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Seasonal Prevalence and Antibiogram Profile of Bacterial Isolates from Bovine Mastitis

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ABSTRACT

The current study examines the prevalence and seasonal occurrence of major bacterial pathogens and effect of different antibiotics on bacteria isolated from 935 bovine mastitic milk samples in northern region of India for characterization and culture sensitivity against different antibiotics. A major proportion of mastitis samples- 412 (44%) were recorded during rainy season, while, samples in equal shares, 276 (28%) and 247 (27%) were documented in winter and summer seasons, respectively. Out of total 935 mastitic milk samples, 889 (95%) samples showed microbial growth, while, 46 (5%) samples were found negative for any bacterial growth. Among total isolated bacteria, Gram positive- 471 (53%) shared a major proportion, followed by Gram negative 341 (38%), while a small part of 53 (6%) and 24 (2%) samples yielded mixed unidentified cultures and Candida species, respectively. In antibiogram study, gentamicin (91.21%), ciprofloxacin (89.60%), enrofloxacin (88.28%) and tetracycline (71.30%) were found to be highly effective antibiotics, while, penicillin (86%), colistin (83.30%), cloxacillin (78.62%), amoxycillin (70.71%) and ampicillin (62.51%) showed least effect against both Gram positive and negative bacteria. The present study showed that there was close association between season, bacterial pathogens and occurrence of of bovine mastitis. Overall, *Staphylococcus* spp., *Streptococcus* spp. and *E. coli* contributed as major mastitis dweller bacteria.

Keywords: Mastitis, bovine, prevalence, antibiogram, antimicrobial resistance

Mastitis is a condition of intra-mammary infection that affects thousands of animals worldwide and leads to huge economic losses (Lightner *et al.* 1988). Being a most economically damaging disease, mastitis severely reduces milk yield, profit margins and affects the quality of milk and milk products in all dairy-producing countries. India is the largest producer of milk in the world and tolerates around INR 16,702/- million losses per annum due to mastitis (Yathiraj, 2006). Clinically, mastitis varies from subclinical to a severe acute febrile clinical form (Lilus and Pesonen, 1990). Mastitis has multi-etiological nature and caused by many bacterial species which include Gram positive; *Staphylococcus* spp., *Streptococcus* spp., *Arcanobacterium* spp., *Bacillus* spp., *Micrococcus* spp., *Mycobaceteria* spp. and Gram negative *Escherichia coli, Klebsiella* spp., *Pseudomonas* spp., *Serratia* spp., *Pasteurella* spp., *Enterobacter* spp., *Leptospira* spp. and *Yersinia* spp. Other organisms like *Mycoplasma* spp., fungi, yeasts, virus and algae are also responsible for mastitis.

Bacteriological examination of mastitis milk and Culture Sensitivity Test of pathogenic bacteria is an important and helpful procedure for mastitis diagnosis and management. Timely and correct disease diagnosis along with identification and antimicrobial resistance pattern of mastitis pathogens helps to prevent major economic

losses occurring due to mastitis in worldwide. Antibiotic resistance among bacterial pathogens is a major challenge of disease control in modern days; therefore, it require pre-knowledge of bacterial pathogens antibiogram profiles for proper treatment of mastitis.

MATERIALS AND METHODS

Study area

Milk samples were collected from mastitis affected cows in district Kangra and adjoining areas of Himachal Pradesh, India. The Kangra is a North-Western Himalayan region having altitude ranging from 427 to 6401 metre with average elevation around 730 metre above mean sea level. The major climatic zones are sub-humid, sub-tropical, humid sub-tropical and wet temperate and temperature ranges from 2-38°C. The samples were collected in winter (October-February), summer (March-June) and rainy (July-September) season. Agriculture and animal husbandry are prime occupations with cattle contributing a major share to the the total livestock population of the region.

Sampling

Between years 2012 to 2014, milk samples from each mastitis affected cows were collected aseptically in 5 ml sterilised vials from Teaching Veterinary Clinical Complex, veterinary hospitals, livestock farms and individual livestock owners. Samples were transported on ice pack and processed within 6 hours for bacterial isolation, characterization and antibiotic culture sensitivity test (CST).

