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EMERGING OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* IN CLINICAL AND SUB-CLINICAL BOVINE MASTITIS IN PESHAWAR

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ABSTRACT : *Bovine mastitis is considering one of the major causes of economy loss in dairy industry. A staphylococcus aureus infection in the mammary glands leads to sub-clinical mastitis and clinical mastitis. S. aureus recovered from bovine mastitis have been found to show resistance against multiple antibiotic agents. This study was designed to evaluate presence of mithicillin resistant Staphylococcus aureus (MRSA) in sub-clinical and clinical mastitis in dairy cattle in Peshawar district. Two hundred milk samples from sub-clinical and clinical mastitis affected cattle were collected in different locality of Peshawar district. The biochemical and sugar fermentation reactions were conducted for the identification and confirmation of S. aureus present in milk samples. Total of 200 milk samples, 68 were contained Staphylococcus aureus with a prevalence of 34.0%. S. aureus isolates in clinical mastitis and sub-clinical mastitis samples were recorded as 44.0 % and 24.0 % respectively. The percentage of MRSA recorded was 6.38%. Similarly in sub-clinical mastitis milk samples the percentage of MRSA recorded was 8.33%. The prevalence of MRSA observed in 68 positive Staphylococcus aureus milk samples was 7.35%. Overall, S. aureus was isolated from clinical and sub-clinical mastitis in dairy cattle. There was evidence of occurrences of MRSA causing bovine mastitis in dairy cattle in Peshawar district.*

Keywords: Mastitis, mithicillin resistant, *Staphylococcus aureus*, cattle, Peshawar

1. INTRODUCTION

Bovine mastitis is considered one of the major causes of economy loss in dairy industry [1]. Various microbes are accountable for causes of mastitis in cows, but the *Staphylococcus aureus* is consider the main causative agent [2]. A *staphylococcus aureus* infection in the mammary glands leads to sub-clinical mastitis and clinical mastitis [3-4]. Inflammation of the mammary glands can exist in clinical and sub clinical forms, but the sub-clinical form is being commonly found in most of livestock farms [5]. Mastitis occurs when white blood cells (leukocytes) are liberating into the mammary gland, usually in response to bacteria subjugate the teat canal. Milk-secreting cells and various ducts throughout the mammary gland are disfigured due to toxins produced by the bacteria. Bovine mastitis is characterized by microbiological, physical and chemical changes in the milk and pathological changes in the mammary glands [6].

Staphylococci bacteria are non-spore forming and non-motile. Usually, *Staphylococci* are found on normal bacterial flora of the skin and mucosal surfaces of respiratory, upper digestive and urogenital tracts of vertebrate [7]. The organism *S. aureus* have been most frequently isolated from infected udder and milk of cattle [8]. *Staphylococcus aureus* is the causal agent of clinical and sub-clinical bovine mastitis and in sub-clinical mastitis cases *S. aureus* acquire resistance to various antibiotics and become a serious problem for milk producers, dairy industries and economy [7, 9]. *Staphylococcus aureus* emits several exclusive virulence factors such as; enterotoxins, exotoxins, Panton-valentine leucocidin and toxic shock syndrome toxin that can impose severe clinical set of symptoms, like septic shock, pneumonia, and complex soft tissue and skin infections [10].

The resistance of *Staphylococcus aureus* to antibiotic agents has been comprehensively studied and it contributed to the treatment failure [11-12]. The injudicious use of antibiotics for treatment of mastitis and without a technical prescription or identification of the pathogen contributes to an increased antibiotic resistance of these organisms and making the treatment of mastitis more difficult. Bacterial resistance to antibiotic is an emerging threat to both human and veterinary medicine and antimicrobial sensitivity testing in pathogenic and non pathogenic bacteria in animals is advised by OIE [13]. Several strains of *S. aureus* recovered from mastitis infected cases have been found to show resistance against multiple antibiotic agents, such as penicillin-G, gentamicin, streptomycin, ampicillin, ciprofloxacin and oxytetracycline [14].

Before the introduction of antibiotics into human and animal health cure, the case fatality rate of S. aureus infection was 80% [15]. With the introduction of penicillin in to human and animal medicine the case fatality rate of *S. aureus* infection decrease to 20%, but not longer than two year the emergence of penicillin resistant *S. aureus* came into existence. The ability of *S. aureus* to become resistant to various antibiotics has led to the emergence of multiple-resistant strains of most important public health concern [16].

Staphylococcus aureus chromosomal *mec*A gene is responsible for expression and encoding of penicillin-binding protein 2a and is accountable for resistance to antibiotics such

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as methicillin and β-lactam. The alteration in Penicillin-binding protein 2a lost its binding ability of methicillin and β-lactam and in result the organism likely to synthesis of cell wall even in presence of these antibiotics. The loss of interaction ability of Penicillin-binding protein 2a to β-lactam moieties and acquirement of *mecA* gene by *S. aureus* become resistant to methicillin in addition to all β-lactam antibiotics as well [17].

