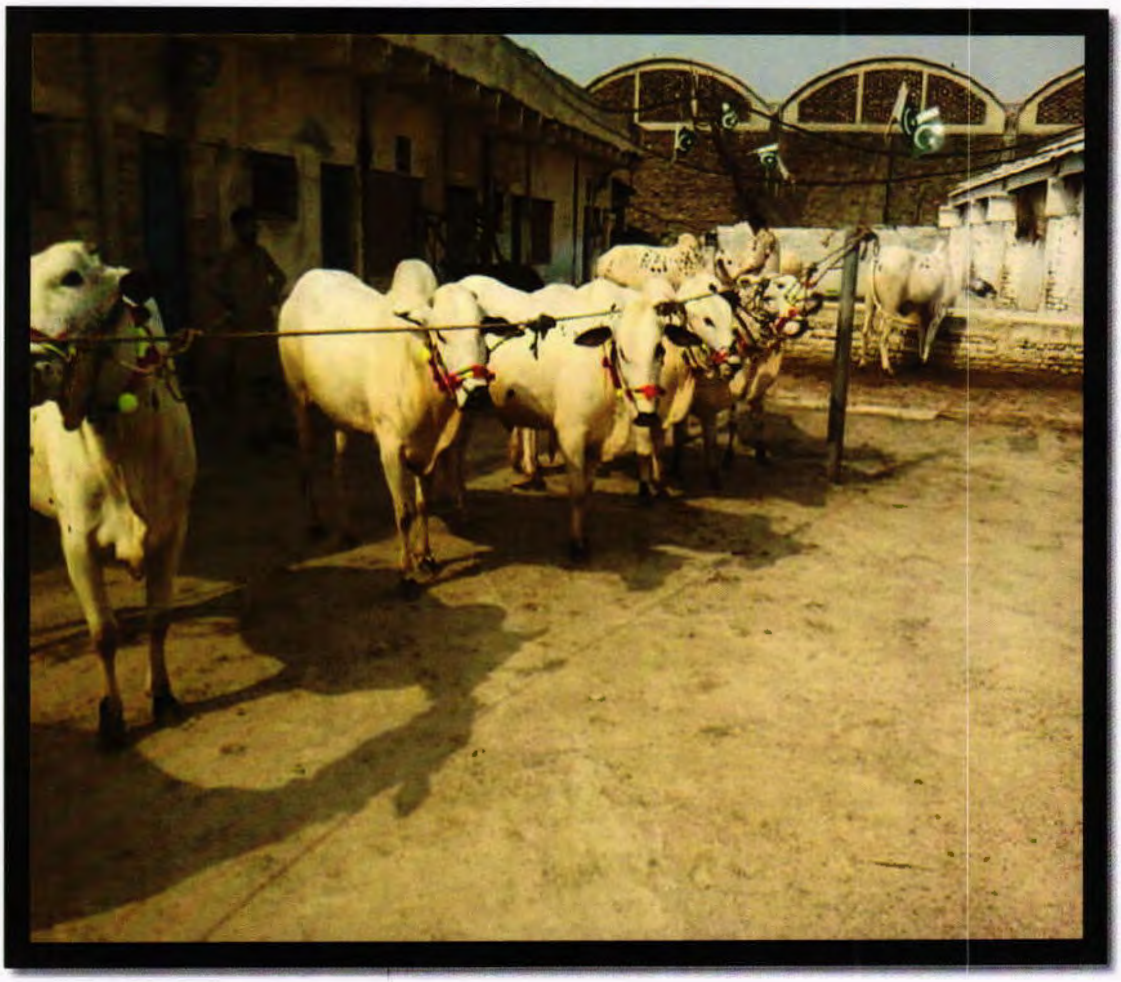
Figures 3.2Dairy Farms (a) (b) (c) (d)

