Research Article

Bacteriology, Antimicrobials Resistance and Susceptibility Profiles of Bacteria from Mastitic Cattle and Buffaloes of Khyber Pakhtunkhwa

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Abstract | Mastitis is one of the major prevailing and costly diseases in dairy cattle and buffaloes. This study describes the current prevalence of mastitis in lactating cattle, and buffaloes in Khyber Pakhtunkhwa during 2019-2020, to identify bacterial pathogens of mastitis, and to determine the antimicrobial resistance. A total of 1013 milk samples belonging to 726 cattle and 287 buffaloes were examined to detect clinical mastitis (CM) and subclinical mastitis (SCM). Mastitic milk samples (*n*=541) were further used to investigate the prevalence of bacteria associated with mastitis and their antimicrobial susceptibilities by standard disc diffusion assay. The results showed that the prevalence of CM and SCM was 21.2% and 62.3% in cattle, while 17.8% and 68.9% in buffaloes, respectively. Additionally, it was noted that CM was higher in cattle, while SCM was higher in buffaloes. Amongst the bacterial isolates from mastitic milk the prevalence of *Staphylococci* spp. (24.6%) was at highest, which was followed by *Escherichia coli* (23.7%), then *Streptococci spp*. (8%) *Salmonella spp*. (5.9%), *Klebsiella spp*. (5.7%), *Candida spp*. (4.1%), and *Proteus spp*. (1.5%). The isolated bacteria were mostly resistant to ampicillin (96.5%), sulphamethoxazole (96.5%), streptomycin (95.1%), oxytetracycline (85%), and amoxicillin (78.1%). However, the isolates were highly susceptible to enrofloxacin (86.2%), gentamicin (83.5%), and florfenicol (82.6%). This study might be helpful to the clinicians and researchers associated with the dairy industry for designing prophylactic as well as therapeutic strategies for the control of bovine mastitis.

Received | March xx, 2021; **Accepted** | April 11, 2022; **Published** | September 21, 2022
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Citation | Ali, T., Kamran, A. Raziq, I.U. Wazir, A. Ali, M.I. Ali M.S. Khan, S. Ullah and S.H. Khan. 2022. Bacteriology, Antimicrobials resistance and susceptibility profiles of bacteria from mastitic cattle and buffaloes of Khyber Pakhtunkhwa. *Sarhad Journal of Agriculture,* 38(4): 1240-1245.
DOI | https://dx.doi.org/10.17582/journal.sja/2022/38.4.1240.1245
Keywords | Mastitis, Bacteriology, Antimicrobials susceptibility, Cattle, Buffaloes

Introduction

Bovine mastitis (inflammation of mammary gland) is one of the most prevailing and economically destructive diseases affecting dairy animals across the globe (De Vliegher *et al*., 2012; Mbindyo *et al*., 2020), including Pakistan (Bachaya *et al*., 2011; Bhat *et al*., 2017; Ali *et al*., 2021). Bovine mastitis imparts huge financial losses, adversely affecting animal's health and production, and welfare; besides, it deteriorates

the milk quality and quantity capacities (De Vliegher *et al*., 2012). Bacterial pathogens are the major causing agents of mastitis. In addition, traumatic and mechanical injuries to the udder are also minor causes of mastitis (Bradley *et al*., 2002). Approximately 135 different bacterial species have been isolated from mastitic animals; however, about 20 types of bacterial pathogens are reported to be mostly associated with mastitis in dairy cattle (Bradley *et al*., 2002; Gao *et al*., 2017; Ali *et al*., 2021). Amongst the bacterial mastitis causing pathogens, coagulase-positive and –negative *staphylococci*, *streptococci spp*. *Escherichia coli, Klebsiela spp*. and *Salmonella spp*. are the main bacteria associated with mastitis (Ali *et al*., 2016; Yang *et al*., 2020; Aslam *et al*., 2021).

Different antimicrobials are commercially used to treat bacterial infections in livestock including intramammary bacterial infections. It has been reported that reproductive and mammary gland diseases are the main reasons to use antimicrobial agents in dairy animals for prolonged and frequent uses (Nobrega *et al*., 2017). These conditions paved the way to produce multi-drug resistant (MDR) bacterial pathogens (Ali *et al*., 2016; 2017). There is always a need of robust antimicrobial resistance surveillance systems of pathogenic bacteria from various sources (WHO, 2014). However, because of the economic constraints, national surveillance programs including monitoring systems of mastitis, the associated bacteria and their resistance profiles, are not present in Pakistan. Although, some regional studies were conducted on these aspects to fill the gaps (Mustafa *et al*., 2011; Ali *et al*., 2014; 2021). Thus, the current study was conducted with the aims to investigate the current prevalence of mastitis in lactating cattle, and buffaloes (*Bubalus bubalis*) in Khyber Pakhtunkhwa during 2019-2020, to detect bacterial pathogens associated with mastitis, and to evaluate their antimicrobial resistance.

