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Research Article

Characterization of bacteria causing bovine mastitis by amplified ribosomal DNA restriction analysis (ARDRA) method

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ABSTRACT

Mastitis is an important constraint that accounts for high economic loss in dairy farms across the world. The prevalence and distribution of mastitis in dairy cows in Bangladesh is well-documented while most of these farms are confronted with problems of clinical and subclinical mastitis. This research was conducted to assess the rapid detection of bovine subclinical mastitis by DNA based ARDRA method and to validate its efficiency to identify other associated organisms. Traditional biochemical tests such as California mastitis test (CMT) was used for initial screening followed by direct DNA extraction from 146 CMT positive milk samples (n=196) collected from 5 different dairy farms in Chittagong. Using the extracted DNA as template, amplification of 16s ribosomal DNA by previously described universal primers (27F and 1492R) was successfully achieved. Subsequent restriction digestion (with Hae-III) of PCR amplicons (n=51) revealed characteristic restriction pattern indicating six different groups of organisms in 38 cases where complete digestion was found. Further sequence analysis from corresponding PCR products and bioinformatic analysis revealed the identity of the responsible pathogens. Plasmid profile also was investigated to develop hypothesis associated with drug resistance patterns. The study shows that, in absence of sequencing facilities, ARDRA can be a useful approach to efficiently characterize the mastitis-causing bacteria indicating diagnostic implications.

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1. INTRODUCTION

Bovine mastitis is the single most common disease syndrome in adult dairy cows, accounting for about 38% of morbidity (Smith, 1996). Annual losses due to mastitis in the dairy industry was approximately 2 billion dollars in USA and 526 million dollars in India, in which subclinical mastitis are responsible for approximately 70% of these losses (Varshney and Naresh, 2004). Globally, the losses due to mastitis amount to about 53 billion dollars annually (Ratafia, 1987).

Subclinical mastitis is more common than clinical mastitis and is responsible for great economic losses in dairy herds (Jasper et al., 1982). If subclinical mastitis is not detected early, it may lead to clinical mastitis which is irreversible in most cases. SCM also represents a constant risk of infection for the whole stock of ruminants. Otherwise, contaminated milk

obtained from the affected cow is unfit for human consumption and provide a mechanism of spread of diseases like tuberculosis, sore-throat, brucellosis, leptospirosis etc. (Sharif et al., 2009). Mastitis is also associated with a number of zoonotic diseases in which milk acts as a vehicle of infection (Jenkins, 1982). So it is of great importance to diagnose subclinical mastitis at an early stage in order to reduce the economic losses caused by this disease in dairy industry and to protect the human consumers from the diseases caused by consumption of contaminated milk from mastitic milch cows.

Milk culture may yield no bacteria from truly subclinically infected glands due to the presence of very low numbers of pathogens when samples are collected (Cai et al., 2003). Negative cultures may also be due to bacteria inhibited by residual therapeutic antimicrobials or leukocytes. Environmental contaminants and intracisternal microorganisms can also represent a major problem in the interpretation of culture results. Moreover, microbiological culture of milk is time consuming. Species identification by standard biochemical methods requires more than 48h to complete (Phuektes et al., 2001).

The use of molecular methods has revolutionised their identification, by improving the quality and effectiveness of this identification due to limitations of conventional culture. Some of these methodologies use either the rDNA spacer region or its target. These techniques are useful for both of the identification and reliable detection of different bacterial species as well as the monitoring of the species (Blaiotta et al., 2008). Currently, there is a wide variety of molecular strategies, such as PCR with specific primers, DGGE, RAPD, PFGE, FISH, RFLP, and PCR-ARDRA (Morris et al., 2002).

However, the organisms involved in mastitis vary from community to community. More than 200 infectious causes of bovine mastitis are known to date (Kader et al., 2002; Sharma, 2008; Sudhan et al., 2005; Yong et al., 2009). So the aim of this study was to establishing a rapid and cost effective protocol for detecting mastitis causing bacteria by ARDRA method and identification of mastitis causing bacteria by DNA sequencing and developing a database for those bacteria. Also, to develop hypothesis associated with drug resistance patterns.

2. Materials and methods

This research work was conducted on bovine mastitis. Milch cows were randomly selected from four local dairy farms of Chittagong, Bangladesh, during the period of 2012-2013. In this study, a total of 196 milk samples were collected from 49 milch cows with subclinical mastitis based on the absence of visible abnormalities of milk secretions and positive California Mastitis Test (CMT).

