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A Role of Staphylococcus aureus in Clinical and Subclinical Bovine Mastitis

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Abstract:

Bovine mastitis is the most economically very important disease affecting dairy cattle worldwide from an economic diagnostic and public health point of view. Bovine mastitis is very common in cow and buffalo all over the world. It is important disease of dairy cattle. Mastitis in dairy cattle is caused by udder infection, usually resulting from bacteria introduced either during the milking process or from environmental contact. S. aureus has relatively high potential for infections as compare to others. There are many severe effects of mastitis and mastitic milk on public health and also cattle health. It affects on milk quality, on milk yield. Mastitis not only negatively affects milk yield production, but also has a negative impact also on milk composition and its physiochemical characteristics.

Keywords*: Bovine mastitis, Bacterial infection, Milk quality, S. aureus*

Introduction:

Milk is a nutrient rich white liquid food produced by the mammary glands of mammals. It is primary source of nutrition for infant mammals (including humans who are breastfed) before they are able to digest other types of food. It contains many other nutrient including protein and lactose

Milk is strategic product in any country as it basic food for children and adults (Agatha, 2015). Milk is nutrient rich, white liquid food produced by mammary gland of mammals. It is the primary source of nutrition. Milk contains protein and lipid amino acid, vitamins, minerals (Haug *et al*., 2007). Additionally, milk contain hormones, immunoglobulin, growth factors, nucleotide, enzymes are significantly contributes to the body requirement (Korhonen *et al.*, 1998; Clare and Swais, 2000). Milk is source of calcium and mineral that important in the development and maintenance of healthy bone. It contains protein, carbohydrates, vitamins, minerals and fate.

The milk yield per cow in the United States, the world's largest cow milk producer, was 9,954 kg (21,945 lb) per year in 2010. In contrast, the milk yields per cow in India and China the second and third largest producers were respectively 1,154 kg (2,544 lb) and 2,282 kg (5,031 lb) per year (FAO, 2012).

The pH of milk ranges from 6.4 to 6.8 and it changes over time. Milk from other bovines and non-bovine mammals varies in composition, but has a similar pH. Normal bovine milk contains 30–35 grams of protein per liter of which about 80% is arranged in casein micelles. Total proteins in milk represent 3.2% of its composition (Harold, 2004).

The composition of milk differs widely among species. Factors such as the type of protein; the proportion of protein, fat and sugar, the levels of various vitamins and minerals and the size of the butterfat globules and the strength of the curd are among those that may vary. For example: Human milk contains, on average, 1.1% protein, 4.2% fat, 7.0% lactose and supplies 72 kcal of energy per 100 grams. Cow's milk contains, on average, 3.4% protein, 3.6% fat, and 4.6% lactose, 0.7% mineral and supplies 66 kcal of energy per 100 grams.

Mastitis

Mastitis occurs when white blood cell are released into the mammary gland, usually in response to bacteria invading the teat canal by chemical, mechanical, or thermal trauma on the udder. Mastitis, also known as udder inflammation, is a common problem in dairy herds causing increase in costs of milk production (Bramley *et al.,* 1996, Halasa *et al.,* 2007) and also have negative impact on milk composition and its technological value (Biggs, 2009). Inflammation of mammary gland or mastitis is response to effects of internal and outside factors.

During last several decades mastitis has become very expensive disease in dairy cows (Bennett *et al.,* 1999; Fourichon *et al*., 2001; Kelmus *et al*., 2006).In the developing countries various research are going on many authors had found there is a high risk of subclinical mastitis in early lactation period and postpartum period high percentage of intra mammary infections (De Vieghe*et al.,* 2005; Milne *et al.,* 2002; Sol *et al.,* 2002). Mastitis occurrence is according to Barkema *et al.* (1999) result of herd management, including rearing condition, nutrition and udder management.

Udder infections can be expressed as clinical or subclinical mastitis. Clinical mastitis is characteristic because of visible changes in milk with appearance of flakes or beads and with signs of inflammation and pain, where as animals with subclinical mastitis do not exhibited. Subclinical do not exhibited any gross change in milk or udder and can be detected only through laboratory tests (Reza *et al.,* 2011).

