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Article

Detection of bacterial species from clinical mastitis in dairy cows at Nilphamari district and their antibiogram studies

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Abstract: The present study was conducted on the rural dairy cows to detect the bacterial species from clinical mastitis in dairy cows with their antibiogram studies during the period from January 2015 to June 2015. For this purpose two upazilla were selected under the Nilphamari district. On the basis of morphology, staining, cultural and biochemical characteristics, the isolated organisms were classified as, *Staphylococcus* spp., *Streptococcus* spp., *E. coli, and Bacillus* spp. .For this study, a total of 48 samples were collected from affected mastitis cows. Out of 48 milk samples, 17 were positive for *Staphylococcus* spp. (35.42%), 9 were positive for *Streptococcus* spp. (18.75%), 7 were positive for *E. coli* (14.58%), 5 were positive for *Bacillus* spp.(10.41%),5 were positive for mixed organisms (10.41%) and 5 were unidentified organisms (10.41%). Antibiogram studies revealed that all of the isolates of *Staphylococcus* spp. were sensitive to gentamicin and were resistant to streptomycin. Gentamicin was sensitive to all of the isolates of *Streptococcus* spp. and was resistant to streptomycin. The isolates of *Bacillus* spp. were sensitive to ciprofloxacin and *Bacillus* spp. was resistant to streptomycin. All of the isolates of *E. coli* were sensitive to ciprofloxacin the isolates were resistant to ampicillin and amoxicillin. Over all sensitivity revealed that ciprofloxacin, gentamicin and enrofloxacin were most efficacious. Thus, it may be recommended that ciprofloxacin, gentamicin and enrofloxacin in optimum doses would resolve most cases of clinical mastitis in dairy cows.

Keywords: clinical mastitis; dairy cows; antibiogram studies

1. Introduction

Milk is a highly nutritious food that is rich in carbohydrate, proteins, fats, vitamins and minerals. However, health risk to consumers can be associated with milk due to the presence of zoonotic pathogens and antimicrobial drug residues (Bradely *et al.,* 2002). The organism is well adapted to survive in the udder and