(a) Rehman Dairy Farm

(a) Gujjar Dairy Farm



(a)Malik Dairy Farm – Rawalpindi



(a)Munna Dairy Farm

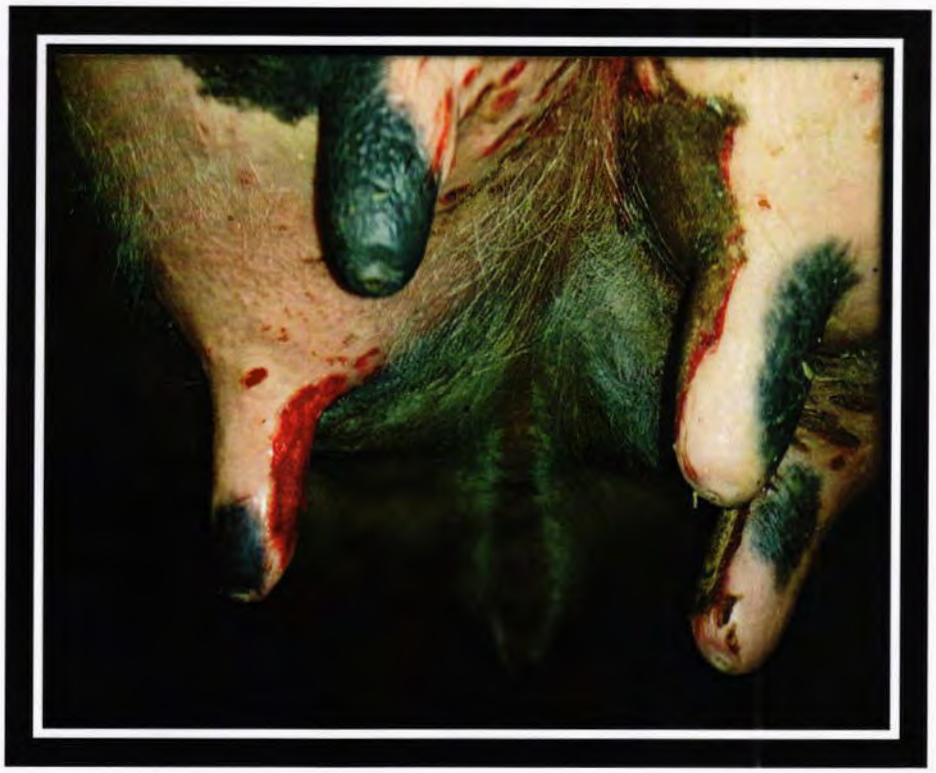


Figure 3.3 Cow suffering from mastitis

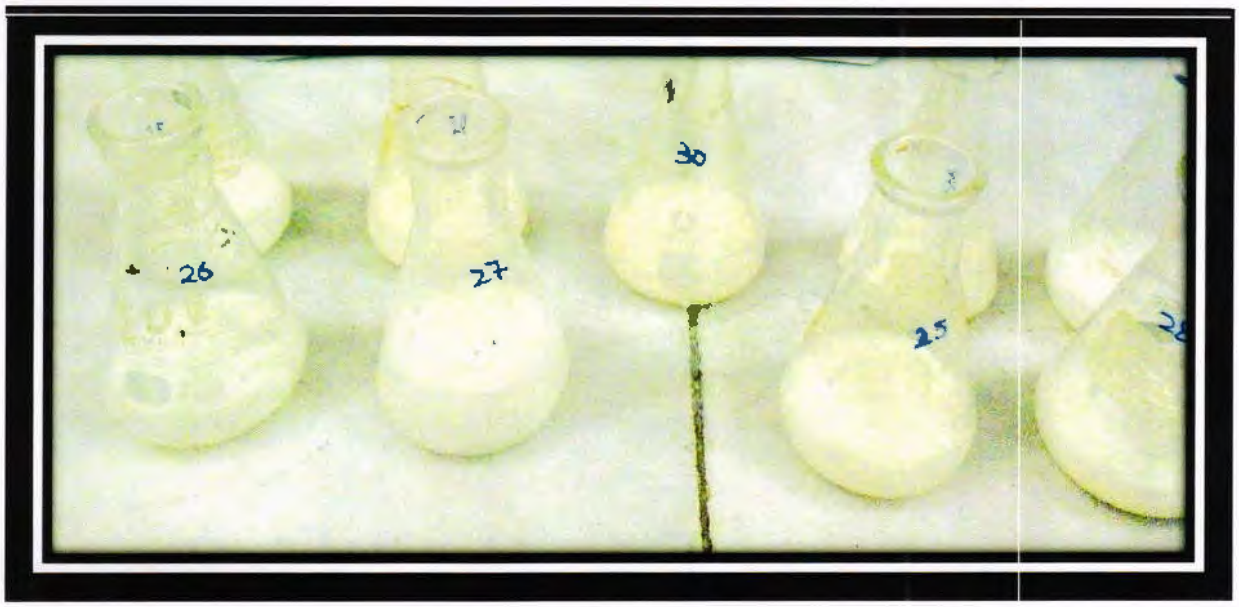
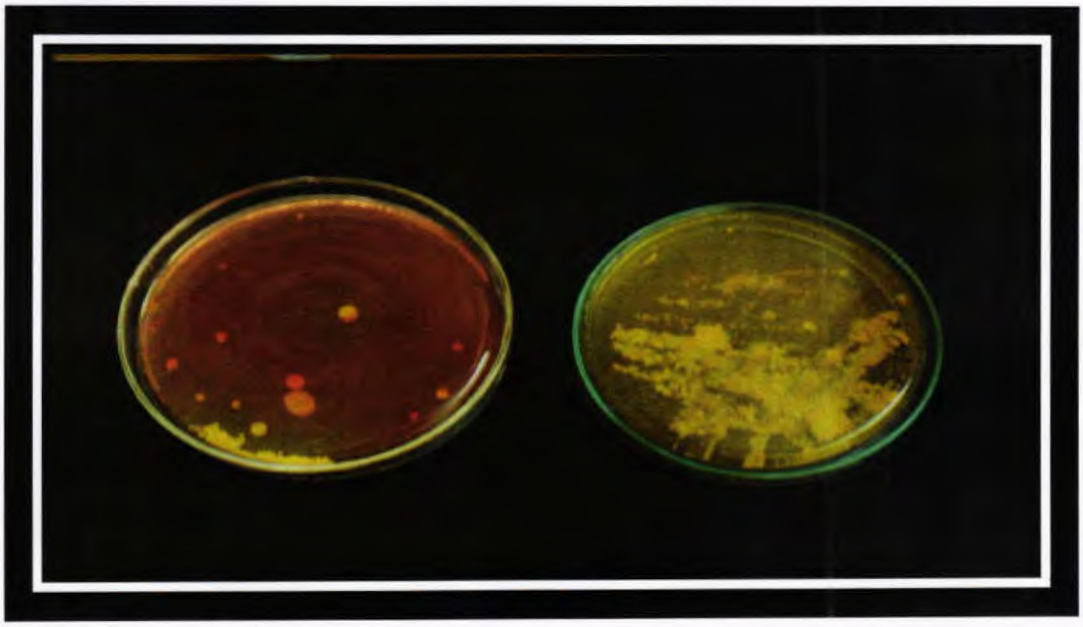