Materials and Methods

Ethical statement

To conduct this study, ethical and technical guidance was obtained from the ethical and technical committee of the Veterinary Research Institute (VRI) Peshawar, Pakistan. Milk samples were obtained from cattle and buffaloes as per the guidelines of the National Mastitis Council (NMC) with the prior consent of the owners and without any harm to the animals.

Sampling and Processing

A total of 1013 milk samples were either collected directly from the animals or brought by the owners/farm managers to detect mastitis. Samples were processed at the Center of Microbiology and Biotechnology (CMB), VRI Peshawar, Pakistan during July, 2019 to June, 2020. Aseptic milk samples were collected from individual small farmers with 01 to 03 cattle or buffaloes. Dairy farmers were guided for the collection of aseptic samples from their animals as per NMC protocols. A total of 1013 milk samples from 726 cattle and 287 buffaloes of different areas of Khyber Pakhtunkhwa (KPK) were brought to our Mastitis Laboratory for detection of clinical mastitis (CM) and subclinical mastitis (SCM) as shown in Table 1 and Table 2. CM was detected with the help of visual observations as well as from the abnormal changes in milk (Ali *et al*., 2014). While, California mastitis test (CMT) kit (ImmuCell, Portland, ME) was used to diagnose SCM.

Bacteriology and antimicrobial resistance profiling of mastitic milk samples

A total of 541 samples from mastitic animals were further cultured to identify the bacterial pathogens. The identification of bacterial isolates was carried out up to the level of genus as described earlier (Ali *et al*., 2021). In addition, antimicrobial resistance and susceptibility profiling of the isolated bacteria from mastitic cattle and buffaloes was carried out on Muller-Hinton agar (Oxoid™, Thermo Scientific Inc. USA). Antimicrobial susceptibility testing of the bacterial isolates from mastitic milk samples was performed on Mueller-Hinton agar (Oxoid™) by the Kirby-Bauer disc diffusion assay according to the recommendations of the Clinical Laboratory Standard Institute (CLSI, 2014). Fourteen different antimicrobial agents were tested, including ampicillin, amoxicillin, augmentin, oxytetracycline, gentamicin, streptomycin, kanamycin, norfloxacin, enrofloxacin, sulphamethoxazole, doxycycline, erythromycin, florfenicol and flumequine.

Results and Discussion

Prevalence of bovine mastitis

The results of this study showed 21.2% and 62.3% of overall prevalence of CM and SCM in cattle and 17.8% and 68.9% in buffaloes, respectively (Table 1**)**. Thus, CM was highest in cattle (21.2%) as compared to buffaloes (17.8%); however, prevalence of SCM

Table 1: *Species-wise prevalence of mastitis in cattle and buffaloes of Khyber Pakhtunkhwa.*

Species	Sample processed	Clinical mastitis		Subclinical mastitis		Negative sample		Blood in Milk	
		n	%	n	%	n	%	n	%
Cattle	726	156	21.2	452	62.3	118	16.3	92	12.7
Buffaloes	287	51	17.8	198	68.9	38	13.2	15	5.2
Total	1013	207	20.4	650	64.2	156	15.4	107	10.6

Table 2: *Area-wise prevalence of subclinical mastitis (SCM) and clinical mastitis (CM).*

Areas	Cow							Buffaloes						
	Total	SCM		CM		Neg.		Total	SCM		CM		Neg.	
	n	n	%	n	%	n	%	n	n	%	n	%	n	%
Peshawar	479	296	40.8	105	14.5	78	66.1	245	176	61.3	44	22.2	25	08.7
Charsada	93	60	08.3	18	11.5	15	12.7	21	11	03.8	05	02.2	05	02.2
Nowshera	26	21	02.9	03	01.9	02	01.7	09	06	03.0	00	00.0	03	01.1
Khyber	49	35	04.8	08	05.1	06	05.1	05	00	00.0	01	0.30	04	01.4
Sawbi	11	09	04.8	01	00.6	01	00.1	03	03	01.1	00	00.0	00	00.0
Mohmand	31	17	02.3	08	05.1	06	05.1	00	00	00.0	00	00.0	00	00.0
Mardan	23	06	00.8	08	05.1	09	07.6	02	01	0.30	00	00.0	01	0.30
Other*	14	08	01.1	05	03.2	00	00.0	02	00	00.0	01	0.30	00	00.0
Total	726	452	62.3	156	21.2	118	16.3	287	198	69.0	51	17.8	38	13.2

other areas include Bajuar, Kohat and Karak

Table 3: *Bacteriology of mastitic milk from cattle and buffaloes.*

Bacteria isolated	Numbers (n)	Percentage (%)
Staphylococci spp	133	24.6
Escherichia coli	128	23.7
Streptococcus spp.	48	8.9
Klebsiella spp.	31	5.7
Salmonella spp.	32	5.9
Proteus spp.	08	1.5
Candida spp.	22	4.1
Culture Negative	161	29.8
Total samples	541	00