usually establishes mild subclinical infection of long duration. Bacteria are shed into milk from infected quarters. Transmission occurs mainly at milking time through contaminated milking machines, clothes and hands of milkers or machine operators (Radostitis *et al.,* 1994). Mastitis remains the most common disease of dairy cattle, causing the biggest economic losses to the dairy industry (Halasa *et al.,* 2007).Mastitis occurs when leukocytes are released into the mammary gland, usually in response to an invasion of bacteria into the teat canal or other microorganisms but also may be caused by stress and physical injuries (Ruegg *et al.,* 2001). Mastitis is also associated with a number of zoonotic diseases in which milk acts as a vehicle of infection (Samad *et al.,* 2008). Mastitis is usually classified as sub-clinical, acute, subacute, chronic and gangrenous based on etiopathological findings and observations. Clinical cases of mastitis are characterized by the presence of one or more of symptoms such as abnormal milk, udder swelling and systemic signs including elevated temperature, lethargy and anorexia (Eriskine *et al.,* 2001; Gharagozloo *et al.,* 2007). Mastitis is one of the most costly and troublesome diseases in dairy cows. Mastitis has been reported a serious health problem of livestock (Ahmad, 2001; Bachaya, *et al.,* 2011) and is responsible for heavy economic losses in dairy industry (Bachaya *et al.,* 2011; Yousaf *et al.,* 2012) as it reduces milk yield, profit margins, and quality of milk and milk products in all dairy-producing countries of the world. Bovine mastitis is a global problem as it adversely affects animal health, quality and quantity of milk and so economics of milk production and every country including developed ones suffer from huge financial losses (Sharma *et al.,* 2004). Mastitis has significantly constrained in the development of the dairy industry in Bangladesh. The most important pathogens involved in bovine mastitis worldwide are *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, *Escherichia coli* and *Klebsiella* spp. (Olde *et al.,* 2008). *Staphylococcus aureus, Escherichia coli, Streptococcus* spp., *Corynebacterium bovis, Klebsiella* spp., *Pseudomonas aeruginosa* etc. are of the major pathogens causing mastitis. Mastitis has economic significance to dairy farms as it causes a reduction in milk quality and loss of production (Lim *et al.,* 2004 and Lira *et al.,* 2004). *Staphylococcus* spp. is a major pathogen for cattle, causing various forms of subclinical and clinical mastitis (Ibrahim *et al.,* 2009). Two groups of virulence factors (leukotoxins and superantigens) are supposed to play an important role in the initiation and exacerbation of this disease (Schuberth *et al.,* 2001). The presence of *E. coli* in milk and dairy products results either from lactating animals through mastitis or contaminated udder or environmental sources through faulty sterilization of utensils, improper preparation of dairy animals and using contaminated water supplies (Bradley *et al.,* 2007). *S. aureus* and *E. coli* are most commonly isolated pathogen from the clinical mastitis (Contreras *et al.* 2003). Coagulase negative *staphylococci* are the most frequently isolated pathogens from the subclinical mastitis in dairy cows (McDougall *et al.,* 2002; Contreras *et al.,* 2003). One key component of better control of this disease is identification of the causative bacterial agent during udder infections in cows. Targeting antimicrobial treatment of animal infections such as mastitis against the causal agent is generally recommended (Constable *et al.,* 2008). One important reason for treatment failure is assumed to be the indiscriminate use of antibacterial drugs without testing in vitro sensitivity of the causal organisms (Garg, 2001). The potential impact of transmission of resistant bacteria to humans via the food chain, as in bulk milk with sub-clinical mastitis is a public health problem (Sol *et al.,* 2000). It is therefore, important to study the sensitivity pattern of different bacteria isolated from mastitis from time to time in different geographical zones of the country in order to formulate appropriate therapeutic measures. Therefore, the objectives of this study are to isolate and identify the causative bacterial species of mastitis in dairy cows at Nilphamari district with their antibiogram profiles in the study area.

2. Materials and Methods

2.1. Collection of samples

The study was carried out on dairy cows at Saidpur upazila and Sadar upazila in Nilphamari district during the period from January 2015 to June 2015.The collected samples were brought to the bacteriology laboratory of the department of microbiology under the faculty of Veterinary and Animal Science, Hajee Mohammad Danesh science and Technology University (HSTU), Dinajpur. A total of 48 milk samples were randomly collected directly from apparently clinically mastitis affected cows then 10 ml of milk were taken in sterilized test tubes with rubber cap, using routine techniques for aseptic infection. The samples were carried to the laboratory in an ice box contained ice and processed for the isolation and characterization of bacteria subsequently and kept in incubator at 37^0C for 24 hours for the isolation and identification.

2.2. Isolation of associated bacteria

Bacteriological examination were carried out using standard method for aerobic bacteria and fungi (Brown, A.E. 2005) for the detection of bacteria, all samples were serially diluted and plated on Nutrient agar and

subsequently incubated at 37^0C for 24 hours. Primary culture was performed in Nutrient agar and Nutrient broth media. For sub-culturing, suspected bacteria were inoculated separately onto different bacteriological agar media under aseptic condition and incubated at 37^0C for 24 hours. Pure cultures were achieved as per procedures described by OIE (2000), Merchant and Packer (1967) and Cowan (1985).

2.3. Identification of associated bacteria

Cultural, morphological and biochemical characteristics were studied in order to identify the bacterial flora. The cultural characteristics or colonial morphology of the bacteria grown on the nutrient and blood agar media were recorded. Gram staining was performed to study the morphology and staining characteristics of bacteria according to the technique described by Merchant and Packer (1967). Biochemical tests, such as sugar fermentation, coagulase, catalase, MR, VP, and indole tests, were performed per standard methods (Cheesbrough, 1985).

