DOI: 10.21276/haya

Haya: The Saudi Journal of Life Sciences **ISSN 2415-623X (Print)**
Scholars Middle East Publishers **ISSN 2415-6221 (Online)**
Dubai, United Arab Emirates
Website: http://scholarsmepub.com/

Research Article

Biochemical Characterization and Antibiotic Susceptibility of *Staphylococcus aureus* from Lame Broiler Chicken and Mastitic Cows of Sylhet City, Bangladesh

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Abstract: Lameness & mastitis are recognized as one of the most important disease affecting the broiler chicken and dairy cattle respectively. The study was performed to observe the biochemical characters and antibiotic susceptibility of *Staphylococcus aureus (S. aureus*) causing lameness in broiler chickens and mastitis in cows. Characters of the isolates were determined by biochemical tests such as mannitol fermentation, catalase, coagulase test. To investigate the antibiogram profiling, frequently used antibiotic discs were tested. From this study, it was observed that at least 84% isolates of *S. aureus* from lame broiler chicken and mastitic cow's milk sample showed positive result in mannitol fermentation test, catalase and coagulase test respectively. But all isolates showed negative result in oxidase and motility test. Interestingly all the bacterial isolates from both broiler and milk sample showed different antibiogram profiling. Antibiotic susceptibility testing suggested that approximately more than 45-65% of the isolates were resistant to penicillin and about 25% resistant to amoxicillin in all isolates. The study reveals that more than 32% isolates were susceptible to methicillin resistant *S. aureus* (MRSA) and 35% chicken isolates were vancomycin resistant. Maximum resistance was observed against azithromycin (68%) of mastitic cow isolates. The present study also showed that *S. aureus* was most sensitive to ciprofloxacin (86-100%). Based on results of this study we conclude that drug resistance of *S. aureus* may vary with the antibiotics being used and with different population.

Keywords: *Staphylococcus aureus*, lameness, mastitis, Antibiotic sensitivity.

INTRODUCTION

Lameness in chickens causes significant economic loss [1]. The term leg weakness is a condition resulting from several causes. It is presumed that purulent arthritis may form a considerable proportion of the cases of leg weakness [2]. Bacterial arthritis in poultry is described to be related with Erysipelothrix, Listeria, Mycoplasma, Staphylococcus, and Escherichia [3]. Disease is mostly caused by *Staphylococcus aureus* which have the veterinary importance in broiler breeders [4].

Staphylococcus aureus is a Gram positive, coagulase positive coccoid cells which falls in the family of Staphylococcaceae [5]. The bacterium is believed to be a normal occupant of the chicken, settled on the skin and feathers and in the respiratory and intestinal tracts. A Staphylococcus contagion, or staphylococcosis, denotes to a mixture of illnesses in poultry caused by Staphylococci bacteria [6]. Roughly 20 species have been isolated, only, *S. aureus*, is of veterinary importance in breeders. *S. aureus* is a crucial opportunist that can cause superficial to severe diseases in a variety of animal species. In poultry, the most common form of infection involves tenosynovitis and arthritis [7, 8]. The pathogen must get into the blood system to cause disease, hence the chances of infection is raised by any injury that supplies the bacteria with a path of entry. The most apparent path of transmission is through an injury in the skin; through the respiratory pathway; and through the gut [6, 9]. *S. aureus* and other microorganisms are found in poultry environment and considered as pathogenic to humans and also may be pathogenic to poultry, making serious contagions that

may conduct to death [10]. *S. aureus* is between the prevalent bacteria required in food poisoning and is a leading cause of gastroenteritis resulting from ingestion of enterotoxins preformed in contaminated food [11]. Whereas most infections can be treated with antibiotics, because of the organism's tendency to develop antimicrobial resistance, it is crucial to continually monitor antibiotic susceptibilities of clinical isolates [12].