Bacteriological analysis

All bacteriological media used were purchased from HiMedia, Mumbai. Mastitic milk samples were processed for bacterial growth by inoculating 10 µl of inoculum on 5% sheep Blood Agar (BA) and MacConkey Lactose Agar plates. All plates were incubated aerobically at 37°C for 24–48 hours and were examined for growth, haemolysis, colony morphology and cultural characteristics. Plates were considered negative to culture, if no growth occurred within 72 hours. Further, bacterial isolates were examined for their staining affinity, morphological and biochemical characters according to the standard methods as described by Quin *et al.* (2002).

Antimicrobial sensitivity test

Antibiotic sensitivity test for isolated bacteria was performed on Muller Hilton Agar (MHA) of pH 7.0 and 5% BA for Streptococcus spp. using in-vitro disc diffusion method described by Baur et al., (1996). Overnight grown bacterial colonies were suspended in nutrient broth and incubated at 37°C to obtain the turbidity equivalent to a 0.5 McFarland standard. Bacterial suspension (100 µl) was spread over the MHA plate and antibiotic discs were transferred aseptically on the surface of inoculated medium. Results were recorded after 12-24 hours incubation at 37°C. The efficacy of antibiotics was determined by measuring the diameter of zone of inhibition. The concentrations of antibiotics used were Amikacin (30 µg), Amoxicillin (10 µg), Ampicillin (30 µg), Cephalexin (30 µg), Cefuroxime (30 µg), Colistin (10 µg), Ciprofloxacin (5 µg), Cephadroxil (30 µg), Cloxacillin (30 µg), Enrofloxacin (30 µg), Gentamicin (120 µg), Ofloxacin (30 µg), Penicillin (10 units), Polymixin-B (300 units), Streptomycin (20 µg) and Tetracycline (30 µg) per disc.

Statistical Analysis

Collected data were analysed for statistical significance and the significant seasonal dominance of collected samples and isolated pathogens was determined by Chi square test with $p < 0.05$ significant level, while one tailed t-test with un-equal variance and with $p < 0.05$ significance level was used to analyse the seasonal variance among isolated pathogens.

RESULTS

A total of 935 milk samples from affected animals were processed bacteriologically in three seasons between years 2012 to 2014. Out of total samples processed, a major proportion i.e. 412 (44%) samples were processed in rainy season while samples about in equal proportions, 276 (28%) and 247 (27%) were processed in winter and summer seasons, respectively (Fig.1).

More number of samples were processed during rainy season, i.e. July (150), August (167) and September