Figure 3.4 Raw milk samples

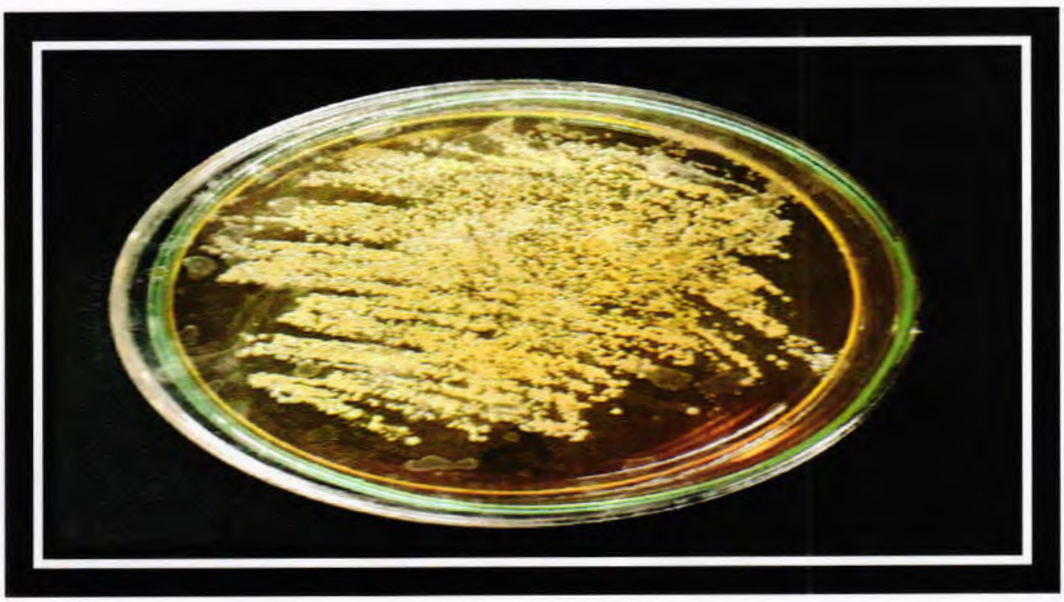
Figure 3.5 MSA media prepared plates

3.6 (a1) **3.6(a2)**

Figures 3.6 (a1)(a2)Plates showing the *Staphylococcus aureus*colonies on Mannitol salt agar (MSA) (positive result)



3.7 (a1) Mastitis sample plates (SA0019 (left), SA0025(right))



3.7(a2) Mastitis sample plate (SA002)

Figure 3.7(a3)Mastitis sample plate (SA0030)

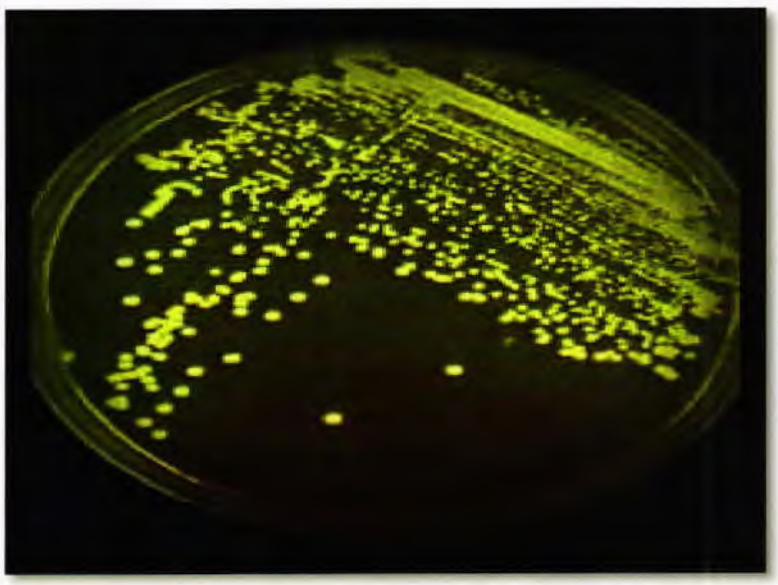
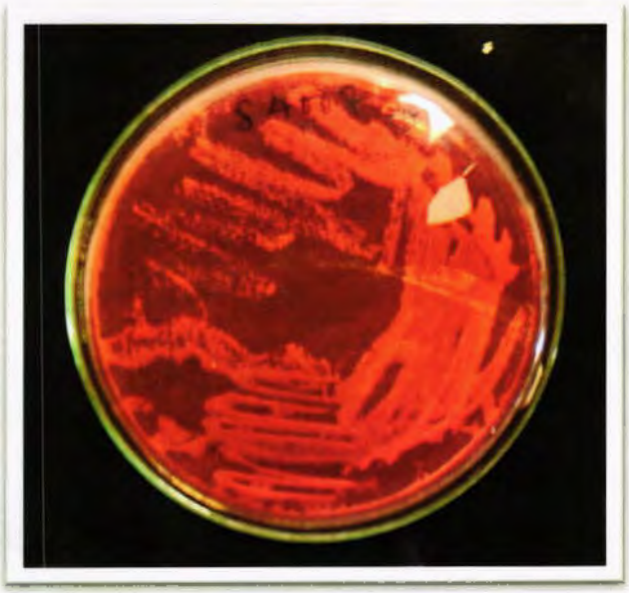


Figure 3.8single colony isolation of *Staphylococcus aureus* on MSAmedia



Figures 3.9 (a1)

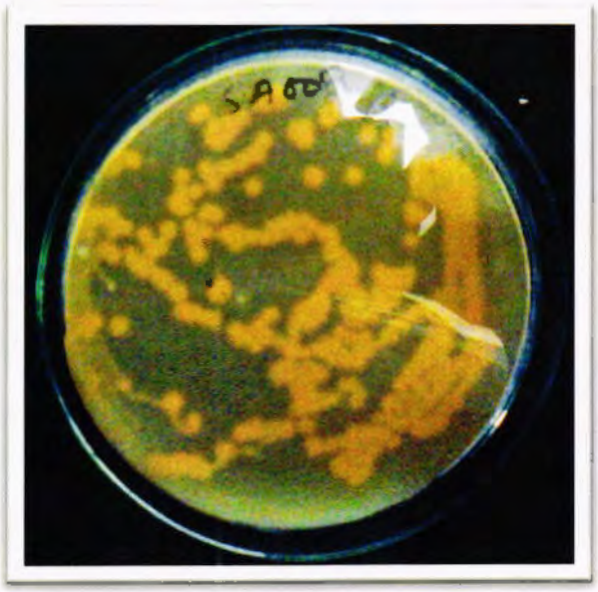


Figure 3.9 (a2)