Mastitis is an inflammation of the mammary tissue, usually caused by bacteria entering the teat canal [13]. Bovine mastitis is a single most common disease syndrome in adult dairy cows, accounting for about 38% of morbidity [14].Mastitis is also associated with number of zoonotic diseases in which milk acts as a vehicle of infection [15]. The composition of the milk is changed as a result of the inflammation. These changes are physical, chemical and microbiological. Somatic cell count (SCC) describes the concentration of body cells, mainly leukocytes present in the milk, which increases in an inflammation. *Staphylococcus aureus (S. aureus)* is one of the most important and frequent causes of both clinical and subclinical bovine mastitis [16]. Identification of pathogens in milk is considered as the definitive diagnosis of intra mammary infections, and it is important for disease prevention and control. *S. aureus* has developed resistance to most classes of antimicrobial agents. In 1994, by destroying the penicillin to penicillinase, *S. aureus* become resistance [17]. More than 90% *S. aureus* strains are resistance to penicillin [18]. Methicillin, a semisynthetic penicillin was used to treat penicillin resistance *S.aureus* but resistance finally emerged in 1962 [19, 20].

Antibiotic use in the treatment and prevention of poultry and cattle diseases is a very common choice of the farmer of Bangladesh. They want to secure the investment and it is expanding day by day because of blooming the poultry industry, availability and low price of drugs, lack of act/regulation for using antibiotic in food animal, improper knowledge and insufficiency of veterinary doctor etc. The use of antibiotics leads directly to the development and spread of resistance. Selection pressure on a bacterial population, such as that from antibiotics, can result in few surviving members who carry resistant genes. These bacteria then multiply, contributing to a growing population of bacteria with antibiotic-resistant genes. Bacteria resistant to one type of antibiotic may exhibit resistance to related antibiotics. These bacteria can spread through a human population [21] if robust enough. Antibiotic resistance cannot be prevented. Every time antibiotics are used, whether they save a life or are used to no effect (to treat viral rather than bacterial infections, for example), the effective lifespan of that antibiotic and perhaps related drugs is shortened [21]. In general, resistant bacteria are increasing in both incidence and virulence, meaning multi-drug-resistant and extensively-drug-resistant strains are increasingly common in the environment. Thus, the aim of this study was to observe the biochemical characterization and antibiotic sensitivity pattern of *S. aureus* against some conventional antibiotics isolated from swollen leg of broiler chicken and milk from cows infected with mastitis.

MATERIAL AND METHODS

Collection of bacterial Isolates:

A total of 50 samples were collected from the different poultry and dairy farms of Sylhet city corporation area for isolation, identification and to investigate the antibiogram of bacterial species. Bacterial samples were collected in a sterile container (falcon tube) and were transferred to the microbiology lab of the department of Genetic Engineering and Biotechnology, SUST, Sylhet, Bangladesh for further biochemical tests and identification. At each time of collection, precautions were taken to prevent or avoid cross contamination of samples.

Identification and characterization of pathogenic bacteria

Birds were assessed for lameness in the farm by observing swollen feet and joint and unable to move properly. The collected broiler samples were dissected at the suspected swollen leg by the sterile surgical blade. Sterile cotton swab was used to collect fibrinopurulent material from the swollen part of the leg in subcutaneous tissue, without any contamination from the outer surface. All swabs were streaked onto the surface of Mannitol Salt Agar plates, labeled and incubated at 37^{o}C for 48 to 72 hours. The various sub-cultures were streaked onto nutrient agar plate surface, incubated at 37^{o}C for 24 hours and then kept in the refrigerator (at 4^{o}C) for further identification and antibiotic sensitivity studies.

A total of 50 received specimens from dairy and poultry farm were subjected to standard microbiological methods and 43 isolates of *S. aureus* species were identified. In both cases, previous history of the use of antibiotic was taken from farmer. In brief, all the isolates were examined morphologically with Gram staining and the motility test was performed according to the hanging drop method. Several biochemical tests like Mannitol fermentation test, catalase test, oxidase test and coagulase test were performed to confirm bacterial species according to the Bergey's manual of determinative bacteriology [22]. Milk sample was collected from cows affected with mastitis by inspecting the condition of udder and history of prevalent mastitis. A reliable and rapid identification of *S. aureus* colonies in cultures from milk samples is a cornerstone in the control of *S. aureus* mastitis. The high specificity and sensitivity of the coagulase test have made it a standard method for the identification of *S. aureus* in milk.

