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Short Communication

Prevalence of Mycoplasma Mastitis in Buffaloes of Pakistan – A Pilot Study in the Centre-West of Balochistan

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Abstract |Mycoplasma are known to cause several diseases in bovine animals including mastitis. The prevalence of mycoplasma mastitis is reported from many parts of the world in farm animals. The response of antimicrobials against this infection is very poor that usually lead to culling of infected animals. In Pakistan, unfortunately no data is available on the occurrence of mycoplasma mastitis in farm animals. Therefore, current field investigation was conducted first time in centre-west Balochistan to get the preliminary data on mycoplasma mastitis. Milk samples (n = 579) were collected from three districts viz., Jaffar abad, Sohbat pur and Naseer abad from mastitis suspected buffaloes. The samples were collected from those animals which had abnormal milk or physical signs (like swelling, abscess, wound etc.) on udder or teats or have suddenly dropped milk production. All samples were cultured on PPLO (pleuropneumonia like organism) media and were found negative for *Mycoplasma* spp. The prevalence of mycoplasma mastitis in this population of dairy buffaloes was recorded <0.23%. The study results suggested that the mycoplasma mastitis did not exist in buffaloes of study area, however, further studies using molecular tools are warranted to validate these findings.

Keywords | Dairy buffalo, *M. bovis*, Mastitis, Mycoplasma, Survey

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INTRODUCTION

Buffalo (also called water buffalo) is known as main dairy animal in many countries, including Pakistan. There are about 27.3 million buffaloes in Pakistan which ranks it at 2nd position in buffalo population in the world (Pakistan Livestock Census, 2021). Buffaloes share in country's total milk production is about 67%. There are two types of buffaloes in Pakistan viz, Khundi and Nili Ravi.

Buffaloes are comparatively more resistant than cows for many infectious diseases and their milk is more nutritious than cow milk (Sedky et al., 2020; Kambli et al., 2019).

Bovine intramammary inflammation is one of the most important endemic and a mainly general infection of dairy animals which causes drastic losses in terms of reduced milk production and quality, costly medication and culling of important animals. Mastitis is responsible for poor milk quality in terms of direct hygienic quality and indirect quality characteristics of milk (Radostits et al., 2007; Bilal et al., 2004). Mastitic animals also have a deleterious

changes in their hemato-biochemical profile and exhibited oxidative stress markers (Abdel-Hamied and Mahmoud, 2020). The most effective strategy to control this infection is appropriate management of all lactating mammals (Halasa et al., 2007). In the dairy sector the presence of bovine udder inflammation and its' associated economical loss are of great interest. It is due to a variety of factors such as bacteria, fungi, mycoplasma, yeast, shape of udder and teats, inheritance of animal, surrounding environment, and milking method (Tiwari et al., 2013). *Staphylococcus aureus, Streptococcus agalactiae, Staphylococcus chromogene, Streptococcus uberis, Corynebacterium bovis* and *Streptococcus dysgalactiae* have been reported as most important and prevalent groups of bacteria to cause bovine mastitis (Ariffin et al., 2019; De Vliegher et al., 2016). Mastitis imposed great threat to human health due to contamination of milk with these pathogenic bacteria that were linked with common diarrheal diseases such as *Streptococcal* intoxication and colibacillosis (Schukken et al., 2016).

Mycoplasma-mastitis is an infrequent type of mastitis which is associated with critical damages to dairy animals (Fujimoto et al., 2020). *Mycoplasma* is commonly found as the microflora of healthy animals in urogenital and respiratory tract where they can possibly be act as pathogens. Most of species of *Mycoplasma* are known to form biofilm which greatly contribute in resistance against antimicrobial agents (Daubenspeck et al., 2020). The specie, *Mycoplasma* having no cell wall, causes the intramammary inflammation in dairy animals such as bovine, caprine, and ovine (De Carlo et al., 2013). Infected animals shed mycoplasma in milk ranging from 10^5-10^8cfu/ml (Pfutzner and Sachse, 1996). Nowadays the existence of mycoplasma-mastitis has been reported in numerous areas all over the world. The pathogenic *Mycoplasma* spp. can be recognized by using the gold standard technique i.e., the direct culturing on mycoplasma agar media as well as using polymerase chain reaction assay (Fox et al., 2005).

In previous studies, mycoplasma were isolated from poultry and pneumonia cases of small ruminants in Pakistan (Ahmad et al., 2021; Gondal et al., 2015), however, no study was carried out on milk or mastitic animals. Thus, keeping in view the above scenario this pilot project was designed to explore the mycoplasma-mastitis in buffaloes of centre-west of Balochistan (Sohbat pur, Jaffar abad and Naseer abad districts). The results of this study will be helpful in formulation of future policies to control the mastitis in dairy animals.