The first MRSA strains were isolated from hospitalized patients just after two years when methicillin has been started to use for the treatment of *Staphylococcal* infections [18]. First bovine MRSA was isolated in Belgium [19]. MRSA emergence causes a severe public health hazard and strains of *S. aureus* when become resistant to β-lactam antibiotics and methicillin are recognized as methicillin resistant *Staphylococcus aureus* [14]. Up to 1990s MRSA were considered to be a pathogen of nosocomial infections and were called HA-MRSA and as time progresses the people who had never in contact with hospitals were detected positive for MRSA. From these patients the strain which were isolated were called CA-MRSA. In 2003 a new strain of MRSA which were not classified through PFGE technique arose in patient in Netherland [20]. These strains were named as non typeable methicillin resistant *Staphylococcus aureus* (NT-MRSA). It was reported NT-MRSA that these patients acquired this strain were in contact with pigs because most the cases were found close to pig farms [21]. Several cases of MRSA were reported in animals such as, chickens, horses, pigs, cats, dogs and cattle [22]. Due to continuous developing drug resistance and emerging of MRSA strains in bovine mastitis, this study was designed to evaluate the presence of *Staphylococcus aureus* and MRSA strain in sub-clinical and clinical mastitis cases in dairy cattle in Peshawar district.

2. MATERIAL AND METHODS

Collection of samples from clinical and sub-clinical bovine mastitis

Two hundred milk samples from sub-clinical and clinical mastitis affected cattle were collected in different locality of Peshawar district aseptically in sterile vials. Sterilized Mccartney bottles (15 ml) were used for sample collection. Prior to collection of samples the udder of cattle was washed with water and dried with paper towel and cotton soaked in 70% ethyl alcohol were used for disinfection of the quarters. First few streams of milk were discarded and then mixed milk sample from animal were collected directly in to Mccartney bottle. The milk samples were tested on spot using California Mastitis Test. CMT Positive milk samples collected in sterilized Mccartney bottles (15 ml) were then labeled indicating Owner name, dairy farm or number of animal and collection date. The samples were placed in a thermal flask having crushed ice and transported to Pathology and Bacteriology section of Veterinary Research Institute Peshawar for isolation and identification of the organism through different biochemical test.

Media preparation

The different culture media such as Mannitol salt agar (oxoid), nutrient agar, Tryptic soy broth (oxoid) Mueller Hinton agar and triple sugar iron agar were prepared and isolation and identification of *Staphylococcus aureus* from bovine sub-clinical and clinical mastitis.

Staining characteristics

Colonies of the well isolated organism were taken on a clean glass slide and stained using Gram's staining procedure. The characteristics of the organism were recorded as Gram-positive or Gram-negative characteristic.

Biochemical characteristics

The biochemical and sugar fermentation reactions were conducted for the identification and confirmation of the *S. aureus* present in milk samples of clinical and sub-clinical mastitis.

Various biochemical properties e.g. coagulase production, aesculin test, catalase production, bile tolerance, gelatin liquefaction, methyl red, urease production, Hugh and Leifson's test, methyl blue Proskauer test, nitrate reduction, oxidase, triple sugar iron utilization, indole production, Simmon's citrate, test, and sugar fermentation efficiencies were conducted and reported by [23-24].

Identification of Methicillin resistant *Staphylococcus aureus* (MRSA)

Oxicillin and cefoxitin disk diffusion test:

For phenotypic detection of MRSA antibiotic susceptibility testing of the all isolated organism were carried-out using the standard protocol [25]. Susceptibility of the organism to oxacillin (OX 1ug) and cefoxitin (FOX 30ug) oxoid UK were determined by kirby-Bauer disk diffusion method. A suspension of the test organism were prepared in sterile saline equivalent to 0.5 McFarland using isolated colonies and were incubated at 37°C for 2 hour. Using a sterile cotton swab the organisms were inoculated on Mueller Hinton agar plate spread evenly over entire surface. Antibiotic discs were firmly dispensed over the surface of the agar, with an equal distance from each other. The agar plates were incubated for 24 hours at 35°C. After 24 hours incubation Methicillin resistant *Staphylococcus aureus* were identified by the diameter of the zone around oxacillin $\leq$ 14 mm and for cefoxitin $\leq$ 21 mm [25]. Cefoxitin disk diffusion test were performed to surrogate oxacillin disk diffusion test for better result.

3. RESULTS AND DISCUSSION

Colony and morphological properties of *Staphylococcus aureus* isolated from mastitis in dairy cattle

Staphylococcus aureus isolates produce colonies with smooth, circular, moist, pinhead, raised, convex having entire margins and yellow in color with yellow zones on Mannitol Salt agar medium. MSA medium have high salt concentration, but *Staphylococcus aureus* grows in that condition and ferment mannitol and produce acid, which convert the Phenol Red a pH indicator in MSA from red to yellow color. Biochemically *S. aureus* were confirmed through is it biochemical properties which includes positive results for catalase, coagulase, methyl red, Vogos-Prosker and gelatin liquefaction and negative for oxidase and indole production [26]. *Staphylococcus aureus* species isolated from bovine mastitis was subjected to Gram's staining and microscopic examination. *S. aureus* strains were recorded as Gram-positive cocci/spherical in shape and forming an irregular graph like clusters.