Note: High prevalence of Staphylococcus spp. (24.6%) was found which was followed by Escherichia coli (23.7%).

was highest in buffaloes (68.9%) in comparison to cattle (62.3%). Additionally, 12.7% of milk samples of cattle were mixed with blood, and 5.3% blood-mixed milk was observed in buffaloes. Table 2 shows the area-wise prevalence of CM and SCM in cattle and buffaloes of different areas of KPK. In districts Peshawar and Charsada, the topmost prevalence of CM and SCM was noted in cattle and buffaloes.

Bacteriology of mastitic milk samples

Isolation of different bacterial pathogens from mastitic dairy animals is presented in Table 3. Amongst the bacterial isolates from mastitic milk, *Staphylococci* spp. (24.6%) were the main prevailing bacteria. Besides, the other bacterial isolates included *E. coli* (23.7%), *Streptococci spp.* (8%), *Klebsiella spp.* (5.9%), *Candida spp.* (4.1%), and *Proteus spp.* (1.5%). Additionally, no bacterial growth was observed in 29.8% of samples from mastitic cattle and buffaloes.

Antimicrobial resistance and susceptibility of bacterial pathogens

Table 4 reveals the antimicrobial resistance and susceptibility profiles of different bacterial pathogens against fourteen antimicrobial agents. The isolated bacteria were highly resistant to ampicillin (96.5%), sulphamethoxazole (96.4%), streptomycin (95.1%), oxytetracycline (85%), and amoxicillin (78.1%). In addition, these isolates were mainly sensitive to enrofloxacin (86.2%), gentamicin (83.5%), and florfenicol (82.6%).

This study reported an overall prevalence of 21.2% and 62.3% of clinical mastitis (CM) and subclinical mastitis (SCM) in cattle; however, it was 17.8% and 68.9% in buffaloes of different areas of KPK. Thus, CM was highest in cows in comparison to buffaloes however SCM was highest in buffaloes. Mustafa

et al. (2011) and our recent study (Ali *et al.*, 2021) also reported higher prevalence of CM in cows than in buffaloes. It has been reported that buffaloes are generally more resilient to different diseases such as bovine mastitis due to their strong immune system (Pal and Chakravarty, 2020). Besides, the other contributing factors of high prevalence of mastitis in cattle might be the harsh environmental conditions of the province, and diminished immunity of exotic and crossbred cows. Also, this study reported higher prevalence of SCM in cows than reported by other studies such as Bachaya *et al.* (2011) reported 35% prevalence of SCM in cows of district Muzafar-Gharr, 30% in cattle belonging to Lahore (Mustafa *et al.*, 2011), and 44% in Punjab province (Ali *et al.*, 2011), and 42% in buffaloes belonging to district Dera Ismail Khan (Ali *et al.*, 2014). This increase in the occurrence of bovine mastitis is worrying, which might be due to the reason that the dairy sector is swiftly growing in the KPK, and large numbers of commercial dairy farms are now developed in this area. Our previous study also reported that with the increase in herd size there is significant increase in the incidence of mastitis in buffaloes (Ali *et al.*, 2014). This study is in line with other studies (Bhat *et al.*, 2017; Ali *et al.*, 2014) that reported a prevalence of 11.5% CM in cattle belonging to Azad Jamu Kashmir, and 11% in buffaloes of district Dera Ismail Khan. In contrast, the studies of Sharif *et al.* (2009) and Mustafa *et al.* (2011) observed the highest prevalence of CM in cows (61%) and buffaloes (61%). Additionally, this study was in line with some other studies (Mustafa *et al.*, 2011: Sharif *et al.*, 2009), we reported that 4.7% milk samples from cattle and 4.0% from buffaloes were mixed with blood. The findings of this study were partially according to other studies across the world, which reported the prevalence of bovine mastitis in India (Sinha *et al.*, 2016), in China (Gao *et al.*, 2017), in Ethiopia (Birhanu *et al.*, 2017), in Poland (Sztachańska *et al.*, 2016), and in Kenya (Mbindyo *et al.*, 2020). Geo-climatic variations in different regions also affect the prevalence of bovine mastitis as investigated in this study and are also in line with other published work (Ali *et al.*, 2011).