Collection and transportation of samples

Before sampling, the teat end was scrubbed with cotton soaked in 70% ethanol. During sampling, the first squirt of milk was discarded. After doing CMT, milk samples from infected teats collected in sterile bottles, placed on ice and transported to Poultry Research and Training Centre (PRTC) laboratory of Chittagong Veterinary and Animal Sciences University (CVASU) for bacteriological examination and molecular characterization. All the vials containing milk samples were labelled with the respective identification number of cow.

California Mastitis Test (CMT)

Classical California mastitis test (CMT) was performed for initial screening of subclinical mastitis. The reaction was scored as trace (+), Weak Positive (++), Distinct Positive (+++), or Strong Positive (++++) and negative (normal), according to the amount of gel formed.

DNA Extraction from CMT positive Milk

DNA was extracted from CMT positive milk samples by modified method of (Romero and Lopez-Goñi, 1999). In this method, frozen milk was thawed at room temperature and 250 µl of sample were mixed with 100 µl of NET buffer and 50 µl of 24% sodium dodecyl sulphate (SDS). After incubation at 80^{o}C for 10 minutes, the mixture was cooled on ice instantly for 10 minutes. 15 µl proteinase k was added to the mixture, and then incubated at 50^{o} for 1.5 hours, followed by treatment with phenol: chloroform: isoamyl alcohol (25:24:1). The pellet was dried and dissolved in 25 micro litres of deionised water and stored at -20^{o}C.

Amplification of 16S rDNA

The 16S rDNA gene was amplified by PCR with a thermal cycler. DNA fragments of approximately 1.5 kbp were amplified using the following universal primers. (Lorena et al., 2010) Forward primer: 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), Reverse primer: 1492R (5'-GGTTACCTTGTTACGACTT-3'). All reactions were carried out in a final volume of 25 µl containg Template 4 (µl), GoTaq Green Master mix (Promega) Contain Taq polymerase, dNTP, MgCl2, buffer (12µl), Forward Primer (2µl), Reverse Primer (2µl), Nuclease free water (5µl). The cycling parametres were: (1) 94°C for 5 min, (2) 94^{o}C for 30 sec, (3) 55^{o}C for 30 sec, (4)

72^{0}C for 30 sec, 30 cycles of step 2 through 4 inclusive, and 72^{0}C for 7 min.

Electrophoresis was performed using Agarose (Promega®) and tris buffer (TAE) solution to visualize PCR product under UV-light (302 nm) in the 1.5% w/v agarose gel (25minutes, 80V) by addition of ethidium bromide.

Restriction digestion

PCR products were digested with Hae-III restriction Enzyme. In order to achieve complete digestion, restriction mixers (20 µl of final volume) will be incubated for 4 hours at 37°C (Lorena et al. 2010). Each reaction tube contained- PCR product (10 µl), 10x NE buffer (2 µl), 100 x Bovine Serum Albumin (1 µl), Hae-III Enzyme (1 µl), Distilled water (6 µl). The resulting digestion products was visualized under UV-light (302 nm) through agarose gel electrophoresis and creating a grouping according to restriction patterns.

PCR product clean up and sequencing

The PCR products of representative strains from each restriction group were purified with FavorPrep PCR Clean-Up Mini Kit. The sequencing performed on an ABI sequencer (ABI, USA) at the Centre for Advanced Studies and Research (CASR) in Biological Sciences at the University of Dhaka where commercial sequencing is routinely conducted.

Nucleotide Sequence Accession Numbers

The sequences were compared with the sequences retrieved from GenBank database using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/), edited and submitted to the GenBank database using the web-based data submission tool, BankIt (http://www.ncbi.nlm.nih.gov/BankIt/).

Plasmid extraction

Plasmid was extracted from CMT positive samples according to the previously described method (Sambrook and Russell, 2001).

CS (Culture sensitivity) Test

The isolated plasmid bearing samples were subjected to CS test and the zone of inhibition was measured with a millimeter scale and interpreted according to CLSI manual, 2010.

3. Results and Discussion

This research was conducted to assess the rapid detection of bovine mastitis by ARDRA method and the prevalence of bovine mastitis in different regions of Chittagong district of Bangladesh. In this regard, CMT screening was the initial step followed by direct DNA extraction from CMT positive milk, amplification of 16s ribosomal DNA by universal primers (27F and 1492R), restriction digestion of PCR positive samples, grouping according to restriction pattern then sequencing for characterizing mastitis causing bacteria. Finally plasmid extraction and CS test were performed to develop hypothesis associated with drug resistance patterns.