Mastitis remains one of the most common diseases of dairy cows and represents a large economic loss to the industry as well as considerable welfare issue to the affected cows. Mastitis has been ranked as number one in the most expensive disease of dairy animal in Pakistan and all over world (Ijaz *et al.,* 2014).

Subclinical mastitis is responsible for 70% of economic loss. The annual losses per cow from mastitis in the United States of America in 1976 were estimated to be US$ 117.35 per cow year (Blosser, 1979).

There are many cause of mastitis. It is commonly associated with bacterial infection, being influenced by many and multiple factor related to managements. Bacterial are usually behind the udder infection, over 140 different

microorganisms have been isolated from bovine mastitis intra mammary infection and majority of infection are caused by *Staphylococcus, Streptococci* and *Enterobacteriacae* (Bansal *et al*., 2015).

In bovine mastitis is a danger that the bacterial contamination of milk from affected cows that may render it unsuitable for human consumption by causing food poisoning and provides a mechanism of spread of disease to humans through consumption of raw milk.

The goal of present study was to isolate and identify the *Staphylococcus aureus* from bovine mastitis by molecular characteristics. The various demographic characteristics, protein estimation and antibiotic sensitivity and resistance pattern was also determined.

Methodology:

Collection of sample: Milk and swabbing the surface area of teats with sterile broth moistened cotton swabs were taken. Samples were collected in sterile container containing nutrient broth and transfer immediately to laboratory for further processing.

CMT (California Mastitis Test): The CMT is simple cow side indicator of the somatic cell count of milk. It operates by disrupting the cell membrane of any cell present in milk sample, allowing the DNA in those cells to react with the test reagent forming a gel.

Protein Estimation: Protein estimation was performed to measure the protein percentage from mastics milk compare to normal milk the protein estimation was performed by Folin Lowry method.

Isolation and Identification of *Staphylococcus aureus* by Phenotypic methods:

Propagation of samples: Sample were transferred in 0.5ml of Nutrient Broth for enrichment and incubated at 37 ^{0}C for 24 hours. Culture medium supports the growth of bacteria (Kaye *et al.,* 2003).

Plating of Enriched Sample: All the samples after enrichment were compared with the control tube. Prior incubation loopful of each enriched culture was then inoculated on the plates of Nutrient media (Hi-media, Mumbai) agar used for the growth of several bacteria. All the samples were inoculated in the triplicate. After inoculation all the plates were kept for incubation aerobically at 37^{0}C for 24 hours.

Cultural characterization (Plating on Selective Media): Colonies of relevant pathogens, with different morphological characters and biochemical characters were selected to analyze their cultural properties and inoculated on respective selective media viz. Blood agar, Mannitol salt agar, Milk agar. All the plates were incubated at 37°Cfor 24 hours. All culture media were procured from Hi-media, Mumbai.

Morphological Characterization: As per the standard literature (Bergey's manual of Systematic bacteriology, 2ndeditition, 1984), next day all the typical colonies on Nutrient media were screened for colony characteristics and

examined microscopically for Gram character using Gram's staining method as well as motility testing using hanging drop method.

Biochemical characterization: All the pathogenic bacteria were then subjected for conventional biochemical analysis as per the following table (Bergey's manual of Systematic bacteriology, 2nd editition, 1984; Dubey and Maheshwari, 2006).

Identification of *Staphylococcus aureus* by Genotypic method (16S rRNA):

Genomic DNA Isolation: Sample obtained was used for genomic DNA isolation by using modified CTAB protocol which was used further in PCR reaction

PCR based 16S rRNA gene amplification and Sequencing: About 200 ng of bacterial DNA was used to amplify 16S rRNA gene applying following 16S universal primers:-

Primer Names	Sequence detail	No. of Base
16S Forward primer	5' AGA GTT TGA TCC TGG CTC AG 3'	20
16S Reverse primer	5' AAG GAG GTG ATC CAG CCG CA 3	20

Phylogenetic Analysis: For the phylogenetics analysis, .DND file obtained from CLUSTAL alignment was used for the phylogram built up by using the MEGA5 software. In an output, built phylogram was documented close homology of the bacteria isolated (showcased with the accession number) with the best matched bacterial sequence and highlighted by marking in a Phylogram.