2.4. The antibiotic sensitivity

To determine the antibiotic sensitivity patterns of different bacterial isolates by disk diffusion method, different types of commercially available antimicrobial discs (Oxoid Ltd., UK) were used. A previously described disc diffusion process, known as Kirby- Bauer method (Bauer *et al.,* 1966; Jakaria *et al.* 2012) was used to determine the susceptibility of the bacterial isolates against selected antibiotic agents. For this purpose, eight different antibiotic discs were obtained from commercial sources (Oxoid Ltd, Baring-stoke, Hampshire, England). The selected antibiotics used were Ciprofloxacin (5 µg/disc), Enrofloxacin (5 µg/disc), Amoxicillin (10 µg/disc), Streptomycin (10µg/disc), Erythromycin (15 µg/disc), Ampicillin (10 µg/disc) and Gentamicin (30 µg/disc). The interpretation on susceptibility was done according to the guidelines of Clinical and Laboratory Standard Institute (CLSI, 2007; formerly known as NCCLS).

3. Results

3.1. Isolation and identification of bacterial species

Isolation of bacterial flora directly from apparently clinically mastitis affected cows was identified as *Staphylococcus* spp. *Streptococcus* spp., *Bacillus* spp., and *E. coli* (Table. 1).

Table 1. Bacterial loads isolated from clinical mastitis milk samples (n=48) from dairy cows at Saidpur and Sadar upazila of Nilphamari district.

Name of the isolated bacteria	No. of isolates	Prevalence
Staphylococcus spp.	17	35.45 %
Streptococcus spp.	9	18.75 %
Escherichia coli	7	14.58 %
Bacillus spp.	5	10.41 %
Mixed organisms:		10.41 %
E. coli + *Streptococcus* spp.	2 2+3= 5	
Staphylococcus spp. + *Streptococcus* spp.	3	
Unclassified	5	10.41 %
Total	**48**	**100 %**

3.1.1. Cultural characterization and isolation of bacterial species

In nutrient agar *staphylococcus spp.* produce circular, small, smooth, convex and gray-white or yellowish colonies and in blood agar media produce beta types of hemolysis (Table 2). *Streptococcus* spp produce circular, small, smooth, convex and gray-white colonies (Table 3). *Escherichia coli* on EMB agar produce smooth, circular, black color colonies with metallic sheen and on MC agar produce bright pink color smooth colonies (Table 4). *Bacillus* spp. on blood agar produce large, creamy, colonies with hemolysis and on nutrient agar produce thick, greyish white or cream colored colonies (Table 5).

3.1.2. Identification of bacteria by biochemical tests

In biochemical test *Staphylococcus spp*. produce acid and gas on dextrose, Maltose, lactose, sucrose and mannitol test. Catalase, MIU, TSI, MR test show positive result but indole test shows negative result (Table 2). *Streptococcus* spp. produce positive result on MR and Catalase test but make negative result on indole test (Table 3). *Escherichia coli* also produce acid and gas on dextrose, Maltose, lactose, sucrose and mannitol test and Catalase, indole, MR, MIU, TSI slant reactor show positive result (Table 4). *Bacillus* spp. produce acid and

gas on dextrose, maltose, lactose, sucrose and mannitol test, catalase and indolent test produce positive reaction but MR test show negative reaction (Table 5).

Table 2. Cultural, morphological and biochemical properties of isolated *Staphylococcus* spp.

Cultural characteristics		**Biochemical Characteristics**		**Staining and morphological Characteristics**
Blood Agar	Nutrient Agar	Tests	Results	Staining properties
ß-Type of hemolysis were produced	Circular, small, smooth, convex and gray-white or yellowish colonies were produced	Dextrose	Acid and Gas	Gram positive cocci arranged' in grape like cluster
		Maltose	Acid and Gas	
		Lactose	Acid and Gas	
		Sucrose	Acid and Gas	
		Mannitole	Acid and Gas	
		Catalase test	+	
		MIU test	+	
		TSI test	+	
		MR test	+	
		Indole test	-	

Legends: MR = Methyl-Red, + = Positive reaction, - = Negative reaction, TSI= Triple Sugar Iron, MIU=Motility Indole Urease

Table 3. Cultural, morphological and biochemical properties of isolated *Streptococcus* spp.

