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Epidemiological Investigations of *Mycoplasma bovis*-Associated Mastitis in Dairy Animals along with Analysis of Interleukin-6 (IL-6) as a Potential Diagnostic Marker

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ABSTRACT

Mycoplasma is a member of the *Mollicutes* group and is the smallest bacteria, which leads to mastitis, arthritis, pneumonia, and reproductive disorders in animals. The major etiological agent behind the scenes is *Mycoplasma bovis*, which is more prevalent and results in major losses but is the least noticeable due to its subclinical infection. The main source of Mycoplasma transmission is contaminated milking or the introduction of sub-clinically infected animals in the herd. Mycoplasma is found all over the world and is also reported from Pakistan. Mycoplasma is one of the mega problems due to its resistance to antibiotics so once the animal gets infection it is very difficult to get rid of it. It can be easily diagnosed by observing the clinical signs or by culturing the bacteria (fastidious or time-consuming). In this study, we collected 384 milk samples and performed Surf Field Mastitis Test (SFMT) to check the presence of mastitis. All these samples were further evaluated for *Mycoplasma bovis* by ELISA (ID Screen® *Mycoplasma bovis* Indirect). Along with this, we targeted the uvrC gene of the *Mycoplasma bovis* through PCR and LAMP for the rapid diagnosis of Mycoplasma. It was observed that 45.57% of animals were positive for mastitis through SFMT and 20.83% prevalence was recorded through ELISA. Only three samples were confirmed for Mycoplasma from all the ELISA-positive animals by LAMP assay. None of the samples was positive for PCR. IL-6 levels were significantly higher in the SFMT and ELISA-positive animals as compared to the healthy ones.

Keywords: Mycoplasma, Mastitis, IL-6, Marker, LAMP, Cattle.

INTRODUCTION

Mastitis is one of the dairy sector's most prevalent and costly problems. Severe economic losses have been reported around the globe with an estimated 53 million US$ due to the reduced milk production, treatment expenditures, labor cost, and ultimately premature culling of the infected animals due to the persistent infection (Sharif and Muhammad 2009; Asfour et al. 2022; Gelgie et al. 2022). Another major factor contributing to the economic losses is that mastitis leads to physical and chemical alterations in the milk properties that are not acceptable to end consumers (Kumari et al. 2018). The shelf life of fresh milk is also decreased due to higher bacterial count so again a big source of loss of income as well as available food for humans. Similarly, many of the pathogens causing mastitis have zoonotic implications, posing a serious risk to human health where fresh milk is preferred. The etiological agents responsible for mastitis include a long list, however, these are considered major culprits including *Streptococci*, *Staphylococci*, and *E. coli.* while *Pseudomonads*, *Nocardia*, *Mycoplasma*, and yeast have been found less frequently associated. Due to the variety of pathogens prevalence of mastitis is high in dairy farms (Sharif and Muhammad 2009; Kumari et al., 2018; Ijaz et al. 2021; Gelgie et al. 2022; Anwar et al. 2022).

Mycoplasma bovis was first diagnosed in the year 1954 as an etiological agent of bovine pneumonia and later in 1962 was isolated from mastitis milk and was known as *Mycoplasma agalactiae* var. *bovis* (Gille 2018). During the last decades, *Mycoplasma bovis* emerged as one of the major culprits leading to mastitis, a member of class *Mollicutes* and family *Mycoplasmataceae.* At the initial stage of infection, *Mycoplasma bovis* initiates an

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immune response (innate) that plays a critical role against itself. The immune system not only recognizes the pathogen but also protects against it (Qin et al. 2019; Ider et al. 2022; Yassin et al. 2023). *Mycoplasma bovis* has been reported to cause respiratory tract infection, arthritis, and reproductive problems in dairy herds. It has been associated with chronic mastitis due to the massive transboundary movement of animals, globally (Gille 2018; Gelgie et al. 2022). Exposure to dairy herds mostly occurs through the introduction of asymptomatic/carrier animals, previously declared negative for mastitis. Among herds, this organism spread through inhalation of respiratory secretions, contaminated milking equipment, and also through the workers from the infected farms (Calcutt et al. 2018).

In the past, isolation and cultivation techniques were used to identify *Mycoplasma bovis*, but these techniques were laborious and time-consuming. Now recent serological techniques like PCR and ELISA are being used. These techniques are more specific, sensitive, and efficient as compared to culture. Anti-Mycoplasma antibodies can also be detected in sera and milk samples, collected from individual animals or even from bulk tank milk samples with the help of ELISA (Parker et al. 2018).