MATERIALS AND METHODS

Study area

This study was carried out in three districts of Balochistan namely Sohbat pur, Jaffar abad and Naseer abad. These three districts are in Naseer abad division of Balochistan and located near to District Jacobabad of Sindh province (Figure 1). All three districts were used to collect milk samples from randomly selected buffalo farms. All study procedures were approved by the Departmental Board of Studies that were endorsed by the Directorate of Advanced Studies, Sindh Agriculture University, Tandojam (Approval Number: No. MICR/034 of 2020).

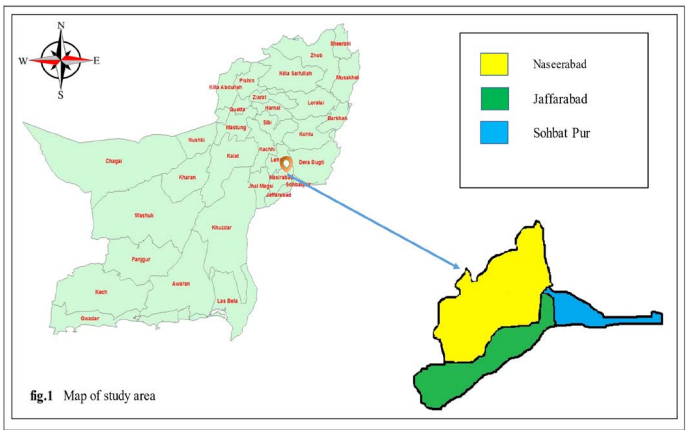

Figure 1: Map of the study area.

Table 1: Details of samples collected during the survey.

District	Total number of farms used to collect sample	Total number of animals on the farms	Number of animals used for sampling
Jaffar abad	81	1932	289
Sohbat pur	50	1233	176
Naseer abad	38	693	114
Total	169	3858	579

Collection of samples

An overall 579 buffalo milk samples were collected from three districts, i.e., Sohbat pur (n=176), Jaffar abad (n=289) and Naseer abad (n=114). The samples were collected from each district from both clinical and subclinical cases of mastitis. The details of sample collected is shown in Table 1. Samples were collected from both small and large herds of buffaloes. According to the previous history taken from farmers only those farms were used for sample collection that have a treatment failure history for the combination of procaine penicillin (200000 IU) and dihydrostreptomycin (200 mg/ml (Penstrip-400). Additionally, only those animals were used for sample collection that have physically abnormal milk or have signs on udder/teat like swelling, abscess, wound etc. Animal-level samples (5-10ml) were collected in sterilized screw-capped plastic bottles following the aseptic procedures recommended by National Mastitis Council (NMC, 2004). During collection of samples, the tips of teats were cleaned through cotton swab

moisten with 70% alcohol and some strips of milk were discarded before collection of sample to cut the chances of environmental contamination. The collected samples were labeled and transported to the laboratory under refrigerated conditions for *Mycoplasma* analysis.

Culturing of Samples

According to the standard operating procedure of OIE (2005), the milk samples were inoculated and streaked on media. During the entire procedure, biological safety cabinet was used, along with all safety measures to avoid any bacterial contamination. Proper sterilized mask, gloves and lab coats were also used. According to the typical microbiological procedures described by NMC, (2004) the isolation of *Mycoplasma* from mastitic milk samples was carried out in the safety cabinet. Using the quadrant streaking method for each sample, a streaking loop with 0.01 ml of milk sample was splashed on pleuropneumonia-like organisms (PPLOs) agar media (Oxoid, UK). After that with the help of wire loop a drop of mastitic milk sample was then inserted into test tube containing broth (liquid) medium. The proper labeling was done and incubated for 48 hours at 37°C in the 10% CO_2 incubator. The culture was then observed after 72 hour, the conformation of *Mycoplasma* growth by detecting any deviation in color of medium from red to yellow with whirl formation. After partial conformation of growth based on visualization, with the help of wire loop utilizing culture was then streaked on prepared agar plate in the biological safety cabinet. The plates were properly labeled and placed again at 37 ^{0}C in the 10% CO_2 incubator for 72 hours.

As per OIE (2005) protocol, all negative samples were remained incubated for up to 7 days. After 7 days, samples having no change (growth) were subcultured in fresh broth by using a 10% (v/v) inoculum of broth into fresh broth as well as on agar media. If no growth seen after 21 days' incubation, the sample considered negative.

Statistical analysis

All data was tabulated in Excel spread sheet and then analyzed using JMP® 5.0.1a Statistical Package Software (statistical software (SAS Institute Inc., Cary, NC). The sampling fraction of each area/district was analyzed using χ^2 tests.

RESULTS

The present study on mycoplasma-mastitis was conducted in detailed for the first time in three districts of province of Balochistan. The milk samples were collected from the mastitis-suspected buffaloes to investigate the problem of mastitis caused by mycoplasma organisms. A total of 579 milk samples were collected from three districts of Balochistan (Jaffar abd, Sohbat pur and Naseer abad) and analyzed for occurrence of *Mycoplasma*. Among collected samples, 358 samples exhibited clinical mastitis while the remaining 221 were found positive for subclinical mastitis. In culture assay, all samples were found negative, thus mycoplasma-mastitis did not occurred in the study area. The χ^2 tests exhibited the <0.23% prevalence of mycoplasma mastitis in the population of dairy buffaloes.