Characters of isolated *S. aureus*

Gram staining

The Gram stain is a very important preliminary step in the initial characterization and classification of bacteria. It is also a key procedure in the identification of bacteria based on staining characteristics, enabling the bacteria to be examined using a light microscope. The bacteria present in an unstained smear are invisible when viewed using a light microscope. Once stained, the morphology and arrangement of the bacteria may be observed as well. The gram staining was performed according to Bergey's manual [22].

Motility test

The motility test was performed according to the method described by (Cowan et al., 1974) to differentiate motile bacteria from the non-motile one. Before performing the test, pure culture of the isolates were allowed to grow in Nutrient Broth [13].

Catalase test

Catalase is an enzyme that catalyzes the breakdown of hydrogen peroxide to oxygen and water. This test is usually used to differentiate *Staphylococci* (Catalase positive) from non-catalase producing *Streptococci* (Catalase negative). An organism is tested for catalase production by bringing it into contact with hydrogen peroxide. 1-2 drops of the 3% Hydrogen peroxide were added to the bacterial cells. Bubbles of oxygen are released if the organism is a catalase producer [19].

Mannitol fermentation test

Phenol red mannitol broth is used to determine whether the microbe can use the sugar mannitol for carbon and energy. The medium was prepared by adding 21 grams in 1000 ml of distilled water. It was heated to completely dissolve the medium and then dispensed into tubes and autoclaved at 15psi pressure at 121°C for 15 minutes. An inoculum from a pure culture is transferred aseptically to a sterile tube of phenol red mannitol broth. The inoculated tube is incubated at 37°C for 24 hours and the results are determined. A positive test consists of a color change from red to yellow, indicating a p*H* change to acidic [14].

Oxidase test

The oxidase test is a test used in microbiology to determine if a bacterium produces certain cytochrome c oxidases. It uses reagent N, N-dimethyl-p-phenylenediamine (DMPD), which is also a redox indicator. The reagent is a dark-blue color when oxidized, and colorless when reduced. A piece of filter paper was placed in a clean Petri dish and two or three drops of freshly prepared oxidase reagent was added. Using a piece of wooden stick, a colony of the test organism was removed and smear it on the filter paper and waited for the development of a blue purple color within 10 seconds for positive result [18].

Coagulase test

The coagulase test was performed using human plasma as an alternative of rabbit plasma. It is reported that human plasma can be used in the routine Tube Coagulase Test (TCT) [29] and considered as definitive test for *Staphylococcus aureus*. Human blood plasma was obtained from blood bank in which 0.1% EDTA was used as anticoagulant. For coagulase test, 1-in-6 dilution of the plasma in saline (0.85% NaCl) was taken and placed 1 ml volumes of the diluted plasma in small tubes. Several isolated colonies of test organism was emulsified in 1 ml of diluted human plasma to give a milky suspension. The tube was incubated at 37°C in water bath for 4 hours. The tube was examined at 1, 2 and 4 hour for clot formation by tilting the tube through 90°**.** Negative tubes was kept at room temperature overnight and re-examined. Results were recorded after 4 and 24 h of incubation at 37°C. Weak coagulase activities were recorded as positive.