Pakistan being an agricultural country mainly relies on the income originating from agriculture and livestock. About 70% of the population residing in rural setup is directly or indirectly making its livelihood through the livestock sector. The 80% of dairy farmers in Pakistan are small farmers who are sharing 90% of total milk production. Out of these 80%, 43% of the farmers had a herd size of 1-2 animals while 37% of them have 3-5 animals (Chandio and Rehman 2017; Anwar et al. 2022). Mastitis is of major concern to dairy farmers and veterinarians in Pakistan, due to its significant effects on the quality and yield of milk, by increasing the culling rate due to reproductive problems and antibiotic resistance. Current losses statistics due to mastitis in Pakistan are not available although in Punjab province, it was estimated about two decades ago. An annual loss of Rs. 240 million per annum was recorded for only Punjab province (Bilal et al. 2004; Ijaz et al. 2021; Ahmed et al. 2022).

Recently, the prevalence of *Mycoplasma bovis* has been reported in Pakistan from slaughtered animals and rearing calves for food purpose and about 9% prevalence has been recorded (Ahmad et al. 2014). The pathogen was confirmed through isolation and histopathology (Mahmood et al. 2017). These few reports confirmed that this organism is prevalent in our livestock population and might be playing a role in the development of mastitis. Hence, this study was planned for the epidemiological investigation of Mycoplasma as the causative agent of sub-clinical mastitis along with an estimation of inflammatory markers. And the prime objective was to investigate a rapid yet confirmatory diagnostic test for the identification of the causative agent at the herd and individual levels.

MATERIALS AND METHODS

Ethical Statement

This study was conducted keeping in view all the research ethics and it has been duly approved by the Graduate Studies and Research Board (GSRB) of the University of Agriculture, Faisalabad, Pakistan.

Sample collection: A total of 384 samples were collected from animals maintained at different cattle colonies of Faisalabad. This number was calculated by following the formula described by Thrusfield (2005) for simple random sampling (at 95% CI and 50% expected prevalence). To correlate the diseases with associated risk factors, the following basic information related to each animal including identity, breed, age, lactation status/number, vaccination, and history of mastitis, if any was recorded. Bulk tank milk samples and samples including milk and whole blood were collected from the animals suspected of mastitis by following the standard protocols.

Field and Laboratory Diagnosis

Herd Screening through Surf Field Mastitis Test (SFMT): The principle of the SFMT is that when a milk sample is mixed with detergent, it leads to the estrangement of somatic cells and the proclamation of DNA and other cell contents occurs. DNA is acidic in nature, while detergent is basic due to alkyl-aryl sulfonate. Detergents and DNA bind to make a salve, whose consistency depends on the number of somatic cells. More the number of cells thicker the gel and vice versa (Muhammad et al. 2010). For the analysis of milk samples, a 3% solution of common household detergent Surf-Excel (Unilever Pakistan Ltd) was made. Then the test solution was mixed with an equivalent amount of milk sample and swirled for one minute. After 1-2min floccules were noted and graded as per their quantity.

In-direct ELISA (I-ELISA) for the diagnosis of *Mycoplasma bovis*

Milk samples that tested positive for mastitis by SFMT were further evaluated by using ID. Vet indirect ELISA kit (Kit catalog # MBOVISS-2P) for *Mycoplasma bovis*. All the standard protocols of the test mentioned in the kit literature were followed. Briefly, in each well of the plate 50µL of dilution Buffer 13 was added. Negative and positive controls were made in a dilution of 1:101 (1µL of the controls in 100µL of dilution buffer 13). Then 100µL of the negative control was added in wells A1, B1, and 100ul of the positive control in wells C1, and D1. After the addition of controls, 100µL of lactose was added to the remaining wells, then covered the plate and incubated at 21°C for 16-20 hours/overnight. After the completion of incubation, washing was done with 300µL of the Wash Solution of concentration of 1X for three times. Then conjugate 1X (100µL) was added to each well. After that incubation was done for 30 min at 21°C. Again, washing was performed with 300µL of the Wash Solution three times. Then in all wells, 100µL of the Substrate Solution was supplemented. Another incubation for 15min at 21°C was given in the dark and a color change was noted. At last, 100µL of the stop solution was added to each well. Optical density (OD) values were recorded through an ELISA reader at 450nm wavelength and results were recorded as S/P%.