Cultural characteristics		**Biochemical Characteristics**		**Staining and morphological Characteristics**
Blood Agar	Nutrient Agar	Tests	Results	Staining properties
ß-Type of hemolysis were produced	Circular, small, smooth, convex and gray-white colonies were produced.	Indole test	-	Gram positive, cocci shape, arranged in chain form
		MR test	+	
		Catalase test	+	

Legends: MR = Methyl-Red test, + = Positive reaction, - = Negative reaction, MR = Methyl-Red

Table 4. Cultural, morphological and biochemical properties of isolated *Escherichia coli.*

Cultural characteristics		**Biochemical Characteristics**		**Staining and morphological Characteristics**
On EMB Agar	On Mac Conkey Agar	Tests	Results	Staining properties
Smooth, circular, black color colonies with metallic sheen were produced	Bright pink color, smooth colonies were produced	Dextrose	Acid and Gas	Gram-negative, pink colored, small rod shaped organisms arranged in single, pairs or short chain
		Maltose	Acid and Gas	
		Lactose	Acid and Gas	
		Sucrose	Acid and Gas	
		Mannitole	Acid and Gas	
		Catalase test	+	
		Indole test	+	
		MR test	+	
		MIU test	+	
		TSI slant reactor	+	

Legends: MR = Methyl-Red, + = Positive reaction, TSI = Triple Sugar Iron, MIU = Motility Indole, Urease

Table 5. Cultural, morphological and biochemical properties of isolated *Bacillus* spp.

Cultural characteristics		Biochemical Characteristics		Staining and morphological Characteristics
Blood Agar	Nutrient Agar	Tests	Results	Staining properties
Large, creamy colonies with ß hemolysis were produced	Thick, grayish white or cream colored colonies were produced.	Dextrose	Acid and Gas	Gram-positive large rod shaped organisms arranged in chain.
		Maltose	Acid and Gas	
		Lactose	Acid and Gas	
		Sucrose	Acid and Gas	
		Mannitole	Acid and Gas	
		Catalase test	+	
		MR test	-	
		Indole test	+	

Legends: MR = Methyl-Red, + = Positive reaction, - = Negative reaction

3.2. Results of antibiotic sensitivity assay of isolated bacteria

Based on the susceptibility to antibiotics, the bacteria were categorized into three groups *viz*. sensitive, intermediate and resistance. Total seven antibiotics used in this study; all of the isolates of *Staphylococcus* spp. was sensitive to gentamicin and was resistant to streptomycin (Table 6). Gentamicin was sensitive to all of the isolates of *Streptococcus* spp. and was resistant to streptomycin (Table 7). The isolates of *Bacillus* spp. were sensitive to ciprofloxacin and *Bacillus* spp. was resistant to streptomycin (Table 8). All of the isolates of *E. coli* were sensitive to ciprofloxacin the isolates were resistant to ampicillin and amoxicillin (Table 9). Over all sensitivity revealed that ciprofloxacin, gentamicin and enrofloxacin were most efficacious (Table 10).

Table 6. Antibiotic sensitivity pattern of *Staphylococcus* spp. (n=17).

Antibacterial agents	Disc concentration (µg /disc)	No. of isolates		Percentages (%)	
		Sensitive	Resistant	Sensitive	Resistant
Streptomycin	10µg	3	14	17.65	82.35
Amoxicillin	10µg	8	9	47.06	52.94
Ampicillin	10µg	9	8	52.94	47.06
Enrofloxacin	5 µg	12	5	70.59	29.41
Erythromycin	15 µg	14	3	82.35	17.65
Ciprofloxacin	5 µg	16	1	94.12	5.88
Gentamicin	30 µg	17	0	100	0

Table 7. Antibiotic sensitivity pattern of *Streptococcus* spp. (n=9).