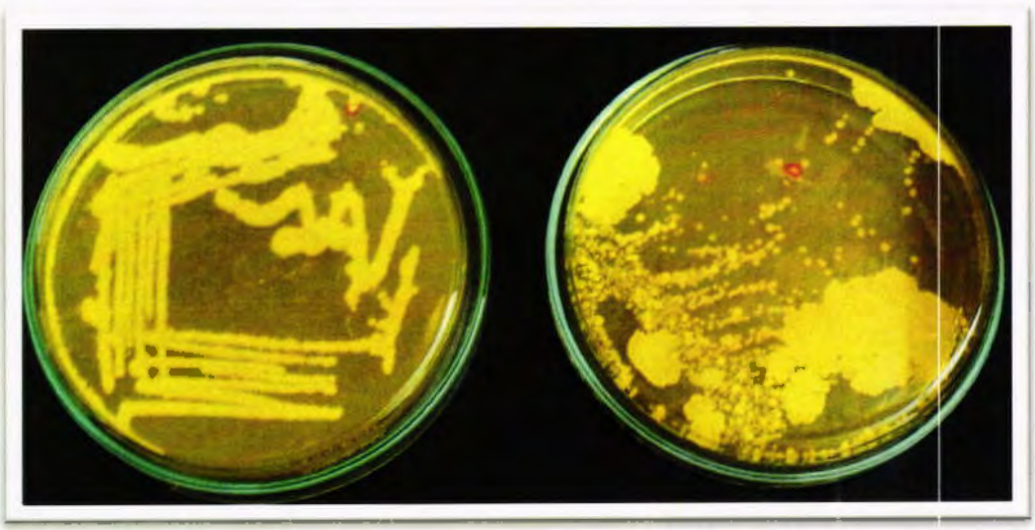


Figure 3.9 (a3)

Figures 3.9 (a1) (a2) Negative results on MSA media.(a3) Positive result on MSA media

Figure 3.10 (a1)

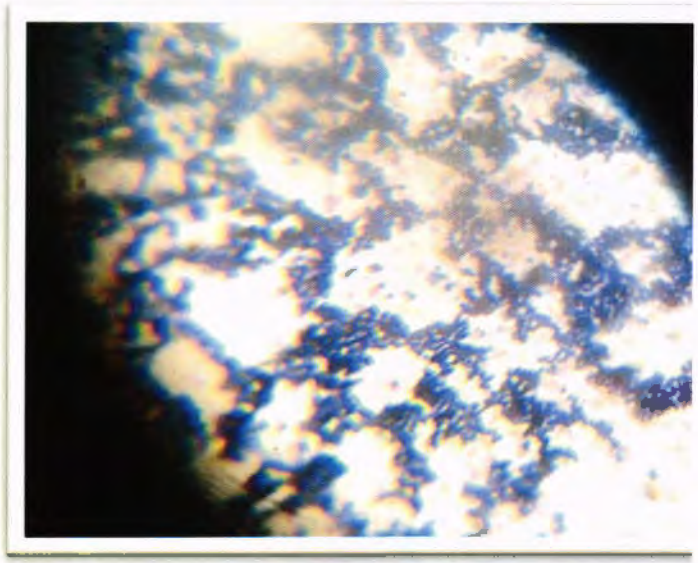


Figure 3.10(a2)

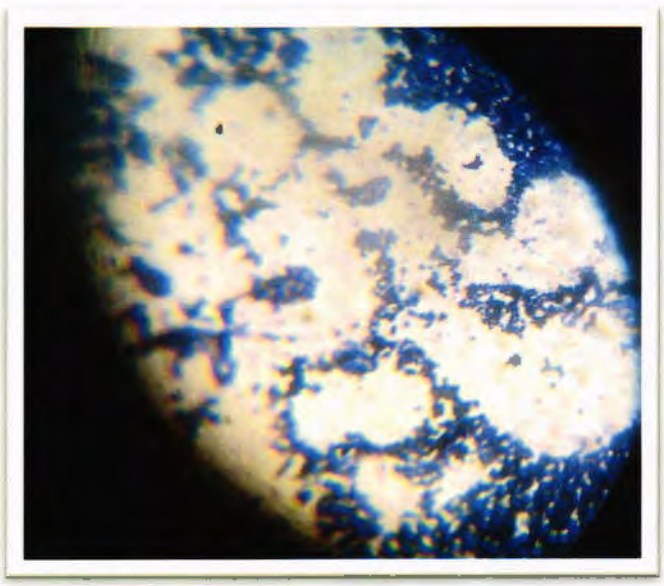


Figure 3.10 (a3)

Figures 3.10 (a1), (a2),(a3) Gram stain positive results

Figure 3.11(a1)

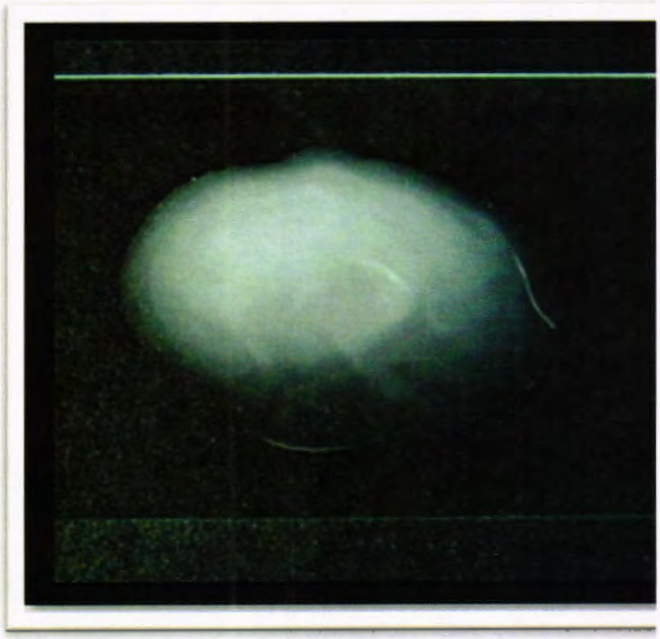


Figure 3.11 (a2)