Detection of *Mycoplasma bovis* through PCR

ELISA Positive samples were subjected to a conventional Polymerase chain reaction (PCR) for the Mycoplasma genus *uvrC* gene that is specie specific by following the procedure described by Ashraf et al. (2018).

The product size was 238bp and the details about the primers have been described in Table 1. Genomic DNA of Mycoplasma was obtained from the blood samples collected from ELISA-positive cattle by using the FAVORGEN kit (FavorPrep Blood Genomic DNA Extraction Mini Kit) and obtained DNA was stored at -20°C and used for amplification.

Briefly, in a thin-walled PCR tube following components were added for each 25µL including 12.5µL PCR Master mix (2.5U of Taq DNA polymerase, 1.5µL of 25mmol/L $MgCl_2$, 200µmol/L dNTPs and 2.5µL of 10X PCR buffer), 1µL of each forward and reverse primers, 5µL of template DNA and 5.5µL of nuclease-free water. The condition followed for amplification has been described in Table 1. The end product was observed through gel electrophoresis using ethidium bromide by using a gel documentation system (Gel Doc™ EZ Imager, BIO-RAD).

Loop-mediated Isothermal Amplification (LAMP) Assay for the diagnosis of *Mycoplasma bovis:* The ELISA-positive samples were also subjected to LAMP assay by following the procedure described by Ashraf et al. (2018). The primers used for the LAMP assay have been described in Table 2. Briefly, for a total 12.5µL of total volume, RNAse free water (4.5 µL of), Betaine (3µL of 5mol/L) Thermopol buffer (1.5µL of 10X solution containing 10mmol/L $(NH_4)2SO_4$, Tris-HCl, of 20mmol/L concentration, 10 mmol/L KCl, 2mmol/L $MgSO_4$, and 0.1% Triton X-100), dNTPs (1µL of 2.5mmol/L of each), primer mix (0.75µL) having different concentrations of outer and inner primers, 0.75µL of 8000U/mL Bst DNA polymerase (New England, Biolabs); and template DNA (1µL) were added.

The incubation of this reaction mixture was done through a heat block and amplification was done at 55-65°C in ESE Quant Tube for an hour. Results in quantitative form were obtained in a couple of minutes and monitored on a computer screen in real-time form for about 40 minutes. For positive samples, the amplification curve was formed in this duration. For qualitative analysis, the reaction mixture was loaded in 2% gel and analyzed through a UV illuminator. At last, the holding was done at 80°C in a thermal cycler for 5min to terminate the reaction and inactivate the enzymes in it.

Determination of cytokines (IL-6) associated with mastitis

For the diagnosis of subclinical mastitis and other inflammatory condition, the markers are playing a very important role. So here the IL-6 was used as a marker and it was determined through a commercial ELISA kit (Invitrogen, ThermoScientific, USA) by following the procedure described by the manufacturer. Briefly, **e**ach component of the ELISA was brought to room temperature and standards were mixed with Reagent Diluent to achieve the final concentrations. 100µL of sample and standards were added into each well and the plate was incubated at 25°C for an hour. Four-time washing was performed with PBS-Tween buffer. 100µL antibody (1:100 dilution) was added to each well and incubated at room temperature with moderate shaking for one hour. Again, washing was done in the same manner. Then, 100µL of streptavidin-HRP conjugate (diluted 1:400) was added to each well, and the plate was incubated at room temperature for 30min. After washing, 100µL of TMB substrate solution was added. And 10min of incubation was done at room temperature. To stop the reaction 50µL of stop solution was added and OD values were recorded through an ELISA reader at 450nm.

RESULTS

Prevalence of mastitis in cattle

Through SFMT, only the prevalence of mastitis was recorded, and later on, whether it is due to mycoplasma or not i-ELISA was performed. An overall prevalence of mastitis was recorded at 45% based on SFMT screening (Table 3). When these positive samples were subjected to the ELISA about 21% were positive for mycoplasmosis. When the data was analyzed to find its association with other risk factors the following detailed results were obtained. Initial screening through SFMT for mastitis based on breeds, the prevalence was recorded at 51, 47, and 35% in Cholistani cross, Sahiwal cross, or in pure Friesian, respectively. A smaller number of cases were recorded in pure breeds. In terms of age, 21, 50, and 71% prevalence of mastitis was recorded in young, mature, and old groups of animals. In terms of source, 51, 54, and 31% prevalence of mastitis was recorded in cattle colony 1, 2, and in cattle colony 3, respectively. Data shows that Mastitis is ubiquitous in nature. Based on SFMT results 51% of the cases (having a head size of more than twenty) were found positive for mastitis. While 40% of the cases were found positive in herds having a head size of less than twenty. By the SFMT test, 56% of animals tested for mastitis in which dry-period therapy was not followed, while 29% of animals tested positive for mastitis in which dry-period therapy was followed.