Antimicrobial sensitivity testing

The antibiotic susceptibility pattern of the identified *S. aureus* isolates was performed *in vitro* by using a modified disk diffusion test of Kirby-Bauer method on Muller-Hinton agar as described by the Clinical Laboratory Standard Institute [24]. Following antibiotic disks were used for the test: Amoxicillin (AMX 30µg disk^{-1}), Azithromycin (AZR 15µg disk^{-1}), Ciprofloxacin (CIP 5µg disk^{-1}), Gentamycin (GEN 30µg disk^{-1}), Methicillin (MET 10 µg disk^{-1}), Novobiocin (NOV 30 µg disk^{-1}), Penicillin (PEN 30µg disk^{-1}), Tetracyclin (TET 30µg disk^{-1}), Vancomycin (VAN 30µg disk^{-1}). The procedure involved measuring the diameter of the zone of inhibition that results from diffusion of the agent into the medium surrounding the disk. The bacterial culture adjusted to desired turbidity (0.5 McFarland standards) was inoculated into Muller Hinton agar plate by streaking the swab over the entire sterile agar surface. Following this, antibiotic disks were evenly placed on the surface of the agar plate by using sterile forceps. These plates were incubated at 37°C for 24 hours. After 24 hrs, clinical interpretation [resistant (R), intermediate (I) and sensitive (S)] of the size of the zone was evaluated based on the diameters of zones of inhibition on petri dish and the isolates were defined according to the criteria suggested by the CLSI [23,24].

RESULTS AND DISCUSSION

S. aureus forms small, shiny, gold colonies on most solid media after 1 day of growth at 37°C. The size and color of the colonies is media and strain dependent (media containing low levels of glycolytic carbon sources will produce colonies that is less pigmented as are some strains harboring specific mutations that affect pigmentation. We had successfully identified 42 isolates as *S. aureus* from the total 50 sample. The biochemical characteristics of presumptive isolates presented in the table 1. Morphologically isolates showed positive cocci shape in clusters, gram

positive having purple color and were non motile. Mannitol Salt Agar is a selective medium for *Staphylococcus aureus* which have high sodium chloride level inhibits most other species with the exception of halophilic Vibrio's. The majority of *S. aureus* ferment mannitol producing yellow colonies, occasional strains of coagulase- negative staphylococci may also ferment mannitol. In our study, some of the isolates showed negative result as other species of staphylococcus such as *S. epidermis* showed light pink color instead of yellow color. Mannitol fermentation test showed positive result of 20 isolates from broiler chicken and 22 isolates from milk sample. The coagulase test was not sensitive after 4 h, and acceptable results were obtained only after 24 h of incubation (Table 1). No species other than *S. aureus* was coagulase positive and finally 42 isolates was confirmed as *Staphylococcus aureus*.

Table-1: Biochemical Characteristics of presumptive *Staphylococcus aureus* from broiler sample (BS) and milk sample (MS)

Isolate no.	Gram Staining	Motility test	Mannitol test	Oxidase test	Catalase test	Coagulase test	Isolate no.	Gram Staining	Motility test	Mannitol test	Oxidase test	Catalase test	Coagulase test
BS01	+	-	+	-	+	+	MS01	+	-	+	-	+	+
BS02	+	-	+	-	+	+	MS02	+	-	+	-	+	+
BS03	+	-	+	-	+	+	MS03	+	-	+	-	+	+
BS04	+	-	+	-	+	+	MS04	+	-	-	-	+	-
BS05	+	-	-	-	+	-	MS05	+	-	+	-	+	+
BS06	+	-	+	-	+	+	MS06	+	-	+	-	+	+
BS07	+	-	-	-	+	-	MS07	+	-	+	-	+	+
BS08	+	-	+	-	+	+	MS08	+	-	-	-	+	-
BS09	+	-	+	-	+	+	MS09	+	-	+	-	+	+
BS10	+	-	+	-	+	+	MS10	+	-	+	-	+	+
BS11	+	-	+	-	+	+	MS11	+	-	+	-	+	+
BS12	+	-	-	-	+	-	MS12	+	-	-	-	+	-
BS13	+	-	+	-	+	+	MS13	+	-	+	-	+	+
BS14	+	-	+	-	+	+	MS14	+	-	+	-	+	+
BS15	+	-	+	-	+	+	MS15	+	-	+	-	+	+
BS16	+	-	+	-	+	+	MS16	+	-	+	-	+	+
BS17	+	-	-	-	+	-	MS17	+	-	+	-	+	+
BS18	+	-	+	-	+	+	MS18	+	-	+	-	+	+
BS19	+	-	+	-	+	+	MS19	+	-	+	-	+	+
BS20	+	-	+	-	+	+	MS20	+	-	+	-	+	+
BS21	+	-	+	-	+	+	MS21	+	-	+	-	+	+
BS22	+	-	-	-	+	-	MS22	+	-	+	-	+	+
BS23	+	-	+	-	+	+	MS23	+	-	+	-	+	+
BS24	+	-	+	-	+	+	MS24	+	-	+	-	+	+
BS25	+	-	+	-	+	+	MS25	+	-	+	-	+	+