Figures 3.11 Coagulase positive result of Staphylococcus aureus (a1) Coagulase negative result of control organism, Staphylococcus epidermidis (a2)

Figure 3.12 Catalase positive result of isolated *Staphylococcus aureus* strains

Biochemical Test for Staphylococcus aureus Confirmation			
Sample code	**Catalase Test**	**Coagulase Test**	**Gram Stain**
SA001	+	+	+
SA002	+	+	+
SA003	+	+	+
SA004	+	+	+
SA005	+	+	+
SA006	+	+	+
SA008	+	+	+
SA009	+	+	+
SA0011	+	+	+
SA0012	+	+	+
SA0015	+	+	+
SA0017	+	+	+
SA0019	+	+	+
SA0020	+	+	+
SA0021	+	+	+
SA0025	+	+	+
SA0026	+	+	+
SA0027	+	+	+
SA0028	+	+	+
SA0029	+	+	+
SA0030	+	+	+
SA0031	+	+	+
SA0032	+	+	+
SA0036	+	+	+
SA0037	+	+	+
SA0040	+	+	+
SA0041	+	+	+
SA0043	+	+	+
SA0045	+	+	+
SA0049	+	+	+

Table 3.1 Biochemical test results for the detection of Staphylococcus aureus in raw milk samples

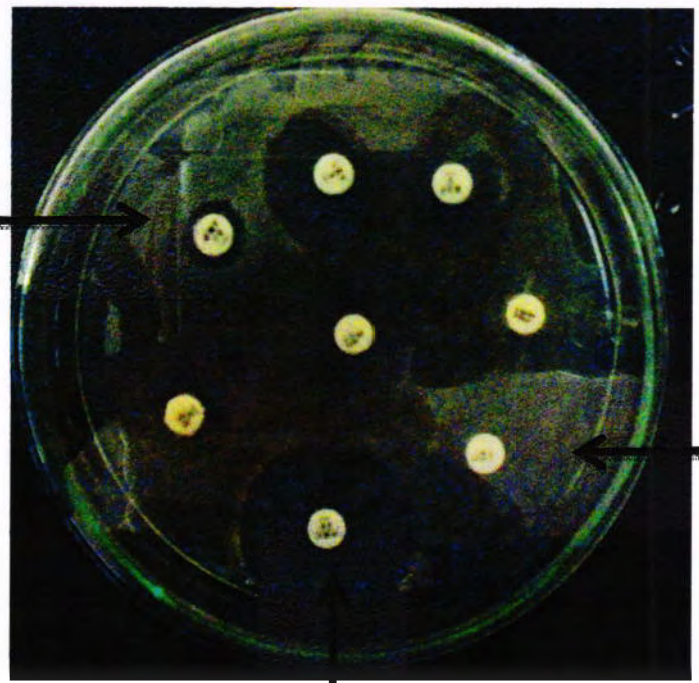


Figure 3.13 Antibiotic resistance profile on nutrient agar plate with antibiotic discs.