For California Mastitis Test milk samples from 49 milch cows (196 quarters) of different dairy farms in Chittagong were collected randomly and aseptically, and examined by CMT. 146 of the total samples showed CMT positive reaction which reflected the total prevalence of mastitis in Chittagong 74.49%. A significantly higher prevalence of mastitis was recorded in milch cows of Super dairy farm (83.33%) than the other dairy farms (QDF-75%; MDF-72.73%; KDF-67.86%) of Chittagong. Thus in total 74.49% of the milk samples were found to be associated with mastitis according to CMT test.

Milk samples from 50 quarters out of 196, were negative to CMT, while milk samples from 146 quarters showed various degrees of reactions in CMT. Milk samples from 20 quarters showed strong (4+) gel formation by CMT and it was about 10.20%. Milk samples from another 20 quarters showed distinct (3+) gel formation making the percentage (%) 10.20. In 40 cases of milk samples, the gel formation was weak (2+) and the percentage (%) was 20.41. 66 of the milk samples showed trace (1+) amount of gel formation in CMT test and it was about 33.67%.

The 16S rDNA gene was amplified by PCR using universal primers (27F and 1492R) (soto et al., 2009). Presence of mastitis causing bacteria was confirmed in 51 samples out of 146 extracted samples (CMT positive cases) through PCR, (Fig. 1).

The antibiotic susceptibility pattern was examined for 32 plasmid containing bacterial isolates by antibiotic disc diffusion method. As revealed, 87.50% and 81.25% bacteria isolated from CMT positive milk samples were resistant to Oxytetracycline and Doxycycline hydrochloride respectively, while Cephalexin showed maximum resistance (65.63%), with Erythromycin (15.63%), Amoxicillin (12.50%) and Ampicillin (9.38%). Least resistance (5.56%) was found towards Gentamycin (6.25%) and Ciprofloxacin (3.13%). The highest susceptibility was found toward Ciprofloxacin (96.87%). 81.25% of those isolates were susceptible to

Erythromycin, 75% to Amoxicillin, 59.37% to Ampicillin, 56.25% to Gentamycin, 31.74% to Cephalexin and 6.25% to Doxycycline hydrochloride and Oxytetracycline (Table 4).

Table 1: Prevalence of mastitis by CMT test

Source	Total No. of cases	No. of positive cases	No. of negative cases	Percentage of mastitis in herd (%)
Molla Dairy Farm	110	80	30	72.73
Quaderi Dairy Farm	16	12	4	75
Kader Dairy Farm	28	19	9	67.86
Super Dairy Farm	42	35	7	83.33
Total	196	146	50	74.49

Table 2: Enzyme cuts 38 from 51 PCR products in 6 patterns. In 13 PCR products no restriction digestion were found. Grouping of restriction pattern is presented below

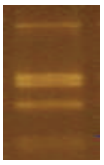
Type							
Sample No	3,4,8, 24,29,32, 33,34,35, 64,76, 133	27,37, 40,42, 46,140	26,65, 131,132, 135, 139	11,56	30,63, 142,146, 148	6,20,41,43, 134,137, 143	16,25,28, 31,36,38, 44,48,53, 82,124, 129,144

Table 3: Antibiotic sensitivity test of plasmid containing 32 bacterial isolates against several commercial antibiotic discs

Isolate	No. of antibiotics subjected to (b) = 8 Drug resistance patterns	No. of antibiotics to which the isolates were resistant (a)	MAR Index (a/b)
SM11	CN, DO, OT	3	0.375
SM 16	CN, DO, OT, GEN	4	0.50
SM19	AMP, CN	2	0.25
SM22	AMX, DO, OT	3	0.375
SM23	AMP, CN, AMX, DO, OT	5	0.625
SM24	CN, DO, OT	3	0.375
SM27	CN, E, DO, OT	4	0.50
SM28	DO, OT	2	0.25
SM34	CN, DO, OT	3	0.375
SM37	CN, DO, OT	3	0.375
SM53	CN, AMX, DO, OT	4	0.50
SM56	CN, DO, OT	3	0.375
SM60	DO, OT	2	0.25
SM63	E, DO, OT	3	0.375
SM64	CN, DO, OT	3	0.375
SM65	CN, DO, OT	3	0.375
SM67	CN, DO, OT, GEN	4	0.50
SM70	CN, DO, OT	3	0.375
SM76	CN, DO, OT	3	0.375
SM82	E, DO, OT	3	0.375
SM84	E, DO, OT	3	0.375
SM111	AMP, CN, AMX, DO	4	0.50
SM112	CIP, DO, OT	3	0.375
SM113	CN, DO, OT	3	0.375
SM147	CN, DO, OT	3	0.375
SM148	CN, E, DO, OT	4	0.50