Nucleotide sequence accession number: The 16S rRNA complete genome sequences of isolate recovered from Bovine Mastitis has been deposited at the DDBJ (DNA Data Bank of Japan) GeneBank sequence database.

Antimicrobial susceptibility testing: After phenotypic characterization, the pathogenic bacteria were subjected for antibiogram. The antimicrobial susceptibility testing was done by the agar Disk Diffusion Method as described by NCCLS 2002, and Kirby Bauer disk diffusion method, now known as the Clinical and Laboratory Standards Institute (CLSI) (Bauer *et al.*, 1966; CLSI, 2015).

Antibiotics used: Gentamicin (10mg/disc), Erythromycin (15mg/disc), Chloramphenicol (30mg/disc), Tetracycline (30mg/disc), Ciprofloxacin (5mg/disc), Amoxyclav (30mg/disc), Ampicillin (10mg/disc), Penicillin (10mg/disc).

Results:

In present study research study 25 samples were collected during period of July 2018 to Dec. 2018 from clinically diagnosed the cattle of bovine mastitis admitted in veterinary hospital and various urban and rural area like

Akola, Akot, Telhara, varul, Bhavrad, Khaparwadi etc. Out of 25 samples bacteria were isolated from 20 samples (Table No.1). The cattle selected were ages, symptoms, and severity from urban and rural area. The major isolates were observed (labeled as A) and other. (Figure 1)

Table No1.: Mastitis samples and bacteria isolate

Sr. No.	Sample no.	Isolated organisms	+ve /- ve sample	Collection date
1	Sample no. 1	A-I, other	+ve	13/7/18
2	Sample no. 2	Na	-ve	19/7/18
3	Sample no. 3	A-II, other	+ve	22/7/18
4	Sample no. 4	A-III, other	+ve	27/7/18
5	Sample no. 5	NA	-ve	31/7/18
6	Sample no. 6	NA	-ve	2/8/18
7	Sample no. 7	A-IV, other	+ve	6/8/18
8	Sample no. 8	A- XIV, other	+ve	14/8/18
9	Sample no. 9	NA		12/9/18
10	Sample no. 10	A-V other	+ve	25/9/18
11	Sample no. 11	NA	-ve	27/9/18
12	Sample no. 12	A-VI other	+ve	10/10/18
13	Sample no. 13	A-VII other	+ve	18/10/18
14	Sample no. 14	A-VIII other	+ve	5/11/18
15	Sample no. 15	A-IX other	+ve	08/11/18
16	Sample no. 16	NA	-ve	10/11/18
17	Sample no. 17	NA	-ve	16/11/18
18	Sample no. 18	A-X other	+ve	26/11/18
19	Sample no. 19	NA	-ve	30/11/18
20	Sample no. 20	NA	-ve	02/12/18
21	Sample no. 21	NA	-ve	02/12/18
22	Sample no. 22	NA	-ve	10/12/18
23	Sample no 23	A-XI other	+ve	15/12/18
24	Sample no 24	NA	-ve	18/12/18
25	Sample no 25	A-XII other	+ve	10/1/19

Isolation and identification of pathogenic from bovine mastitis by phenotypic methods

The present study focus on phenotypic characteristics of pathogenic bacteria associated with bovine mastitis. Basic physiological, morphological and biochemical test were conducted to confirm the preliminary characterizations of the stains.

Table No. 2 : Demographic study

No of sample	Area	Location	Age	Symptoms	Severity
1	Urban	GaurakShansantha,	Cow -40	Redness, pain	Mild
2	Rural	Hospital	Buffalo -20	Swelling	Mild
3	Rural	Hospital	Buffalo- 22	Fever	Moderate
4	Urban	Hospital	Cow -15	Redness, fever	Moderate
5	Urban	Hospital	Buffalo -17	Pain swelling	Mild
6	Urban	Hospital	Buffalo- 35	Wound, bleeding	Severe