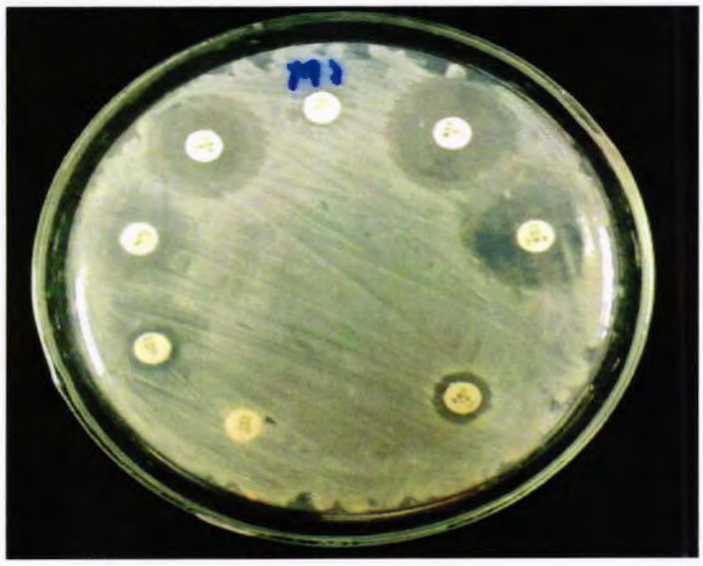


Figure 3.14 (a1) Antibiotic resistance pattern of mastitis sample

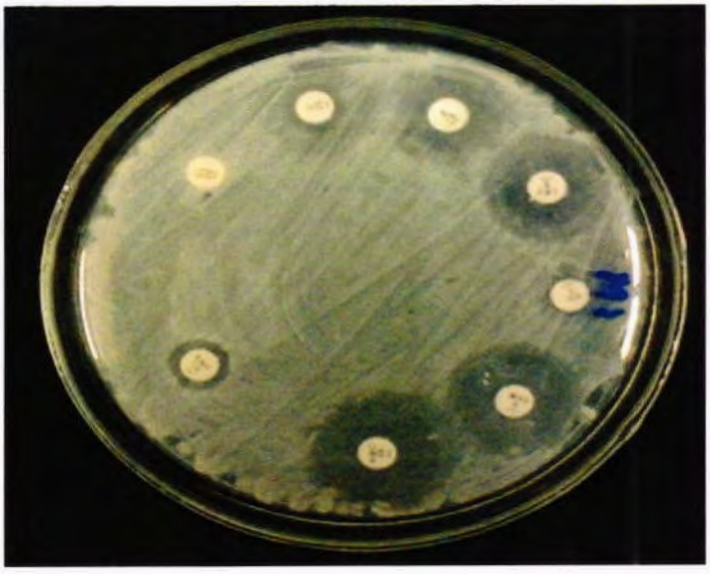


Figure 3.14 (a2) Antibiotic resistance pattern of mastitis sample

SAMPLE CODE	FOX	C	AMP	CN	E	DA	SXT
SA001	R	R	R	R	S	R	S
SA002	R	R	R	R	R	S	R
SA003	R	R	R	S	S	R	R
SA004	S	R	R	R	S	S	S
SA005	R	R	S	R	R	S	R
SA006	S	R	R	R	S	R	R
SA008	R	R	R	R	R	R	R
SA009	R	R	S	R	R	S	S
SA0011	S	R	R	R	R	S	S
SA0012	S	R	S	S	S	R	S
SA0015	R	S	R	R	R	R	S
SA0017	R	R	S	R	S	S	R
SA0019	R	R	R	R	R	R	R
SA0020	S	R	R	R	R	S	R
SA0021	R	S	S	R	S	R	S
SA0022	R	S	R	S	S	S	S
SA0023	S	R	R	R	S	R	R
SA0025	R	R	R	R	R	R	R
SA0026	R	R	S	R	R	R	R
SA0027	S	S	S	R	R	R	S
SA0028	R	R	R	R	R	R	R
SA0029	R	S	S	R	R	R	R
SA0030	R	R	R	R	R	R	S
SA0031	R	R	R	R	R	R	R
SA0032	R	R	R	R	R	S	S
SA0036	R	S	R	R	S	R	S
SA0037	S	R	S	S	R	R	R
SA0039	S	S	S	R	S	S	S
SA0040	R	R	R	R	S	R	S
SA0041	R	R	S	S	R	R	R
SA0043	R	S	R	R	S	S	S
SA0045	S	R	R	R	R	S	R
SA0049	R	R	R	R	R	R	R

Table 3.2 Antibiotic resistance profile of confirmed staphylococcus strains previously isolated

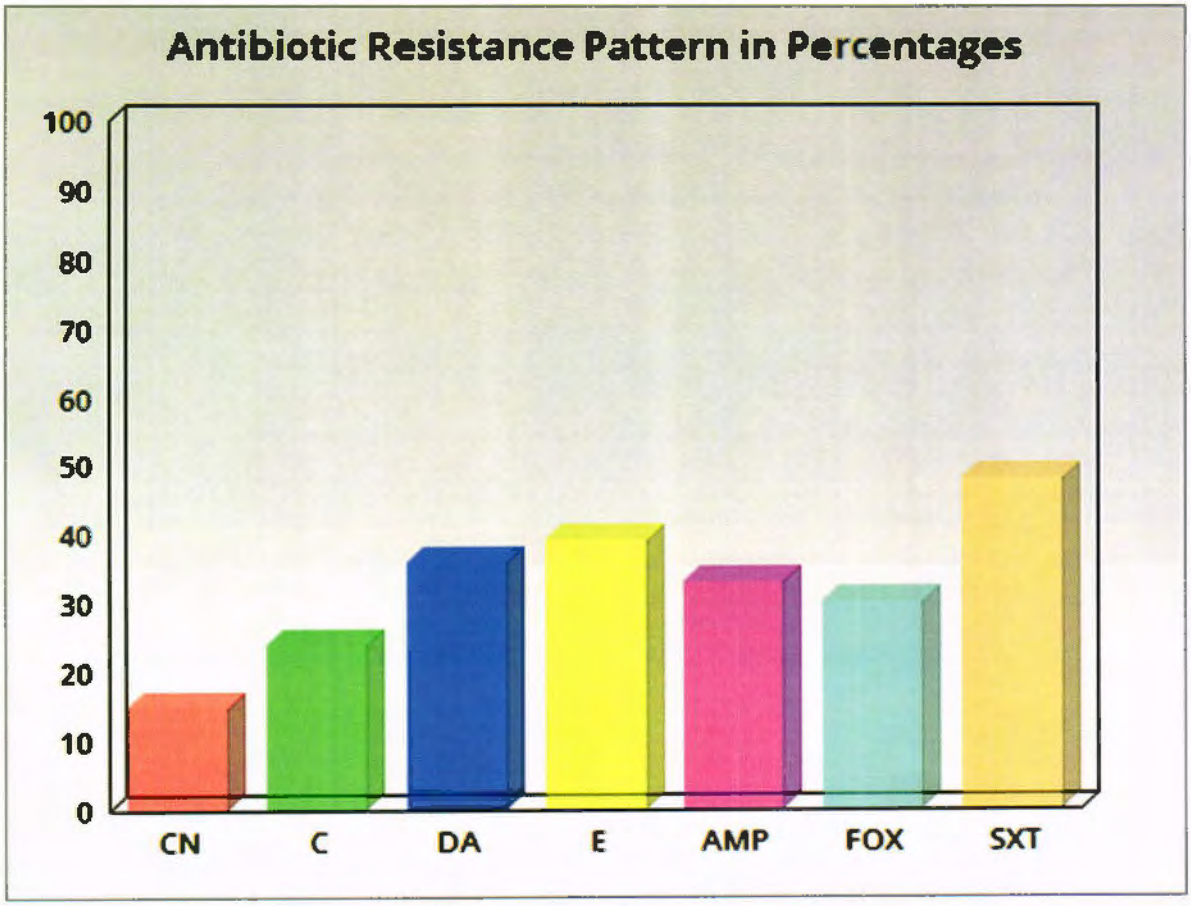


Figure 3.15 Antibiotic resistance profile of isolated staphylococcus strains in percentages.