MAR: Multiple Antibiotic Resistance, SM: Sample Milk, AMP: Ampicillin (25µg/disc), CN: Cephalexin (30µg/disc), CIP: Ciprofloxacin (5µg/disc), AMX: Amoxicillin (30µg/disc), E: Erythromycin (15µg/disc), DO: Doxycycline hydrochloride (30µg/disc), OT: Oxytetracycline (30µg/disc), GEN: Gentamycin (10µg/disc)

Table 4: Susceptibility of bacterial isolates to 8 different antibiotics

Antibiotic	Resistant R	(%)	Intermediate I	(%)	Susceptible S	(%)
AMP	3	(9.38)	10	(31.25)	19	(59.37)
CN	21	(65.63)	1	(3.13)	10	(31.74)
CIP	1	(3.13)	0	(0)	31	(96.87)
AMX	4	(12.50)	4	(12.50)	24	(75.00)
E	5	(15.63)	1	(3.13)	26	(81.25)
DO	26	(81.25)	4	(12.50)	2	(6.25)
OT	28	(87.50)	2	(6.25)	2	(6.25)
GEN	2	(6.25)	12	(37.50)	18	(56.25)

AMP: Ampicillin (25µg/disc), CN: Cephalexin (30µg/disc), CIP: Ciprofloxacin (5µg/disc), AMX: Amoxicillin (30µg/disc), E: Erythromycin (15µg/disc), DO: Doxycycline hydrochloride (30µg/disc), OT: Oxytetracycline (30µg/disc), GEN: Gentamycin (10µg/disc)

Table 5: BLAST search results after searching against non redundant database

Group No.	Sample No.	Name of Bacteria	Accession No.
Group-1	C-1 (sm-11)	*Streptococcus parauberis (95%)*	JN630844
	C-9 (sm-56)	*Streptococcus parauberis (64%)*	JQ945266
Group-2	C-2 (sm-20)	*Micrococcus luteus (81%),*	AB079788,
		Macrococcus caseolyticus (81%),	KC212014,
		Staphylococcus sciuri (40%)	JX519590.
	C-7 (sm-43)	*Streptococcus agalactia (62%)*	KC510212
Group-3	C-3 (sm-26)	*Streptococcus uberis (89%),*	NR074912,
		Streptococcus pseudoporcinus (89%),	JN57842,
		Streptococcus porcinus (89%),	JN578462,
		Streptococcus seminale (83%),	AB370975,
		Streptococcus paruaberis (90%),	JQ945266,
		Streptococcus ictaluri (89%),	DQ462419,
		Streptococcus iniae (90%)	EU622512.
	C-11 (sm-65)	*Streptococcus dysgalactia (55%)*	KC510219
Group-4	C-4 (sm-29)	*Staphylococcus chromogenes (79%),*	HM367779,
		Staphylococcus hyicus (79%),	KC212040,
		Staphylococcus agnetis (79%),	HM484986,
		Staphylococcus microti (79%),	EU888120,
		Staphylococcus haemolyticus (81%),	HE800828,
		Staphylococcus aureus (43%),	JN687472,
		Staphylococcus pasteuri (43%),	AY857686,
		Staphylococcus saprophyticus (44%)	JF935125.
	C-12 (sm-76)	*Staphylococcus aureus (67%)*	KC494367
Group-5	C-5 (sm-30)	*Streptococcus urinalis (92%),*	JQ307003,
		Streptococcus canis (92%),	JX876612,
		Streptococcus dysgalactia (92%),	EU075034,
		Streptococcus agalactia (93%),	HQ658089,
		Streptococcus uberis (54%),	AB370974,
		Streptococcus ictaluri (54%),	DQ462419,
		Streptococcus pyogenes (54%)	NR074091.
	C-10 (sm-63)	*Streptococcus dysgalactia (67%),*	KC510218,
		Streptococcus ictaluri (67%),	DQ462419,
		Streptococcus iniae (67%),	DQ462419,
		Streptococcus agalactia (61%),	JQ039376.
Group-6	C-6 (sm-40)	*Streptococcus agalactia (78%)*	HQ658089
	C-8 (sm-46)	*Streptococcus agalactia (76%)*	HQ658089