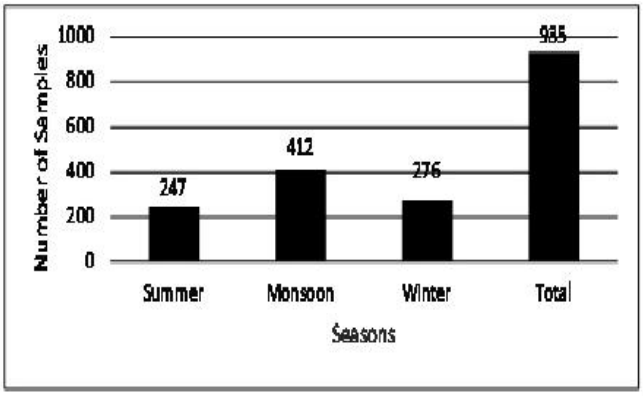

Fig. 1: Total number of samples processed in three different seasons

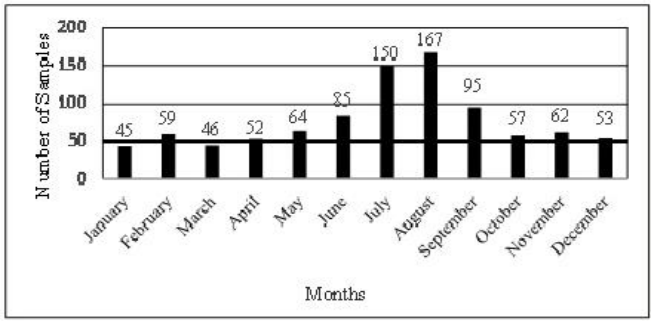

Fig. 2: Month-wise collection of mastitis samples in different years

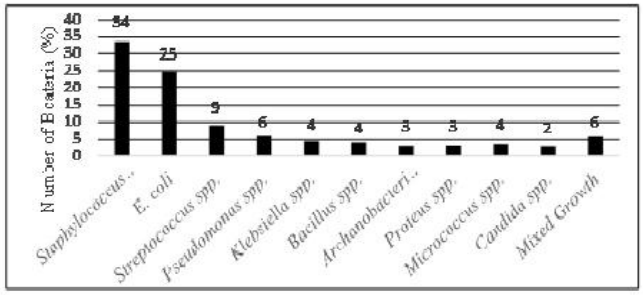

Fig. 3: Percentage recovery of different bacterial species from processed mastitis samples

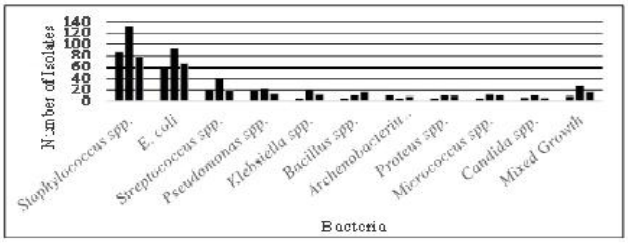

Fig. 4: Seasonal occurrence of different mastitis pathogens

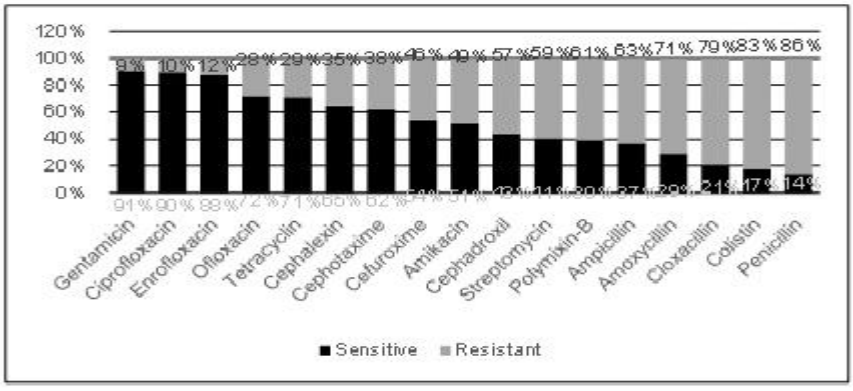

Fig 5: CST profile of different antibiotics against bacteria isolated

(95) as compared to winter, i.e. October (57), November (62), December (53), January (45) and February (59) and summer, viz. March (46), April (52), May (64) and June (85). Occurence of isolated bacteria in three different seasons showed many of bacteria were encountered in rainy season as compared to other two seasons which have

little difference. The difference of occurrence in rainy season as compared to summer and winter was higher for Staphylococcus spp., E. coli and Streptococcus spp. than other isolated bacteria.

Out of 935 mastitis samples, 889 (95%) samples showed bacterial growth, out of which, 471 (53%) were Gram positive bacteria, 341 (38%) were Gram negative bacteria, 53 (6%) were un-identified (mixed) bacterial species and 24 (2%) belongs to *Candida* spp. Staphylococcus spp. 299 (34%) were the most encountered bacterial species among Gram positive bacteria, followed by Streptococcus spp. 81 (9%), Bacillus spp. 35 (4%), Micrococcus spp. 31 (4%) and *Arcanobacterium pyogenes* 25 (3%), while among Gram negative bacteria *E. Coli* 221 (25%) was the most prevalent, followed by *Pseudomonas* spp. 55 (6%), *Klebsiella* spp. 39 (4%) and *Proteus* spp. 26 (3%) shown in Fig. 4. *Streptococcus* spp. accounted for 9% of the total isolated pathogens. The study documented higher incidences (41/81) of *Streptococcus* spp. in rainy season as compared to winter (20/81) and summer (20/81).