It was observed that the isolates of *S. aureus* collected from different farm specimen exhibits varying degree of sensitivity to different antibiotics. Significant numbers of the isolates were found multidrug resistant. For the convenience of our study we examined all 42 isolates confirmed as *S. aureus* with nine available antibiotic disc. The antibiogram profile of the isolates was presented in the table 2.

In cases of *S. aureus* strains tested in this study it could be noticed that the highest sensitivity was to ciprofloxacin (100%) followed by the sensitivity to amoxicillin (70%), gentamicin (65%), Vancomycin (65%), Azithromycin (60%), Tetracycline (60%), Methicilin (55%), Novobiocin (45%) and Penicillin (30 %) from broiler isolates (figure 1). A highest percent of isolates were resistant to penicillin (65%) followed by Novobiocin (50%), Vancomycin (35%), Methicilin (35%), Azithromycin (30%) and amoxiciline (25%) from the chicken isolates. The next *in vitro* effective antibiotics were ciprofloxacin (86%), followed by amoxicillin (59%), tetracycline (59%), methicillin (55%), gentamycin (50%), penicillin (27%), azithromycin (23%) and novobiocin (23%) from the milk isolates of mastitic cows (figure 2). However, resistant isolates also showed as alarming condition in mastitic cows. It was accounted that 68% isolates were azithromycin resistant followed by penicillin (46%), Novobiocin (41%), methicillin (32%), tetracycline (32%) and amoxicillin (32%).

Table-2: Antibiogram of isolated *S. aureus* against nine antibiotics

Name of antibiotics (disc concentration in µg)	No. of isolates Broiler + milk (42)	Sensitivity pattern of isolated *S. aureus* (%)					
		Broiler sample			Milk sample		
		R	I	S	R	I	S
AMX(25)	20+22	25.0	5.00	70.0	31.8	9.1	59.1
AZN(15)	20+22	30.0	10.0	60.0	68.2	9.1	22.7
CIP (5)	20+22	-	-	100.0	4.5	9.1	86.4
GEN(30)	20+22	10.0	25.0	65.0	9.1	36.4	50.0
MET(10)	20+22	35.0	10.0	55.0	31.8	13.6	54.5
NOV(5)	20+22	50.0	10.0	45.0	40.9	36.4	22.7
PEN (6)	20+22	65.0	5.00	30.0	45.5	27.3	27.3
TET (30)	20+22	15.0	25.0	60.0	31.8	9.1	59.1
VAN(30)	20+22	35.0	-	65.0	N/T	N/T	N/T

Legend: Amoxicillin – AMX, Azithromycin – AZN, Ciprofloxacin – CIP, Gentamycin – GEN, Methicillin – MET, Novobiocin – NOV, Penicillin – PEN, Tetracycline – TET, Vancomycin – VAN, % R – Resistant percent, % I – Intermediate percent, % S – Susceptible percent. N/T – Not Tested