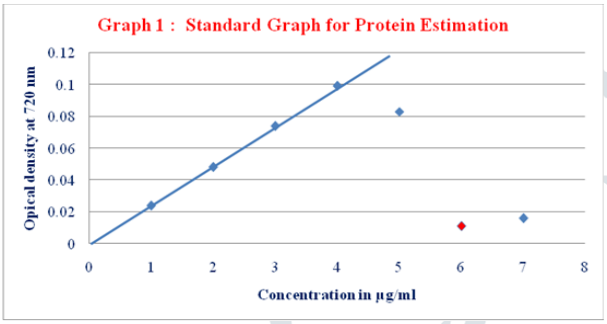
7	Rural	Domestic	Cow -12	No symptom	Mild
8	Rural	Domestic	Cow -8	Fever, Wound	Severe
9	Rural	Hospital	Cow -5	Wound	Moderate
10	Urban	Domestic	Cow -10	Rednees	Mild
11	Rural	Domestic	Buffalo -30	No symptom	Mild
12	Rural	Domestic	Buffalo -18	Swelling	Moderate
13	Rural	Domestic	Buffalo- 40	Pain	Mild
14	Urban	Hospital	Cow -25	Rednees, fever	Severe
15	Rural	Domestic	Cow -9	No symptom	Mild
16	Urban	Hospital	Cow -24	No symptom	Moderate
17	Rural	Domestic	Buffalo- 20	Fever	Moderate
18	Urban	Hospital	Cow -10	Redness	Moderate
19	Rural	Domestic	Cow -13	Pain	Mild
20	Rural	Domestic	Cow -6	No symptom	Mild
21	Urban	Hospital	Buffalo -9	Swelling	Moderate
22	Rural	Domestic	Buffalo-25	Rednees fever	Severe
23	Urban	Domestic	Buffalo-12	No symptom	Mild
24	Urban	Hospital	Cow-11	Redness	Mild
25	Rural	Domestic	Buffalo-10	No symptom	Mild

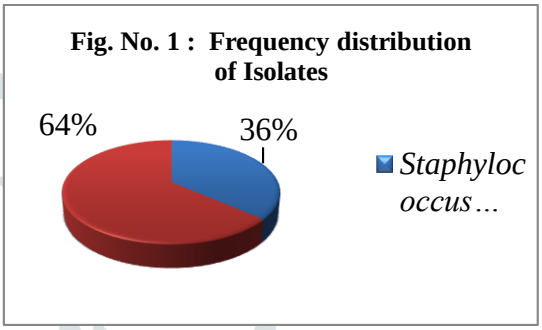
Morphological Characteristic: The organism was identified on the colony morphology characteristic. XIV isolate was a non motile Gram positive cocci shaped and arranged in cluster from.

Cultural characterization: The organism was identified on the basis of colony morphology and biochemical reaction. XIV isolate was showing yellowish colony coloration on nutrient agar and yellow coloured pigmentation on mannitol salt agar, golden yellow colonies on Milk agar and black coloured colonies on Baird Parker agar.

Biochemical characterization: Biochemical characterization of A isolates from bovine mastitis. A Isolate was fermenting all the sugar without gas production. These isolates were positive for amylase, catalase and coagulase test. Two isolates showed slide test positive and 10 isolates showed tube test positive.

Protein estimation: Protein estimation was perform to measure the protein content from mastitic milk compare to normal milk. The result showed that protein content in mastitic milk decrease as compare to normal milk. The protein estimation was performed by Folin Lowry method. The result showed that the protein content in normal milk sample was observed to be 0.016µg/ml which reduced significantly in mastitic milk to 0.011µg/ml that is it reduced by 0.05µg/ml. (Graph 1)