Antimicrobial susceptibility against all the bacterial isolates was higest for gentamicin (91%), followed by ciprofloxacin (90%), enrofloxacin (88%), ofloxacin (72%), tetracyclin (71%), cephalexin (65%), cephotaxime (62%), cefuroxime (54%), amikacin (51%), cephadroxil (43%), streptomycin (40%), polymixin-B (39%), ampicillin (37%), amoxicillin (29%), cloxacillin (21%), colistin (17%) and penicillin (14%).

On Chi square test ($p<0.05$) the significant values were found for total collected samples in three different seasons were 0.85 in summer, 0.86 in monsoon and 0.99 in winter, while for isolated mastitis pathogens a significance of 0.25, 0.78 and 0.52 for summer, monsoon and winter was observed in respective manner. One tailed t-test with un-equal sample variance ($p<0.05$) was applied to analyse the seasonal variance among isolated pathogens. A highly significant variation of 0.0005 and 0.001 was found when samples of summer/monsoon and monsoon/winter were compared, while no significance variation (0.223) was observed for summer/winter samples.

DISCUSSION

Total of 935 mastitic milk samples were processed bacteriologically for the years 2012 to 2014 and data were obtained for seasonal occurrence of bovine mastitis and associated pathogens. All the bacterial isolates, 889 in total, were subjected to *in-vitro* antimicrobial susceptibility testing using disc diffusion method (Bauer *et al.,* 1966).

Comparatively, a higher proportion of samples (412, 44%) were processed during rainy season in comparison to winter (276,29%) and summer (247, 27%). These findings are in consonance with the findings of Dhakal *et al.* (2007) who reported an incidence of bovine mastitis around 37.3% during rainy season. The possible reason for increased incidences of mastitis cases during the rainy season is humid weather, higher calving rate and peak lactation. Due to high lactation, the immunity of mammary glands decreases and become highly susceptible for bacterial pathogens. Contrarily, Ranjan *et al.* (2011) showed less (7.37%) number of cases of mastitis in a single year study conducted in Jharkhand state of India.

In this study, Gram positive bacteria were the predominant pathogens (471, 53%), followed by Gram negative bacteria (341, 38%). Of the recovered isolates, *Staphylococcus* spp. (34%), followed by *E. coli* (25%) and *Streptococcus* spp. (9%) were the predominant pathogens, whereas, Arcanobacterium pyogenes and *Proteus* spp. were the least pathogens isolated. Therefore, *Staphylococcus* spp. are widely accepted as a major cause of mastitis. The findings of this study was closely resembles those of Bedada *et al.* (2011) and Sumathi *et al.* (2008). A previous study conducted by Sharma *et al.* (1993) also showed higher prevalence of Staphylococcus spp. *Staphylococcus* spp. because these organisms bind to epithelial cells of the ductile and alveoli in mammary glands and are major inhabitant of infected udder skin and milk (Olmsted and Norcross, 1992). In current study, *E. coli* (25%) and Pseudomonas spp. (6%) were the most prevalent Gram negative bacteria. Similarly in a study conducted by Ranjan *et al.* (2011) reported 9% and 8% prevalence of *E. coli* and *Pseudomonas* spp., respectively from 190 mastitis samples. Nagal *et al.* (1999), however, reported lower prevalence for both these bacteria and instead *Streptococcus* spp. were encountered more. Poor hygienic conditions lead to mastitis due to *E. coli* (Sumathi *et al.,* 2008) and *Pseudomonas* spp. (Redaelli and Perini, 1960), which suppress streptococcal mastitis.

This study found that more than 50% of the mastitis in this region is caused by two major pathogens, i.e. *Staphylococcus* spp. and *E. coli*. Sharma *et al.* (1993) and (Sharma and Prasad, 2003) also reported a higher prevalence (>50%) of *Staphylococcus* spp.. Previous studies to decipher the etiology of mastitis from Asian countries has revealed that *Staphylococcus* spp. and *E. coli* together share 60-90% proportion of total prevalence of isolated mastitis pathogens (Sharma, 2012). Contagious staphylococci adapted to survive in host mammary glands and spread subclinical intra-mammary infections from cow to cow and respond poorly to antimicrobial therapy in chronic mastitis. Staphylococcus aureus can transmit readily through the lactating herd despite excellent hygiene at the time of milking and mastitis control procedures (Smith *et al.,* 1998). There are different *E. coli* genotypes with unknown pathogenicity mechanisms which are responsible for subsequent intra-mammary infections in same animal repeatedly (Dogan *et al.,* 2006).