DISCUSSION

Mycoplasma causes various sicknesses in bovine animals like mastitis, pneumonia, reproductive diseases, arthritis, bovine respiratory disease, otitis etc., (Fox, 2012). In large herds, *M. bovis* is known to cause mycoplasma-mastitis more frequently than other *Mycoplasma* species (Timonen et al., 2020). The prevalence of mycoplasma-mastitis is reported in many parts of the world (Junqueira et al., 2020; Timonen et al., 2020; Ulloa et al., 2021), however some studies reported its' zero percent prevalence and declared the herds/regions as free from mycoplasma-mastitis (Arcangioli et al., 2011; De Carlo et al., 2013). The true economic impact of this udder problem was studied in United States of America, where the estimated annual losses by this infection were estimated as high as 108 million dollars (Nicholas et al., 2008). Globally, there is no effective vaccine against this disease, and response of antimicrobials against this infection is also very poor that usually lead to culling of infected animals (Calcutt et al., 2018). To the best of our knowledge, in Pakistan no conclusive study was carried out to explore the mycoplasma-mastitis in buffaloes. Therefore, current study attempted to explore the mycoplasma-mastitis in Balochistan province of Pakistan. This province is comprises 26 districts with collective buffalo population of 0.31million (Pakistan Livestock Census, 2021).

The current study was carried out during August to December 2020. A total of 579 buffalo milk samples from mastitis suspected animals were collected from three districts of Balochistan i.e, Jaffar abad, Sohbat pur and Naseer abad and analyzed for the *Mycoplasma* spices. Initially, all buffalo farms were visited and screened for history of treatment failure for combined therapy of procaine penicillin and dihydrostreptomycin. Farms with such treatment failure history were chosen for sample collection. Additionally only those animals were used for sample collection that have abnormal milk or physical signs (like swelling, abscess, wound etc) on udder or teats or have suddenly dropped milk production.

In current investigation, all buffalo milk samples (n=579) were found negative for *Mycoplasma* spp. Initially, 1 sample exhibited colony on mycoplasma media (PPLO agar), however, during repetition/validation it was also found

negative. In agreement to our results, De Carlo et al. (2013) also reported a study from southern Italy with zero percent prevalence of mycoplasma-mastitis. The authors collected 60 cow and 20 buffalo milk samples from a dairy farm with mastitis issue. All samples were investigated using culture technique as well polymerase chain reaction (PCR) assay, however, none of the sample was found positive for *Mycoplasma* spp. Similarly, a study published in New Zealand Veterinary Journal reported the 0% occurrence of mycoplasma-mastitis in southeast of France (Arcangioli et al., 2011). The authors carried out 2 preliminary studies to find out the prevalence of *M. bovis* infection. In first study, 345 bulk tank milk samples (representing the 345 herds) were collected, and in 2nd study, 828 milk samples (representing the 217 herds) were collected from clinical mastitis. In both studies, none of the sample exhibited the *M. bovis* via culture or PCR assay. Thus, study concluded that the prevalence of *M. bovis* udder infection in dairy herds of southeast of France is very low (Arcangioli et al., 2011).

In our investigation, milk samples were transported to the laboratory immediately after collection, however 4-6 hours on average were consumed in transportation. The samples were kept cool (4°C) during this period. It has been reported that 10^5-10^8cfu/ml milk is a usual level of *Mycoplasma* spp. excreted in clinical cases of mycoplasma-mastitis (Pfutzner and Sachse, 1996). A loss of 10^3cfu/ml *Mycoplasma* spp. is expected during storage (Biddle et al., 2004). Thus, even after this loss, milk still would have a threshold of detection of culture (10^1-10^2cfu/ml milk; Sachse et al., 1993). Therefore, our negative results suggested that all the collected samples were free from *Mycoplasma* infection.

In a most recent study, the effectiveness of control measures against *M.bovis* infection were tested in Finland during 2020 (Haapala et al., 2021). It was reported that control measures including, early detection and culling of mycoplasma mastitic animals, segregation of new calves from older animals, avoidance of nose-to-nose contact, and strict hygienic measures mostly related to feeding buckets, calf pens, milking and teats significantly minimize the risk of spread of *M.bovis* infection to naive herds. Thirteen out of 19 herds were found negative for *M.bovis* infection after adaptation of these control measures.

CONCLUSION

From the findings of this pilot study it could be concluded that the prevalence of mycoplasma-mastitis is as low as could not be detected by culture assay in the centre-west of Balochistan. This data could be used in formulation of future studies at national level. Moreover, large-scale studies are warranted to investigate the status of mycoplasma-mastitis around the country using culture as well as molecular techniques.

AUTHORS' CONTRIBUTION

Author SS collect samples and carried out the experiment, while AAK conceived the research. HB, and RB supervised study and helped in write-up. AMM and GMS assisted the lab work and statistical analysis.

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