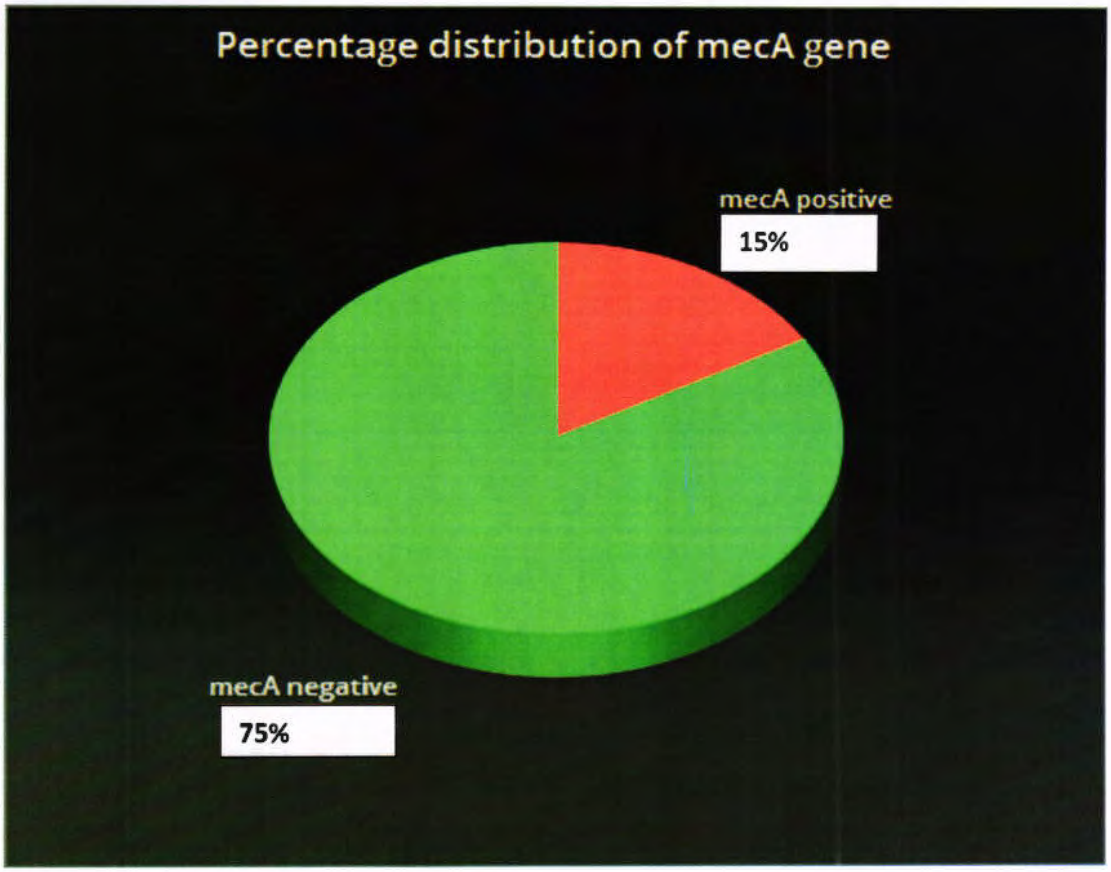
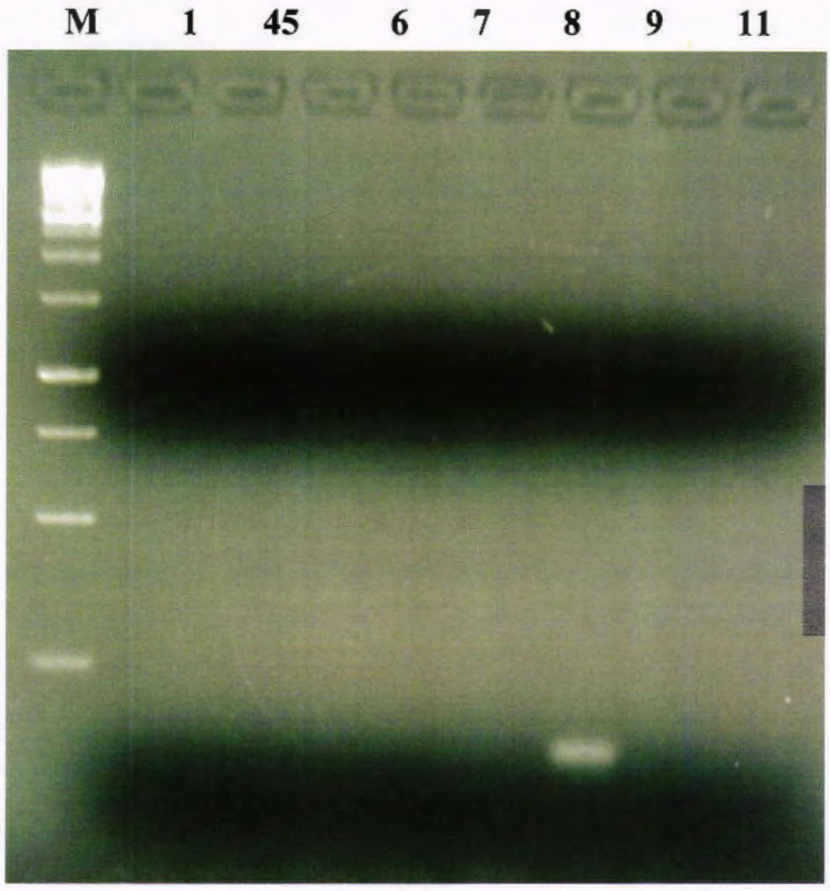
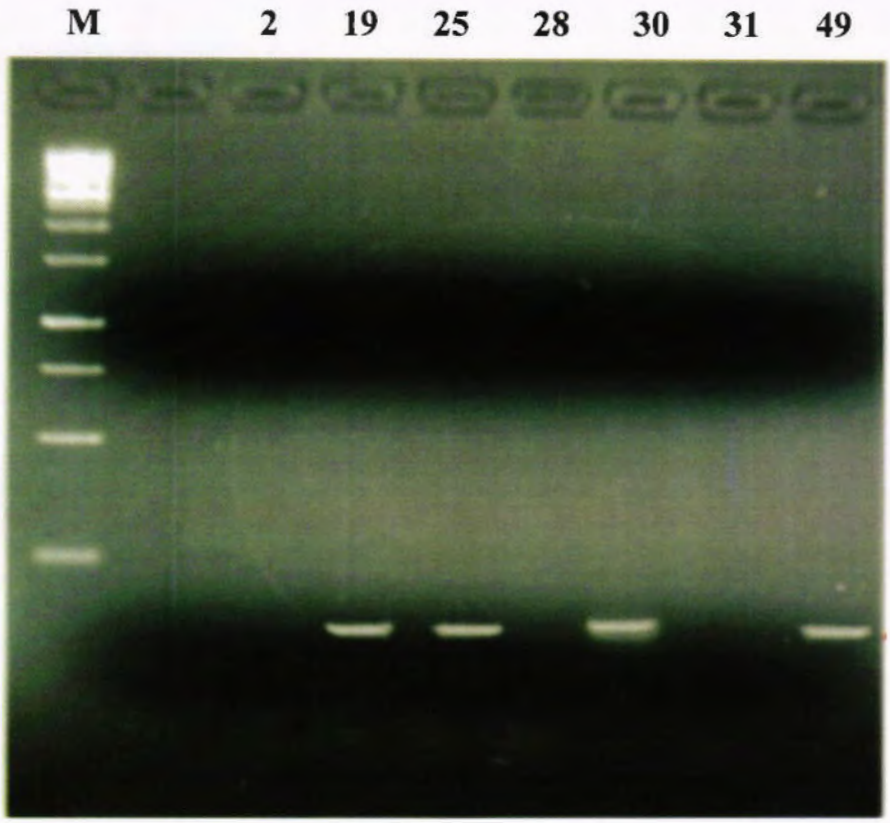