Table 1: PCR Primer set for the diagnosis of *Mycoplasma bovis*

Gene Name	Sequence (5'-3')		Product size
uvrC 1	TAATTTAGAAGCTTTAAATGAGCGC		238bp
*uvrC*2	CATATCTAGGTCAATTAAGGCTTTG		
DNA amplification conditions			
Step	Temperature (°C)	Time	Number of Cycles
Initial denaturation	95	5min	1
Denaturation	95	45sec	30 cycles
Annealing	54	30sec	
Extension	72	45sec	
Final extension	72	10min	1
Infinite hold	4	∞	-

Table 2: LAMP Primers for *Mycoplasma bovis*

Primer Name	Corresponding Gene	Sequence (5'-3')
B3		AAGCACCCTATTGATTTTTACTC
F3		AGAAACAGACAAAAAATTAGTTCAC
FIP	*Uvrc*	GATTTTTGCATAGCTTTTAAAGTGATTT-TGAAGGCAAACTAAGAAACATAAAAGG
BIP		GACGCTTCAGTTGAAGAATTATCA-TTTTAATCCTTATTTTTAATGCTTTTGGC

Table 3: Prevalence of *Mycoplasma bovis* in dairy animals based on risk factors

Risk Factors		Total	SFMT Positive	Prevalence (%)	ELISA Positive	Prevalence (%)
Breed	Cholistani X Friesian	135	69	51.11	27	20
	Sahiwal X Friesian	156	73	46.79	20	12.82
	Friesian	93	33	35.48	33	35.48
Pregnancy status	Pregnant	184	72	39.13	37	20.1
	Non pregnant	200	103	51.5	43	21.5
Age (Years)	2-4	140	30	21.42	27	19.28
	4-6	135	67	49.62	20	14.81
	Above 6	109	78	71.55	33	30.72
Area	Cattle colony 1	128	65	50.78	25	19.53
	Cattle colony 2	128	70	54.68	40	31.25
	Cattle colony 3	128	40	31.25	15	11.71
Stocking density	>20	190	97	51.05	53	27.89
	<20	194	78	40.2	27	13.91
Dry period therapy	Yes	154	45	29.22	16	10.38
	No	230	130	56.52	64	27.82
History of Mastitis	Exposed	127	93	73.22	27	21.25
	Not Exposed	257	82	31.9	53	210.52
Disinfection protocol	Yes	88	33	37.5	13	14.77
	No	296	142	47.97	67	22.63
History of Replacement of Animals	Yes	91	62	68.13	63	69.23
	No	293	113	38.56	17	5.8

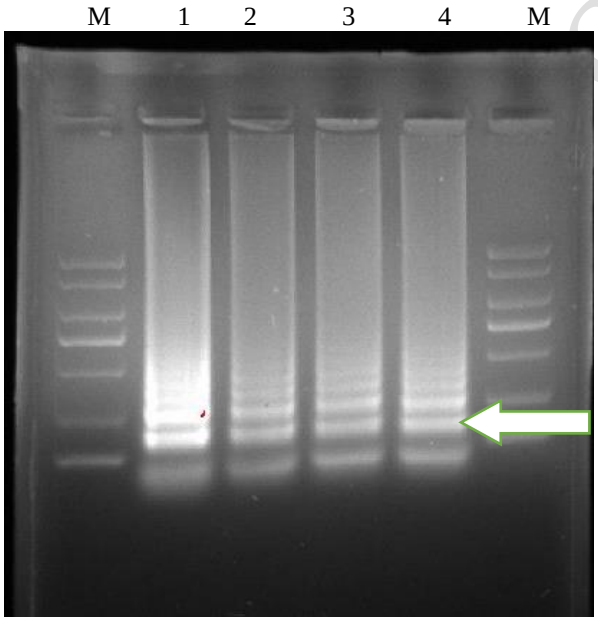

Fig. 1: Band-like Pattern (urvc gene) of amplified products of samples on gel upon LAMP. Lane Description: M is DNA Ladder (100bp), 1 is control positive and 2, 3 and 4 are field positive samples for *Mycoplasma bovis*.