Antibacterial agents	Disc concentration (µg /disc)	No. of isolates		Percentages (%)	
		Sensitive	Resistant	Sensitive	Resistant
Streptomycin	10µg	1	8	11.11	88.89
Amoxicillin	10µg	5	4	55.56	44.44
Ampicillin	10µg	5	4	55.56	44.44
Enrofloxacin	5 µg	6	3	66.67	33.33
Erythromycin	15 µg	7	2	77.78	22.22
Ciprofloxacin	5 µg	8	1	88.89	11.11
Gentamicin	30 µg	9	0	100	0

Table 8. Antibiotic sensitivity pattern of *Bacillus* spp. (n=5).

Antibacterial agents	Disc concentration (µg /disc)	No. of isolates		Percentages (%)	
		Sensitive	Resistant	Sensitive	Resistant
Streptomycin	10µg	1	4	20	80
Amoxicillin	10µg	2	3	40	60
Ampicillin	10µg	3	2	60	40
Enrofloxacin	5 µg	4	1	80	20
Erythromycin	15µg	4	1	80	20
Ciprofloxacin	5 µg	5	0	100	0.00
Gentamicin	30 µg	5	0	100	0.00

Table 9. Antibiotic sensitivity pattern of *E. coli* (n = 7).

Antibacterial agents	Disc concentration (µg /disc)	No. of isolates		Percentages (%)	
		Sensitive	Resistant	Sensitive	Resistant
Ampicillin	10µg	0	7	0	100
Amoxicillin	10µg	0	7	0	100
Streptomycin	10µg	2	5	28.57	71.43
Erythromycin	15 µg	2	5	28.57	71.43
Enrofloxacin	5 µg	5	2	71.43	28.57
Gentamicin	30 µg	6	1	85.71	14.29
Ciprofloxacin	5 µg	7	0	100	0

Table 10. Antibiotic sensitivity assay of the isolated bacteria obtained from clinically affected mastitis cows.

Name of the isolates	GN	CIP	ENR	ERY	AML	AMP	S
Staphylococcus spp.(n=17)	+++	+++	++	++	++	++	-
Streptococcus spp.(n=9)	+++	+++	++	++	++	++	-
*Bacillus spp.(*n=5)	+++	+++	++	++	++	++	-
E. coli (n = 7)	+++	+++	++	++	-	-	++

Legends: (ENR= Enrofloxacin, ERY = Erythromycin, AMP= Ampicillin, CIP= Ciprofloxacin, GN= Gentamicin, S = Streptomycin, AML=Amoxycillin)
(+++) = sensitive; (++) = moderate sensitive; (-) = resistant