Figure Legends

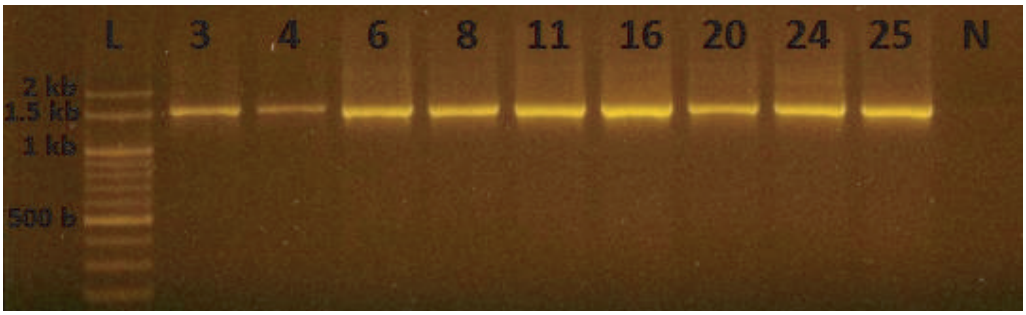


Fig. 1. Representative samples after electrophoretic (1.5% Agarose) separation of 16s rDNA gene fragment, amplicon size: ≈ 1500 bp, L = Ladder (marker), N = Negative Control

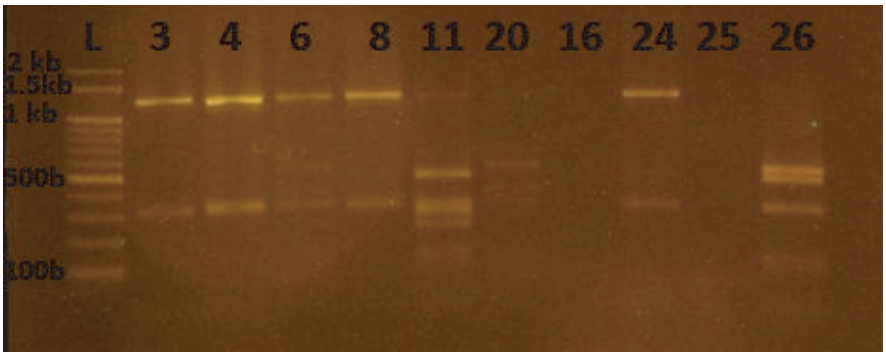


Fig. 2. Representative samples after electrophoretic (1.5% Agarose) separation of restricted gene fragment, L = Ladder (marker)

Twenty six out of thirty two bacterial isolates showed multiple antibiotic resistances. Among these, one isolate resisted five types of antibiotics. Six isolates resisted four types of antibiotics. Sixteen isolates resisted three types of antibiotics and two types of antibiotics were resisted by three isolates (Table 3 and 4). The MAR Index of an isolate is defined as a/b, where a represents the number of antibiotics to which the isolate was resistant and b represents the number of antibiotics to which the isolate was subjected (Sathishkumar et al., 2012). From the result of CS test it has been found that 26 of these 32 plasmid containing isolates are multiple drug resistant (MDR). One of the rest 6 isolates is susceptible to all of the tested antibiotics. Plasmids with two different sizes were observed. The size of the plasmids was found to be >10 kb.

The PCR product was sent for DNA sequencing and partial sequences of 12 bacteria were found. The sequence data were used to conduct BLAST analysis (in the NCBI website, http://blast.ncbi.nlm.nih.gov/Blast.cgi), to characterize the bacteria. From the search result, we found that C-1, C-9 have same restriction pattern and they match only to Streptococcus paruaberis (JN630844=95% and JQ945266=64%). C-6, C-8 have same restriction pattern and they match only to Streptococcus agalactia (HQ658089, C-6=78% and C-8=76%). C-7 match to Streptococcus agalactia (KC510212=62%), C-11 match to Streptococcus dysgalactia (KC510219-55%), C-12 match to Staphylococcus aureus (KC494367=67%). Remaining C-2, C-3, C-4, C-5, C-10 samples match to different bacteria at different percentage. It may be due to partial sequence and presence of multi-infections (Table 5).

Six sequences from each group (C-1, C-3, C-4, C-5, C-6, and C-7) were submitted to the GenBank database using the web based data submission tool, BankIt (http://www.ncbi.nlm.nih.gov/BankIt/) and got the accession numbers, are KF055826, KF055827, KF055828, KF055829, KF055830, and KF055831, respectively.

The ARDRA protocol described in this paper allowed the identification of a high number of isolates avoiding subculturing. Compared to the existing procedures, the

ARDRA here described allowed a very good and rapid identification of the species in the subclinical mastitis; this potential of identification was confirmed by 16S-rRNA gene sequencing. The use of PCR-ARDRA for bacterial identification offers a considerable potential as rapid method and combines speed and reliability features.

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