Prevalence of Mycoplasma associated Mastitis in cattle

On the basis of ELISA, 20, 13 and 35% prevalence of Mycoplasma was recorded in Cholistani cross, Sahiwal Cross and in pure Friesian, respectively. In this study, Mycoplasma cases much reported in pure animals as compared to the cross. Similarly, in terms of age 19%, 14% and 30% prevalence of Mycoplasma was recorded in young, mature and adult groups of animals. On the basis of sources, about 19, 31 and 11.7% prevalence of Mycoplasma was recorded from milk samples collected from various cattle colonies numbered as 1, 2 and 3, respectively. The highest prevalence was recorded in cattle colony two, that have the history of purchasing new but untested animals. In terms of stocking density, 28% of the animals were found positive for Mycoplasma kept in herds having a head size of more than twenty. While only 14 % of the animals tested positive for Mycoplasma kept in herds having a head size of less than twenty. Data analysis considering dry therapy practice as a risk factor indicated a higher incidence of Mycoplasma (28%) in animals who did not undergo dry-period therapy as compared to those in which it was followed properly where it was only 10% (Table 3).

Confirmation through PCR and LAMP

For the confirmation of M*ycoplasma bovis* ELISA-positive samples were subjected to PCR and LAMP assay. DNA was extracted and each DNA was subjected to PCR amplification using reported primers for specific detection of *Mycoplasma bovis*. However, all the screened samples were found negative during this study. The same samples were subjected to LAMP and out of eighty only 03 were positive for LAMP assay (Fig. 1).

IL-6 as an Inflammatory marker in Clinical and sub-clinical mastitis

A total of 80 samples were subjected for the IL-6 analysis through ELISA (40 from healthy and 40 from *Mycoplasma bovis* ELISA positive). Mean serum values for the IL-6 in healthy animals were recorded lower (0.36±0.18) as compared to the mastitis positive samples (0.61±0.36), those were statistically significant.

DISCUSSION

Mycoplasma bovis is one of the most perilous and contagious pathogens that can adversely affect the existence of dairy farmers. It leads to severe economic losses. Previously, it was known as *Mycoplasma agalactiae var. bovis* (Gille 2018). In acute cases, it alters the consistency of milk and makes a sudden and severe drop in milk production. Mostly, the consistency varies from purulent to watery along with fibrinous sandy residues that are a persistent character in mycoplasma-associated mastitis. This disease also results in therapy-resistant mycoplasma affecting all four quarters and about 2-10 days are required to have the clinical picture of mastitis (Al-Farha et al. 2017). Microorganism shedding from the teats is there even before the clinical symptoms of the diseases. Hence the control of the disease mainly will depend on the early detection of the carrier animals as compared to the conventional diagnostic techniques (Calcutt et al. 2018).

During this study, the following results were obtained. An overall prevalence of mastitis was recorded at 45% based on SFMT screening. When these positive samples were subjected to the ELISA about 21% were positive for mycoplasmosis and that was an indicator this organism is prevalent in our dairy herds and contributing towards mastitis along with other pathological implications. Recently, this organism was reported in calves having clinical illnesses like arthritis by Mahmood et al. (2017) in Pakistan. Just taking into account the mastitis only, irrespective of the etiology it was recorded that in the cattle population, this problem is manyfold higher in cross-bred bred animals as compared to the pure breed animals. This might be due to the resistance of pure breeds to specific pathogens.

While considering various associated risk factors, the following observations have been recorded during this study: animals undergoing multiple parities were more prone to this problem as compared to animals with earlier parities. Similarly, older animals have the highest prevalence as compared to all other age groups. The sample collected from cattle colonies located at different sites has a visible difference in percent prevalence. The samples collected for each colony were the same, so it proves that mastitis is ubiquitous in nature. Similarly, herd size also has a significant effect on the prevalence of mastitis. The smaller the herd, the lesser the percentage that has been recorded. In dairy herds, a very common practice to keep the fertile animals healthy and productive dry period therapy is done so almost half the percentage was recorded in the herds where this practice is still followed as per standard protocols.

ELISA (indirect) has been used for the early detection of *M. bovis* in mastitis outbreaks. Based on i-ELISA, a higher prevalence (21%) was recorded as compared to the previous reports where it was less than 20%. As the ELISA detects the antibodies that can be formed and remained in the body once the exposure has occurred to the organism and be used for longitudinal studies (Byrne et al. 2000; Hazelton et al. 2018; 2020; Dudek et al. 2020). In terms of associated risk factor analysis, the highest prevalence of mycoplasma-associated mastitis was recorded in pure-bred exotic animals as compared to the other crossbreeds. The factor responsible for this higher prevalence is mainly improper hygienic measures on the farm. The farms adopting good hygienic practices suffer less and the other major factor contributing is the replacement of animals due to a decrease in milk production (Gille 2018).