In the present study, contagious mastitis pathogens *Staphylococci* spp. (24.6%) were the most prevalent bacteria, followed by environmental mastitis pathogens *E. coli* (23.7%). The other bacterial isolates were *Salmonella* spp. (5.9%), *Klebsiella* spp. (5.7%), *Candida* spp. (4.1%) and *Proteus* spp. (1.5%). Several studies from Pakistan (Iqbal *et al.*, 2004, Ali *et al.*, 2011; Mustafa *et al.*, 2011; Bhat *et al.*, 2017; Aslam *et al.*, 2021) and other countries (Ali *et al.*, 2017; Birhanu *et al.*, 2017) have also reported these bacterial pathogens from cases of bovine mastitis; although the bacterial prevalence was slightly different in different regions. The findings of Bhat *et al.* (2017) were in agreement with our findings. They also reported 61% prevalence of *S. aureus*, 13% *E. coli*, 13% coagulase-negative streptococci (CNS), 9% *Streptococcus dysgalactiae*, and 4% *Streptococcus uberis* in cattle of Jammu and Kashmir. Nevertheless, in contrast, researchers in China investigated 14% prevalence of *E. coli,* 10% CNS, 10% *Staph. aureus*, 11% *Strep. dysgalactiae*, 3% *Strep. agalactiae,* and 2% *Strep. uberis* (Gao *et al.*, 2017). The reasons of this variation might be that the prevalence of bacterial pathogens associated with bovine mastitis is varying with topographical conditions and managemental practices.

Table 4: *Antimicrobial susceptibility of bacterial isolates from mastitic cattle and buffaloes.*

Antimicrobial agents	Concentrations (µg)	Susceptible isolates		Resistant isolates	
		n	%	n	%
Ampicillin	10	08	03.5	220	96.5
Amoxicillin	20	52	21.9	185	78.1
Augmentin	20/10	74	58.7	52	41.3
Oxytetracycline	30	36	15.0	204	85.0
Gentamicin	10	101	83.5	20	16.5
Streptomycin	10	07	04.9	135	95.1
Kanamycin	30	48	35.3	88	64.7
Norfloxacin	10	152	65.2	81	34.8
Enrofloxacin	05	213	86.2	34	13.8
Sulphamethoxazole	300	06	03.6	163	96.5
Doxycycline	30	62	40.0	93	60.0
Erythromycin	15	20	15.8	107	84.3
Florfenicol	30	147	82.6	31	17.4
Flumequine	30	37	43.5	48	56.5

In our most recently published work (Ali *et al.*, 2021), we isolated different bacteria from mastitic cattle and buffaloes, which were highly susceptible to enrofloxacin (86.2%), gentamicin (83.5%), and florfenicol (82.6%). These isolates were resistant to ampicillin (96.5%), sulphamethoxazole (96.4%), streptomycin (95.1%), oxytetracycline (85%), and amoxicillin (78.1%). Similarly, the findings of Iqbal *et al.* (2004) also suggested that several antimicrobial drugs such as aminoglycoside, enrofloxacin, and nor-

floxacin were highly effective drugs against bacterial isolates recovered from mastitic animals in Pakistan. Also, gentamicin, enrofloxacin, norfloxacin and ciprofloxacin were also found susceptible against bacterial pathogens in several studies (Sharif *et al*., 2009; Ali *et al*., 2011; Aslam *et al*., 2021). Strikingly, gentamicin and enrofloxacin are also in the approved and listed for veterinary usage by World Organization for Animal Health (OIE) and World Health Organization (WHO, 2016).

Conclusions and Recommendations

Our study revealed that contagious mastitis and environmental mastitis due to *Staphylococcus spp*. and *E. coli* were highly prevalent in cows and buffaloes of KPK and the recovered bacteria were mostly susceptible to enrofloxacin, gentamicin, and florfenicol. This study might be helpful to clinicians and researchers for designing and implementing prophylactic and therapeutic plans for bovine mastitis. In addition, this study recommends national level surveillance study on prevalence of bacterial pathogens associated with bovine mastitis and their resistance profiles across the country.

Acknowledgments

We highly appreciate the assistance of our para-Lab staff at the Center, especially, Mr. M. Rauf, Mr. Raham Dad, Mr. Haseeb Gul and Mr. Qaisar Khan.

Novelty Statement

This is an important surveillance study on bovine mastitis, the prevalence of different bacteria associated with mastitis and their antimicrobial resistance and susceptibility profiling. Such studies are always needed by the clinicians and researchers associated with dairy industry to design and implement strategies in order to control and prevent mastitis in cattle and buffaloes.

Author's Contribution

Tariq Ali, Abdur Raziq and Muhammad Ijaz Ali: Designed and perceived this study.
Tariq Ali, Kamran, Anwar Ali and Inam Ullah Wazir: Performed the experimental work.
Muhammad Shuaib Khan, Shakeeb Ullah and Inam Ullah Wazir: Performed data analysis and wrote this article.
Abdur Raziq, Muhammad Ijaz Ali, Shakeeb Ullah and Sher Hayat Khan: Critically reviewed and revised the article.
All authors had read and approved the final draft for submission.

Conflict of Interest
No conflict of interest is declared by the authors.

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