Figure 3.16Percentage distribution of mecA gene among isolated staphylococcus strains



Key : lane labeled M contained 1kb DNA ladder , lane labeled 8(SA008B) was mecA positive and was confirmed MRSA , while rest of the samples were mecA negative.

Figure 3.17mecA gene amplification by PCR using 1kb DNA ladder



Key :lane labeled M contained 1kb DNA ladder , lanes labeled 19,25,30 and 49 (mastitis samples - SA0019, SA0025,SA0030, and SA0049) weremecA positive and were confirmed MRSA , while lanes labeled 2,28,and 31 (mastitis samples - SA002, SA0030 and SA0031) weremecA negative.

Figure 3.18 mecA gene amplification by PCR using 1kb DNA ladder for mastitis samples that had tested positive for staphylococcus aureus.

DISCUSSION

Major objective of this study was to analyze the level of contamination of raw milk , which is a major food source in Pakistan with MRSA in the specified region of pothhar.

Though for the last few decades the pharmaceutical industry has produced numerous new antibiotics , however resistance to such antimicrobial agents by microbes is also on the rise. Microorganisms have the hereditary ability to gain and transmit resistance to medicines (Gislene et al.,2000). Staph aureus is notorious for developing quick resistance to antibiotics. It is a gram positive bacterium responsible for morbidity and mortality (Liu et al.,2011). They are in general resistant to multiples antibiotics including aminoglycosides, Chloramphenicol macrolides, Clindamycin, Flouroquinolones, Sulfamethaxozole, and beta-lactamases due to acquirement of the mecAgene(Corrigan et al.,2009) . In this study it was observed that out of all the strains , 48% of strains showed resistance to sulfamethoxazole , which indicate confirmation of MRSA strains.

The gene mecA was responsible for *staphylococcusaureus* developing resistance to antibiotics (Muto et al.,2003). It was also reported by Rybak in 2005 that mecA gene is carried on a mobile hereditary component called SCCmec, Staphylococcal chromosome cassette. PCR is a more effective, reliable and accurate as compared to conventional methods for detection of clinically relevant antibiotic resistance gene of staph aureus (Sajith et al., 2012).

In this study the most resistant MRSA were screened for mecA gene by PCR. It was observed that mecA gene was present in 15 % (5 out of 33 – 4 out of 7 mastitis samples and 1 sample from the rest of the total samples collected), of the strains that were confirmed as MRSA on the basis of biochemical characterization and antibiotic sensitivity profiling.This result is in agreement with (Sajith et al.,2012) which demonstrated the effectiveness of PCR for detecting antibiotic resistance Staph aureus.

Investigation in other countries reviled similar results as to the presence of *S.aureus* in raw milk samples , as obtained in this study. Farhan and Salk (2007) studied studied on 130milk samples in Palestine and found 48 (36.9%)samples were contaminated with *S.aureus*. Around 18.18% of milk samples had tested positive for *S.aureus* while studying 66 samples in Turkey (Ekici et al., 2004). In Morocco , 27 samples were studied and 40% were contaminated with *S*.aureus (Bendahou et al., 2008). This study also shows that milk from cows suffering from mastitis had a greater contamination ofMRSA strains which contained the *mecA* gene i.e 4 out of 7 total mastitis samples while 1 out of the general data contained the *mecA* gene. This is an alarming situation as resistant bacterial strains are present in apparently healthy cowsmilk , which is a threat to public safety and health.

CONCLUSION

Based on observations made throughout the collection of samples in this study, it can be concluded that improper hygiene practice and poor management before and during milking may have contributed to the contamination of milk with *S. aureus*. It is not just the presence of *S.aureus*which is alarming but the fact that most of the potential MRSA strains isolated in this study were also resistant to most of the drugs employed in the study. *S.aureus*that is resistant to multiple drugs can cause serious health problems and can quickly become a threat to public safety and health .

It was also observed in this study that *S.aureus* strains isolated from mastitis raw milk samples had a higher percentage of*mecA* gene as compared to regular samples , as well as being resistant to almost all of the antibiotic drugs used in the study.

RECOMMENDATION

1. Hygienic conditions of dairy farms can be strictly monitored on the basis of contamination assessment of live stock.
2. Drugs and antibiotics targeting the inactivation of *mecA*gene eliciting pathogenicity to the bacteria can be developed.
3. Proper care and handling of raw milk and other milk based products.
4. Comparison of raw milk , pasteurized and boiled milk and other milk based products can be conducted to investigate whether such products are just as much susceptible to contamination by pathogenic bacterial strains.
5. Detection of *mecA* gene can be used to detect whether pathogenicity can be passed between bacterial species hence leading to the emergence of resistant strains .

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