In different age groups, older animals have a more prevalent percentage of mycoplasma-associated mastitis as compared to the other age groups. As this might be due to the reason as the animal gets older, the aging induces changes in teat anatomy particularly the sphincters are loosened and the barrier for the entry of microorganisms remains no longer protective (Mahmood et al. 2017). It has also been reported that Mycoplasma affects all age groups of cattle including neonates to adults and all sectors including dairy, beef or rearing, etc. (Nicholas et al. 2008; Punyapornwithaya et al. 2010; Hazelton et al. 2020).

Based on sources, highest the prevalence was recorded in cattle colonies in which animal movement was frequent without any prior screening. There was a history of purchasing new animals in most of the herds. As this has been reported as the most common cause of *M. bovis-induced* mastitis due to the induction of the carrier/sub-clinically infected but non-lactating animals including dry animals, heifers, calves, and breeding bulls (Hazelton et al. 2018; Gelgie et al. 2022). Similarly, another important risk factor associated with *M. bovis* prevalence in dairy herds is the rearing of future dairy crops with dairy animals (Nicholas et al. 2016)

In terms of stocking density, herds having head sizes less than twenty have less positivity as compared to larger herds. High population density contributes to positivity which is alarming for the countries where large size dairy herds are present including the United States. Lack of awareness among dairy farmers is the main contributing factor (Aebi et al. 2015).

To evaluate the sensitivity of the PCR and LAMP for M*ycoplasma bovis*, ELISA-positive samples were subjected to both of these tests, and it was recorded that none of the samples was positive through PCR, however, 03 samples were positive through LAMP. LAMP appeared to be the more sensitive and specific test due to the involvement of multiple gene targeting which has also been endorsed previously by many researchers (Ashraf et al. 2018; Appelt et al. 2019; Gelgie et al. 2022) and the current study results are in accordance with previous findings. Using such techniques before the microbial culture not only improves the diagnostic efficiency as well saves resources and time (Andr´es-Lasheras et al. 2020).

For the diagnosis of sub-clinical infection in many infectious diseases inflammatory markers have been proven to be specific markers. In *Mycoplasma bovis* infections the cellular response is mainly initiated by the

Th1 cells and macrophages through the release of interferon-gamma (INF-γ) and tumor necrosis factor (TNF-α), which ultimately causes apoptosis or degradation of the mycoplasma (Askar et al. 2021). The role of interleukins, particularly (IL-4, IL-5, IL-6, IL-8, IL-12, IL-17, and IL-22) are also here very much important because these are involved in the differentiation of the T-cells in Th-1 cells, enhances the recruitment of neutrophils that ultimately destroys the pathogen (Annunziato et al. 2015; Gondaira et al. 2015; Jimbo et al. 2017; Askar et al. 2021). Another phenomenon where the role of interleukin has been documented is in type 2 immunity, coordinated through the Th2 cells through the activation of B-cells into plasma cells by the release of IL-4 and IL-5. In this study, the level of interleukin-6 was evaluated and found elevated in mycoplasma-infected dairy animals. The reason to choose this particular interleukin is that when *Mycoplasma bovis* interacts with the epithelial cells as a pro-inflammatory mechanism, IL-6, IL-8, and TNF-α are being secreted to provoke the immune mechanisms mentioned above. Thus, it can be stated that IL-6 can be used to detect the carrier animals (Zbinden et al. 2015; Jimbo et al. 2017; Askar et al. 2021).

Conclusion

In the modern era of dairy farming disease monitoring is of prime concern as the diseases are changing their patterns tremendously. With every passing day, due to the rapid connection of the world, diseases don't have boundaries, they can be seen around the globe due to the movement of animals and their products across borders due to corporate sector investment. Their epidemics can only be controlled if diagnosed at a very early stage. Continuing monitoring of the diseases is also required to prevent the disaster. Acute phase proteins like IL-6 can be very helpful for the early detection of the pathomorphological alterations induced by these pathogens.

Author's Contribution

All the authors were actively involved for conceiving the idea, execution of field sampling, write up and data analysis of this research project.

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Conflict of Interest

All the authors declare no conflict of interest.

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