4. Discussion

In this experiment, 4 different types of bacteria were isolated from a total of 48 milk samples that were collected from cows. The isolated bacteria were *Staphylococcus spp. Streptococcus spp. Escherichia coli* and *Bacillus* spp. Among 48 milk samples, 17 were *Staphylococcus* spp. (35.41%), 9 were *Streptococcus* spp.(18.75%), 7 were *E. coli* (14.58%), 5 were B*acillus* spp. (10.41%), 5 were mixed organisms (10.41%), and 5 were unidentified organisms (10.41%) and Cultural examination expressed that *Staphylococcus* spp.(35.41%) to be predominant organisms followed by *Streptococcus* spp. (18.75%), *E. coli* (14.58%) and *Bacillus* spp. (10.48%).Our findings are in agreement with findings of Abdel-Radyand Sayed (2009) in which they isolated *Staphylococcus* spp., *bacillus* spp.. *and Escherichia coli* from the positive mastitis samples with prevalence 52.5%, 31.5% and 16.25% respectively. Similarly Mahbub-E-Elahi *et al.* (1996) reported that *Staphylococcus aureus* (31.33*%), Streptococcus* spp.(14%), *Eschecrichia coli* (16.00%), *Bacillus* spp. (4.67%) were responsible for mastitis. Rashad- Munir *et al.* (2003) isolated *staphylococci (Staphylococcus aureus*, *Staphylococcus epidermidis* 46.8%, 89 samples), *Streptococcus* spp. (18.4%), *Escherichia coli* (21.0%, 40 samples). Khan and Muhammad (2005) also report that *Staphylococcus aureus* showed the highest (45%) frequency followed by *Streptococcus* spp.(23%), *E. coli* (18%) and *Bacillus* spp. (14%) are responsible clinical mastitis. Above all reports are more or less similar this experiment. The frequency distributions of different species of bacterial isolates in different milk samples were found variable. Result of the present study indicates that all the three different types of bacteria were not present in the same milk sample collected from the clinical mastitis cows. *Staphylococcus* spp. have been recorded of the main pathogens of clinical mastitis in cows. The different isolates of *Staphylococcus* spp., *Streptococcus* spp., *E. coli and Bacillus* spp. showed identical results in different biochemical tests including sugar fermentation, catalase, methyl red and indole test. The actual causes for which the manifestation of an identical result in biochemical tests by the three groups of known identified isolates were not clear. It is not unlikely that almost all isolates in the present study possess some common genetic materials which are responsible for the manifestation of similar type of biochemical reaction as reported by Ahmed and Kanwal (2004). The in vitro antibiotic sensitivity test of two different types of bacterial isolates to 7 different antibiotics such as gentamycin, ciprofloxacin, erythromycin, ampicillin, amoxicillin, Streptomycin, enrofloxacin were studied. A major variation was noticed in the results of sensitivity of isolates against 7 different antibiotics used. Overall effective drugs against isolated organisms in order by ciprofloxacin, gentamicin and erythromycin but resistance isolates to amoxicillin, ampicillin, and streptomycin were observed. The variation in the sensitivity of common antibiotics could be the result of extensive and indiscriminate use of these in the treatment of udder infection. Maximum sensitive to gentamicin, ciprofloxacin, erythromycin, enrofloxacin might probably be due to its rare use in the treatment of clinical mastitis. Different bacteria isolated from clinical mastitis in this study at Saidpur upazila and Sadar upazila in Nilphamari district showed that

Staphylococci were the most common, followed by *Streptococcus* spp., *E. coli* and *Bacillus* spp. It is therefore, important for effective treatment of bovine mastitis, medicinal formulations should preferably contain antibiotics having good spectrum of inhibition against all species of bacteria. In this context, it is interesting to note that gentamicin, ciprofloxacin, erythromycin, enrofloxacin could be the antibiotics of choice and as such their use in the treatment of bovine mastitis in preference to conventional formulae is likely to yield the best possible result. Indeed, gentamicin or ciprofloxacin and enrofloxacin could cover most of the prevalent bacteria causing clinical mastitis. Therefore, these antibiotics appear to be promising for the treatment of clinical mastitis in Bangladesh. The results of isolation, identification, biochemical test, frequency distribution and antibiotic sensitivity of the bacteria isolated from milk of clinical mastitis of cows in the present study, indicated that the microbial factors play an important role for the development of clinical bovine mastitis. Detailed further epidemiological study about the extrinsic and intrinsic factors, which might have direct or indirect influence on the development of bovine mastitis in association with microbes are required.

5. Conclusions

Escherichia coli, *Staphylococcus* spp., *Streptococcus* spp. and *Bacillus* spp., were isolated from clinically mastitis affected dairy cows at Saidpur upazila and Sadar upazila in Nilphamari district of Bangladesh. Prudent use of antibiotics should be considered in dairy farm (where permissible) since many strains are resistant to common antibiotics as described in this study. Potential drug resistant pathogens in otherwise normal dairy may be a serious concern for public health. Current findings warrants further studies with the isolated strains of bacteria. The present study has demonstrated the existence of alarming levels of resistance of *Staphylococcus* spp. to commonly used antimicrobial agents in the study farms and the results are suggesting a possible development of resistance from prolonged and indiscriminate usage of some antimicrobials. It is therefore, very important to implement a systemic application of an *in vitro* antibiotic susceptibility test prior to the use of antibiotics in both treatment and prevention of intra-mammary infections.

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Conflict of interest

None to declare.

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