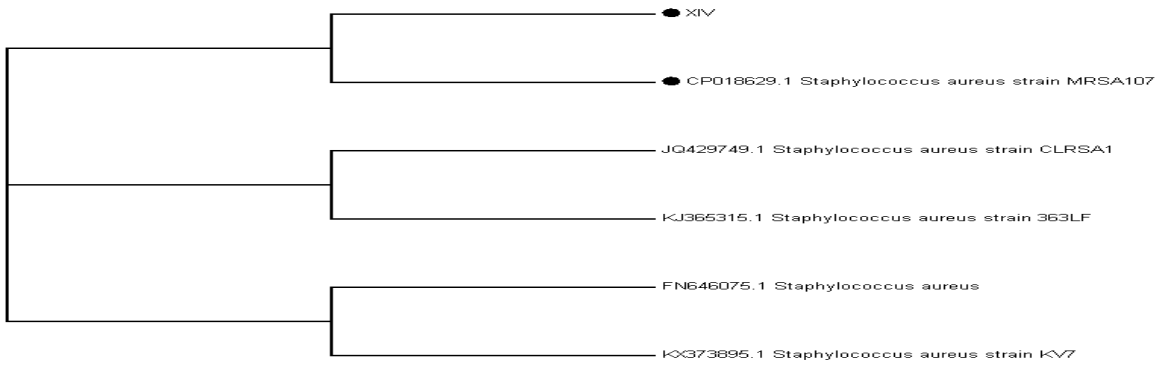
Isolation and Identification of Pathogenic Bacteria by Genotypic Methods

The primary identification of isolate coded as XIV was carried out based on morphological, cultural biochemical characteristics but to accuracy in the assignment of gene and species. Molecular study was carried out by using 16S rRNA analysis at Sai Biosystems Private Limited, Nagpur, Maharashtra, India.

Molecular characterization by 16S rRNA sequencing method:

Isolate was sequenced by using universal primers by giving name such XIV (*Staphylococcus aureus).* Sequence data was aligned and analyzed for identifying the isolate genetically. The 16S rRNA sequence was blast using NCBI blast similarity search tool. The sequence of isolates was compared with sequence of closely related species in GeneBank by multiple sequence alignment, using the program MUSCLE. NCBI database provide as more information regarding nucleotide sequence Phylogenenic relationship was determine by using the neighbor joining method. Taxonomy was carried out which helps classified and arranging diversity of bacteria into groups or taxa on the basis of their similarity and evolutionary relatedness.

Phylogram

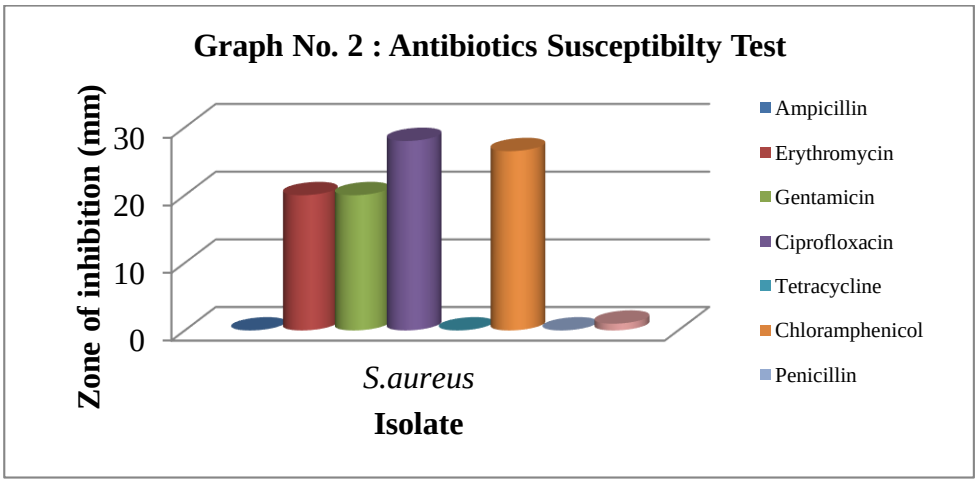


Sequencing of the 16S rRNA of the isolate showed there was 85%.Simillarity between the 16S rRNA sequence of XIV isolate of present study and *Staphylococcus aureus* stain MRSA 107 (GeneBank accession no. CP018629.1).

The 16S rRNA complete genome sequence of *staphylococcus aureus* gram positive cocci isolate from Bovine Mastitis has been deposited at NCBI (DDBJ) Genebank sequence database under accession no. LC475102.