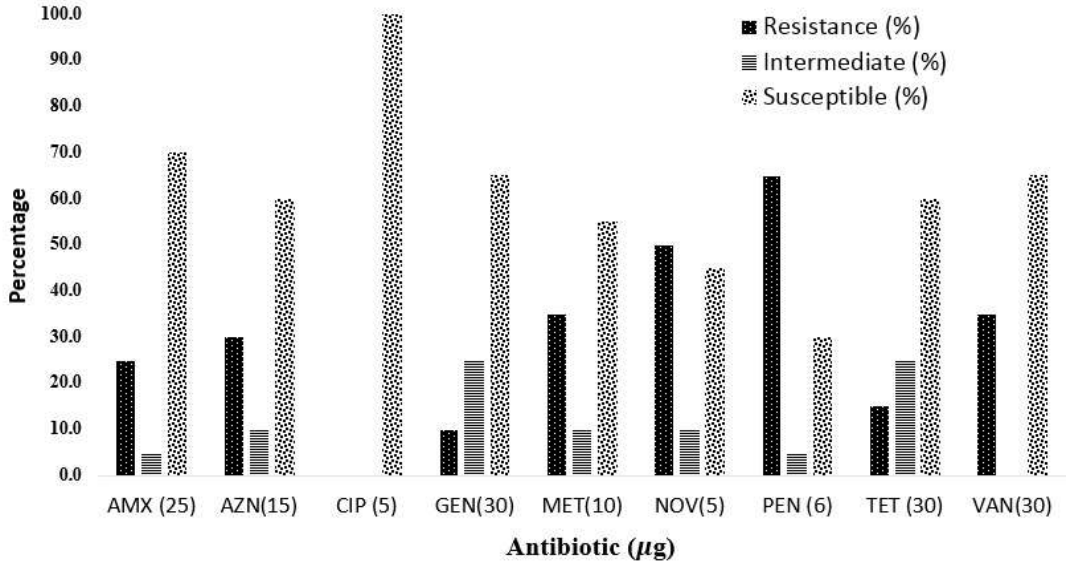

Fig-1: Susceptibility patterns of the tested antibiotics against *S. aureus* from broiler isolates

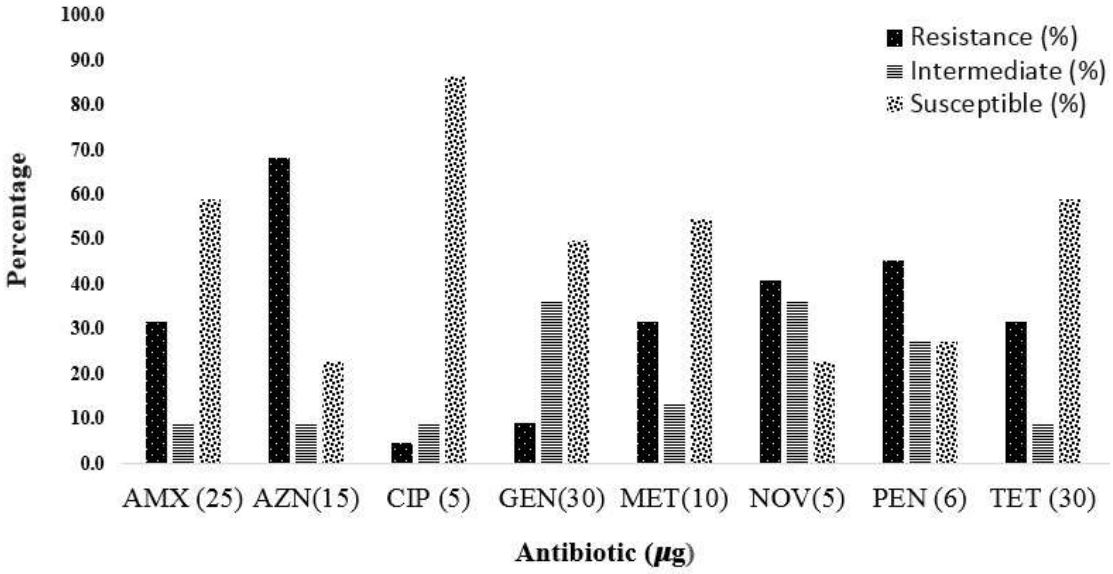

Fig-2: Susceptibility patterns of the tested antibiotics against S. aureus from milk isolates

Accurate identification of bacteria is a crucial step for antibiogram profiling. All the culture isolates were confirmed as *Staphylococcus aureus* genus by various tests, i.e. gram staining, catalase and oxidase test [29]. Catalase positive, gram positive and oxidase negative isolates were defined as Staphylococcus. Further test by mannitol salt agar fermentation of the isolates and positive coagulase tests indicated the presence of *Staphylococcus aureus.* We also examined the conventional tube coagulase test (TCT) with replacement of human blood plasma instead of rabbit plasma. This test would be useful in future studies.