Of all the streptococcal isolates, almost 50% were recovered during rainy seasons and rest 25% each during winter and summer. Buddle *et al.* (1988) found higher incidences of S. uberis during winter with high rain fall and low average day temperature. Unhygienic conditions often lead to Streptococcal intra-mammary infection in lactating and non-lactating cows. Streptococci infection often remains subclinical during long periods of time and in the absence of treatment, this causes serious losses in milk production (Khan *et al.,* 2003).

Present study reportes 6% incidences of Pseudomonas spp. which is an environmental contaminant, the most common means for infection are contaminated water used to wash the teats and contaminated antimicrobial agents and clinical equipment's in mastitis cure (Frank, 1997). Data show that intra-mammary Pseudomonas infections often lead to outbreaks in dairy cow herds (Malmo *et al.,* 1972; Erksine, 1987 and Daly, 1999).

Klebsiella spp. recognised as the most common bovine mastitis causing coliforms along with *E. coli* and becoming more common cause of mastitisin dairy animals. Around 4% isolates of *Klebsiella* spp. in the present study is similar to the studies of Dhakal *et al.* (2007) and Ranjan *et al.,* (2011). Feaces, contaminated water, soil, sawdust and shaving are thr main reservoirs of *Klebsiella* spp. and causes teat canal infection.

Among the isolated mastitis pathogens, 3% were Arcanobacterium pyogenes. A. pyogenes cause summer mastitis and frequently found with other pathogens. Cow to cow transmission of intra-mammary infection due to A. pyogenes are facilitated by flies, dry period and wet environmental conditions. Out of 17 isolates of A. pyogenes, 9 were isolated in summer season.

Antibiogram study revealed gentamicin (91%) to be most effective drug, followed by ciprofloxacin (90%), enrofloxacin (88%), ofloxacin (72%), tetracycline (71%), cephalexin (65%), cephotaxime (62%), cefuroxime (54%) and amikacin (51%) against these microbes. Similar antibiogram pattern were reported by Bedada *et al.,* 2011 and Kumar *et al.,* 2002. The highest efficacy of gentamicin, ciprofloxacin, enrofloxacin, and ofloxacin could be due to less commonly used before and employed recently for mastitis treatment in the area of investigation. Gentamicin, ciprofloxacin and enrofloxacin proved to be the antibiotics of choice in this study. Similar findings were also reported by Dhakal *et al.,* 2007 and Kumar and Sharma, 2002. Large number of isolates showed resistance to penicillin (86%), colistin (83%), cloxacillin (79%), ampicillin (63%), polymixin-B (61%), streptomycin (60%), and cephadroxil (57%). These results are in consonance with the observations published by Dhakal *et al.* (2007) and Sumathi *et al.* (2008). The high resistance to penicillin is in accordance with the results reported by Aarestrup *et al.* (1995), where 75% of the isolated bacteria were resistant to this antibiotic due to its indiscrimate use and production of plasmids mediated beta-lactamase enzymes. Similar observations were also made for polymixin and streptomycin (Sumathi *et al.,* 2008). Mastitis is the single most common animal disease due to which highest amounts of antibiotics are used in dairy farming. The high use of antibiotics in dairy farming is a major cause of antimicrobial resistance among animal pathogens and are responsible for food borne and zoonotic diseases. In order to reduce the antibiotics load in mastitis treatment, proper hygienic condition, appropriate feeding, housing and milking practices must be adopted. Affordable, effective vaccines against major mastitic pathogens such as staphylococci and *E. coli* could result in alleviating the economic losses and prevent spread of antibiotic resistance.

CONCLUSIONS

Current study concludes higher number of bovine mastitis incidences as monsoon gives rise to unhygienic conditions in cattle shed or farms. *Staphylococus* spp. (34%) were found major inhabitant of infected bovine udder milk, followd by *E. coli* (25%). Gentamicin (91%) and ciprofloxacin (90%) were the most effective drugs.

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