Antimicrobial susceptibility testing: *Invitro* antimicrobial susceptibility test has an important role in suggesting proper antimicrobial chemotherapy. After molecular characterization all the bacteria were subjected to antibiotic sensitivity and resistance test. In present antibiotic susceptibility studied for 12 isolate viz. *Staphylococcus aureus* isolates from bovine mastitis and examined toward a panel of 7 antibiotics by using disc diffusion method on Mueller Hinton Agar. The results of antibiotic susceptibility testing were categorized into two groups (sensitive and resistance) von the basis of zone of inhibition.

In present study *Staphylococcus aureus* isolates were highly resistant to Ampicillin, Tetracycline and Penicillin, intermediately sensitive to Erythromycin, and Gentamicin and it's highly sensitive to ciprofloxacin and chloramphenicol.



Discussion:

Milks is also popularly called the complete balanced food. If we are unable to consume food on a particular day and compensate it with a glass of milk, the nutrients that the body ought to receive through solid food can be easily generated by milk itself. Milk provided all the essential nutrients to provide healthy bones and muscles to our body. It strengthens the immune system. Milk contains calcium that is very essential for our body. It provides healthy bones and aids in body development.

Bovine mastitis is the persistent, inflammatory reaction of the udder tissue due to physical trauma or microorganism's infection. Mastitis is a potentially fatal mammary gland infection. Mastitis is a versatile disease in milk animal and is caused either by pathological, genetically, physiological or environmental factors. Milk from cows suffering from mastitis has an increased somatic cell count.

Salihand Ahemed (2012), have collected mastitis milk sample in certain area at Khartoum State (Eltebna, Falasteen, Shabat, Hilat Kuku, Elhalfaia, Elsamrab and The University of Khartoum farms) from May/2006 to April/2008 to determine the type of *Staphylococcus spps.* In present study sample collected from various area like Akola, Akot, Telhara, varul, Bhavrad, Khaparwadi etc.

According to Btavani *et al.*, 2006 have studied the protein content in normal 4.31 in SCM 4.32 and in clinical Mastitis 4.26 respectively. In present study, the protein content in normal milk was found 0.16 and Mastitis milk 0.11.

In present study the 25 samples were collected during period of July 2018 to January 2019 from clinically diagnosed the cattle of bovine mastitis. Similarly In 2011, Yadav and Kumar stated that mastitis is serious problem in dairy sector and among various etiological agents, the incidence of *Staphylococcus* and *Streptococci* remain high in milking animal. They have focused on detection of *Staphylococcus* and *Streptococci* in winter season.

Elsyed in 2000 isolated *Staphylococcus aureus* and Staphylococcus from 499 milk samples from different domastic animal cows, sheep goat camels. Alayies, (2004) isolated *Staphylococcus aureus* (73.7%) and *Staphylococcus hycus* (6%) from 100 bovine mastitis milk samples. In present study 25 milk sample collected from different location like Akola, Akot, Khaprwadi etc. from cows and buffaloes. We have isolate *Staphylococcus aureus* with other bacteria.

This study revealed a higher prevalence of subclinical mastitis (60.61 %) than clinical mastitis (39.39). A similar result was found by Mewkibib *et al.*, 2009, who reported 22.4% and 48.6 % for clinical and subclinical mastitis.

According Shathele, 2009 reported that incidences of mastitis was higher in colder months in Hoslstein-Friesian and showed significant effect of season on incidence of mastitis on new case. In these study incidences of mastitis was higher in raining reason.

In 2011, Supre *et al*., stated that milk samples from 25 cows in each herd as well as samples from clinical mastitis were collected over a 13 month period. In present study collected 25 samples from clinical and subclinical mastitis in period of July to January.

S. aureus usually acts as a commensal of the human microbiota it can also become an opportunistic pathogen, being a common cause of skin infections including abscesses, respiratory infections, such as sinusitis and food poisoning.

Pumipuntu *et al.,* 2017, In that study a total of 224 subclinical bovine mastitis milk samples were collected from four provinces of Thailand and determined *S. aureus* using conventional method and also subjected to the screening test, MSA and DNase test. In present sample collected from rural and urban area and determine the *S. aureus* by using the MSA test DNase test.

According to Yadav and Kumar (2011), present study is on agreement as the rate of mastitis incidence was more in cow as compared to buffaloes. In present study cows were infected more as compared to buffaloes.