It is also necessary to confirm the identity of presumptive S. aureus colonies by other means e.g. DN'ase, thermonuclease or 16S rRNA gene (*rrs*) sequencing method. But all these methods are expensive, time consuming and rarely done. With continuously improving technologies and decreasing costs, genetic identification methods like *rrs* gene sequencing will soon find a place in routine veterinary diagnostics. It was reported that *S. aureus* populations from cattle and from humans are different [30]. It might be reflected in our study because bacteria from chicken and cows population showed different antibiogram pattern.

Even though the bacterial isolates were confirmed as *S. aureus,* there may be variation in antigenic determinants with changes in their antibiotic susceptibility pattern leading to emergence of new strains within the bacterial species. Antibiotics are used in animal farming for treatment, control, prevention and growth promotion. Because of prolonged treatments with same antibiotics frequently is noticed the emergence of resistant variants of bacterial strains. *S. aureus* has been tested in meat and poultry products to assess microbiological safety, sanitation conditions during processing, and storage quality of products [26]. In fact, poultry meat has been frequently associated with food borne illness in which initial contamination is traceable to food handlers [27]. One study demonstrate that Neonatal staphylococcal osteomyelitis should be considered when turkey flocks experience increased mortality, especially if they develop severe swelling and inflammation of toes following trimming and have enlarged swollen feet, tendons, or joints [28].

The result of our present study indicated that most of the *S. aureus* isolates possesses considerable resistant to the antibiotics such as penicillin, Novobiocin, azithromycin, methicillin. But the resistance pattern of chicken isolates was different with the milk isolates. This might be different bacterial strain population. When animals are administered an antibiotic that is closely related to an antibiotic used in human medicine, cross resistance occurs and disease causing bacteria become resistant to the drug used in human medicine. But vast majority of antibiotic used either in people or in animals not both. A report from American Meat Institute showed that penicillin, cephalasporins, sulfa drugs and quinolones are widely used in human whereas tetracyclines and lonophores are used in animals [31]. Lonophores antibiotic are never used in human medicine. Methicillin-resistant *Staphylococcus aureus* (MRSA) known as superbug first identified in the pig farms in Netherlands during 2004-05. This livestock-associated MRSA strain had colonized in chickens, dairy cattle and the people who handle them and may also be emerged as a food safety risk. In our study more than 32 % of isolates was methicillin resistant which is alarming. Vancomycin, which is one of the antibiotics most often used to treat MRSA also 35% resistant in our study.

With comparison of above mentioned results it has been revealed that outbreak of staphylococcal infections in food animal is high and because of its epidemiological importance, measures must be taken in this field to minimize the zoonotic disease transmissible from livestock to humans.

CONCLUSION

The current study has provided important data on the antimicrobial susceptibility patterns of *S. aureus* to several antibiotics, including the prevalence of methicillin resistance. No single antimicrobial drug is appropriate for every lameness and mastitis-causing organism. Thus, when selecting antibiotics for treatment, it's important to understand how the results obtained in the laboratory (in vitro) can be used to treat animal (in vivo). The findings from this study can be used to guide the choice of empirical treatment of *S. aureus* infections and may also inform the review of treatment guidelines by the veterinarians. We concluded that there was a high level of resistance to penicillin, novobiocin, methicillin, tetracycline among *S. aureus* isolates at different farm, treatment of *S. aureus* infections with these antibacterial agents, without antibiotic sensitivity testing would therefore be unrealistic. A national antibiotic resistance surveillance of this organism is recommended as methicillin resistant MRSA strains developed.

ACKNOWLEDGEMENTS

This work was conducted and supported by the Microbiology laboratory, Department of Genetic Engineering and Biotechnology, Shahjalal University of science and technology, Bangladesh. H. M. Rejwan and Ajay Shah was involved in all aspects of the experimental works, sample collection, analysis and interpretation. M .Z. Alam design the experiment, prepared manuscript and S. M Abu Sayem thoroughly checked data, manuscript and experimental works.

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