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Prevalence of *Staphylococcus aureus* detected from sub-clinical and clinical mastitis in dairy cattle

Milk is a complete balanced diet having the right amount of sugar, amino acids, fats, vitamins and minerals. Different types of bacteria flourish and as a consequence diminish the quality of milk. The infectious agents present in milk are very important for community health. The contaminated hands of worker, the equipment used for milk handling, environment, and the cow herself are responsible for pathogenic bacteria to contaminate the milk [27]. *S. aureus* mastitis in cattles can either be manifested in sub-clinical or clinical form. The sub-clinical form of mastitis in cattle is of worldwide importance in dairy cattle production system According [28].

In this study, methicillin resistant *Staphylococcus aureus* from bovine clinical and sub-clinical mastitis, 200 milk samples were collected and cultured on MSA to isolate the pure colonies *S. aureus*. Of all cultured, only those were selected and picked up having typical characteristic growth and colony morphology of *S. aureus* on MSA medium. Total of 200 samples, 68 isolates were found *Staphylococcus aureus* positive with a prevalence of 34.0% (Table-1). *Staphylococcus aureus* isolates in clinical mastitis and sub-clinical mastitis were 44/100 with 44.0 % and 24/100 with 24.0 % respectively. Previously, *Staphylococcus aureus* was isolated from humans was 52.3%, sheep 42.5%, goats 30.0% and cattle 37.5% Adamu *et al*. (2010) [29]. Our finding is nearly related to their results. Similarly, El-Seedy *et al*. (2010) [30] carried bacteriological study on milk samples. They observed out of 232 milk samples 76 samples were found sub-clinical cases of bovine mastitis. Further, they observed 71 samples of the clinical mastitis and 22 of the sub-clinical mastitis milk samples were found positive for *Staphylococcal* species with an incidence of 30.6% and 28.9% respectively. The percentage of *S. aureus* isolates from clinical mastitis were recorded 17.7% and considered as major pathogen causing mastitis while the percentage *S. aureus* in sub-clinical mastitis were found 15.8%. They concluded that *Staphylococcus aureus* was the major causative agent of bovine sub-clinical and clinical mastitis because it represented 17.7% and 15.8% of the total isolated organism from bovine mastitis respectively. The results of this study are in agreement with other conducted studies [31-32] in which the predominant causative agent of sub-clinical and clinical was *S. aureus*.

Table-1. Prevalence of *Staphylococcus aureus* isolated and identified from sub-clinical and clinical mastitis in dairy cattle in Peshawar.

Source of milk samples	No. Of milk samples culture	No. Of milk samples with –ive growth	No. Of milk samples with +ive growth	No. Of pure *Staph. aureus* isolates	%age Prevalence
Clinical mastitis	100	54	46	44	44.0
Sub-clinical mastitis	100	55	45	24	24.0
Total	200	109	91	68	34.0

Prevalence of MRSA isolated and identified from sub-clinical and clinical mastitis in dairy cattle in Peshawar

This table illustrates the examination of 200 milk samples, 100 milk samples for each clinical and sub-clinical cases of mastitis in dairy cattle from dairy farm in Peshawar. After observation, it was found that 44 clinical and 24 sub-clinical of the total milk samples were positive for *Staphylococcus aureus* specie. High incidence of *S. aureus* is most likely due to the widespread dissemination of the microorganism on the skin of animals and inside the mammary glands [33].

Table- 2. Prevalence of MRSA isolated from milk samples of sub-clinical and clinical mastitis in dairy cattle in Peshawar

Source of *S. aureus*	No. of milk samples examined	No. of *S. aureus* Isolates	No. of MRSA Isolates	%age
Clinical	100	44	3	6.28
Sub-clinical	100	24	2	8.33
Total	200	68	5	7.35

In this study, clinical mastitis milk samples the percentage of MRSA recorded was 6.38%. Similarly, in sub-clinical mastitis milk samples the percentage of MRSA recorded was 8.33%. The overall percentage prevalence of MRSA observed in 68 positive *Staphylococcus aureus* milk samples was 7.35% (Table- 4). From CMT positive cows a high proportion of *S. aureus* were isolated because the cows were kept in a very bad and muddy housing environment this high rate of *S. aureus* is due to this organism is environmentally very harsh and persisting in a wide range of heat and humidity. Also colonization of organism takes place readily in teat orifices and causing detrimental effect on epithelium [34]. The transmission of the organism take place through milker's hands, utensils, towels and the environment especially floor in which the cows are kept and infected udder the cows. In chronic and sub-clinical infections *S. aureus* has adapted to persist in the udder and from where it makes it way into milk and become a source of contamination for other cows during milking process [35].

4. CONCLUSIONS

In summary, *S. aureus* was isolated from clinical and sub-clinical mastitis in dairy cattle. There was evidence of occurrences of Methicillin resistant *Staphylococcus aureus* in bovine mastitis in livestock farms in Peshawar district.

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