In present study the screening the *Staphylococcus* isolated from bovine mastitis by using the MSA, Coagulase test. The resulted similar to *Kateet et al*., who have clinical *Staphylococcus* Isolated from human by using MSA and combination methods (MSA/DNase). Many studies reported that DNase test showed lower sensitivity than MSA compare the results with TCT Makwana *et al.,* 2012.

The isolated 12 *Staphylococcus aureus* isolates were confirmed on the basis of phenotypic tests. Randomly one isolate (isolate no XIV) selected and analyzed for 16S RNA sequencing. The 16S RNA was composed of nucleotide. There was 85% similarity between the 16S rRNA sequence of XIV isolate of present study and *Staphylococcus aureus* (GeneBank accession no.CP018629.1). This accession no. NCBI, provide 85% identity BLAST analysis. In 2014, Rizwan *et al.,* have done the molecular characterization of *Staphylococcus aureus* and found 85% of similarity with 95%.

Staphylococcus aureus is a widespread commensal bacterium and pathogen. Approximately 50% to 60% of individuals are intermittently or permanently colonized with *S. aureus* and, thus, there is relatively high potential for infections. Indeed, *S. aureus* is among the most prominent causes of bacterial infections in the United States and other industrialized countries. For example, *S. aureus* was the most frequently recovered bacterium from inpatients among 300 clinical microbiology laboratories in the United States from 1998 to 2005. *Staphylococcus aureus* ranked second (after *Escherichia coli*) among bacterial isolates recovered from bacteremias in Europe in 2008, and the prevalence of *S. aureus* bacteremias increased from 2002 to 2008. Recently, *S. aureus* has been reported to be second only to *Clostridium difficile* as a cause of health care–associated infections in the United States

Antibiotics are powerful medicines that fight certain infections and can save lives when used properly. They either stop bacteria from reproducing or destroy them.

The first antibiotic was penicillin. Penicillin-based antibiotics, such as ampicillin, amoxicillin, and penicillin G, are still available to treat a variety of infections and have been around for a long time.

Several types of modern antibiotics are available, and they are usually only available with a prescription in most countries. Topical antibiotics are available in over-the-counter (OTC) creams and ointments

β-lactam are bacterial, cell wall active agents that target the peptidation step of the peptidoglycan synthesis. This is achieved by binding and inactivating the trans peptidase domain od PBPs (Penicillin binding protein) in the cell wall (Chabers, 2004).

β-lactamase are protein with enzymatic activities that are reposible for their resistance to beta- lactam antibiotics like Penicillin Cephalosporin and Carbapenems. These antibiotics have a common element in their molecular structure a four atom ring known as a beta-lactam. β-lactamase bind β- lactams, which results in formation of the β-lactam ring. The inactivated β-lactam antibiotics and active β-lactamase are released (Frere,1995).

According Bansal *et al.*, 2015, observed sensitivity to gentamicin in staphylococcus which is in constrat to the finding of our study reported susceptibility of *Staphylococcus aureus* to ciprofloxacin.

Conclusion

Bovin mastitis is a disease of the most prevalence and costly diseases in dairy industry. Cow or some livestock milk industries with losses lead to reducing of milk production, low quality milk and change in milk composition. Bovin mastitis is a wild spread disease. The subclinical occurrence of the mastitis remains a substantial problem for dairy producers. The relatively high prevalence reported in this study was clearly indicated lack of strategic control measures against the disease as well as poor surveillance measures. Lack of maintenance of strict hygiene and good sanitary environment may be contributory factors in the cause of subclinical mastitis. It is therefore important that farmers should ensure strict personal hygiene and that of animals and general personal hygiene and that of animals and general sanitary condition of the farms should be improved and maintained.

CMT finding represent valuable diagnostic tool in the detection of cattle with secretion disorder whose no. clinical signs of disease. This study confirmed the role of *Staphylococuus aureus* as a most causative agent of Bovine Mastitis. The antibiotic sensitivity test showed that *Staphylococcus aureus* was highly sensitivity to Ciproflaxacin and highly resistant to Penicillin. In current study the bacteria sequence was submitted to NCBI GeneBank got accessed.

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