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Seroprevalence and determination of serogroup-specific antibodies of *Leptospira* in cattle and buffaloes in Karnataka, India

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ABSTRACT

This study sought to evaluate seroprevalence and distribution of *Leptospira* serovars among bovines experiencing reproductive disorders across four revenue divisions in the state of Karnataka, India. A total of 582 serum samples, consisting of 314 cows and 268 buffaloes, were randomly collected from Bengaluru, Belgaum, Gulbarga, and Mysore divisions of Karnataka. Microscopic Agglutination Test (MAT) was employed to assess these samples against the reference panel of serovars comprising of eight pathogenic *Leptospira* namely Hardjo, Pomona, Canicola, Icterohaemorrhagie, Hebdomadis, Grippotyphosa, Pyrogenes, and Autumnalis. The findings indicated an overall seroprevalence rate of 28% (163/582), with specific antibodies against the employed serovars: Hardjo (34.35%), Pomona (16.56%), Canicola (11.66%), Icterohaemorrhagie (10.43%), Hebdomadis (10.43%), and Autumnalis (9.81%). No specific positive reactions were observed for Grippotyphosa and Pyrogenes serovars. Further, buffaloes exhibited a higher seropositivity (29%) as compared to cattle (27%). Serovar Hebdomadis was most prevalent in both cattle and buffaloes. Furthermore, among the 163 MAT reactive positive samples, the majority (62.58%) were linked to a history of abortion, followed by repeat breeding (28.22%), while the remaining cases (9.2%) were associated with other reproductive disorders. Bengaluru, Mysore, and Belgaum divisions displayed higher seropositivity and a greater diversity of serovars, potentially due to increased risk factors in these regions compared to Gulbarga division of Karnataka. These results underscore the necessity for enhanced surveillance and diagnostic efforts, particularly in animals with a history of abortion, to address leptospirosis in bovines. Furthermore, identifying prevalent serovars may be useful for targeted interventions in particular geographical areas, and may be of use in the reference panels of antigens in the MAT in disease diagnostic laboratories which pay the way for more accurate, efficient, and timely diagnosis of leptospirosis, and ultimately contribute to the management of 'One Health' concerns.

Keywords: Bovine leptospirosis, Karnataka, MAT, Seroprevalence

Leptospirosis is caused by pathogenic spirochetes, *Leptospira* and poses a substantial global health threat to both humans and animals. This ailment has gained prominence, particularly in countries like India, boasting vast populations of livestock, rodents, and wildlife. In bovines, leptospirosis is frequently linked to reproductive disorders, with abortion as a prevalent clinical manifestation (Elis 2015, Balamurugan *et al.* 2018, Murugavelu *et al.* 2022, Dharmashekar *et al.* 2021, 2023). Consequently, leptospirosis not only affects animal health but also carries substantial economic implications for the agricultural sector (WHO 2017).

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These adaptable spiral bacteria, possess an internal flagella, and are categorized into serovars based on their cell surface antigens and fall within the Genus *Leptospira*, of the family *Leptospiraceae*, in the Order *Spirochaetales*. Given the severity and prevalence of the disease, comprehending its epidemiology, modes of infection, carrier organisms, prevalent serovars, and diagnostic methods is crucial for effective prevention and control. The diagnosis of leptospirosis is intricate, often necessitating specialized reference laboratories for precise detection and identification of leptospires. Although laboratory isolation tends to be less successful, gold standard serological tests like microscopic agglutination test (MAT) are integral to leptospirosis diagnosis, detecting antibodies specific to serovars involved in an outbreak or endemic to a particular geographical area (Ismail *et al.* 2006; WHO, 2011, Senthilkumar *et al.* 2022). Routine screening of animal samples, especially those with a history of abortion, can provide valuable insights into the landscape of the disease.

With the Southern peninsular states of India reporting

an increased number of positive cases, the urgency for comprehensive seroprevalence studies has never been more apparent. The current study aims to investigate the prevalence of circulating serovars and antibody status in bovines in the Karnataka state of India, utilizing MAT for a comprehensive understanding of the regional leptospirosis scenario. By testing a wide array of serovars, our goal is to enhance our understanding of serovar distribution in this particular area. Further, to comprehend the epidemiology of leptospirosis, understanding the restricted prevalence of various pathogenic leptospires and knowledge regarding the prevalent serovar(s) in reservoir and carrier hosts can serve as a valuable indicator of transmission to incidental or accidental hosts within a specific geographical niche. This study was conducted to determine the frequency distribution of *Leptospira* serovars and the seroprevalence of Leptospirosis in Cattle and Buffaloes in Karnataka, India.

MATERIALS AND METHODS

Study area and samples: Four divisions of the Karnataka state, namely Bengaluru (comprising 5 districts), Belgaum (with 3 districts), Gulbarga (encompassing 4 districts), and Mysore (covering 3 districts), were chosen for the collection of samples. In this study, 582 samples were collected from clinical cases of reproductive disorders (314 cows and 268 buffaloes), 279 samples from animals experiencing abortions after more than 5 months of gestation, 214 samples from repeat breeders, and 89 samples from animals displaying other reproductive clinical symptoms such as retention of placenta and stillbirth (Fig. 1). Serum from blood samples was carefully separated and stored at -20ºC until use.

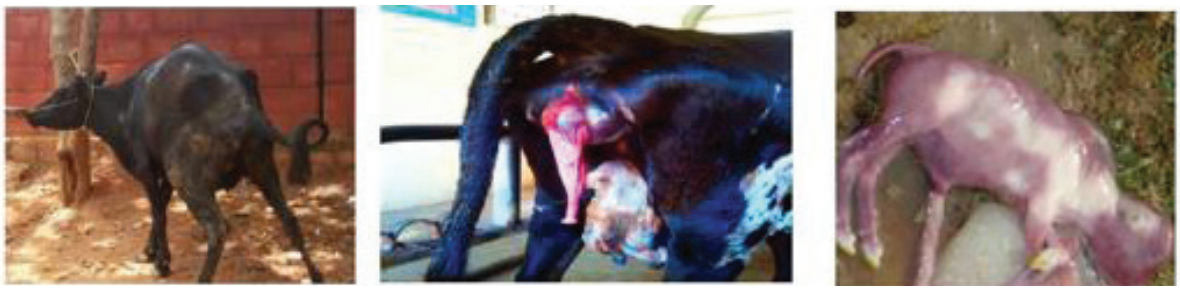
Leptospira culture: All these serum samples underwent MAT using reference pathogenic *Leptospira* serovars, namely Hardjo, Pomona, Canicola, Icterohaemorrhagie, Hebdomadis, Grippotyphosa, Pyrogenes, and Autumnalis (Supplementary Table). These serovars are maintained in the laboratory in Ellinghausen, McCullough, Johnson, and Harris (EMJH) semisolid and liquid media (Difco-BD). The modified EMJH media, (addition of Tween 80 and albumin Ellinghausen and McCullough, 1965), was employed for the growth of the culture and used for the MAT. Additionally, hyperimmune serum samples from the Southern Regional Disease Diagnostic Laboratory (SRDDL), IAH & VB, Bengaluru, India, having Titre Range of 6400- 12800 were utilized as a positive control in MAT, with known negative samples (Murag *et al.* 2021). The selection of serovars for this study was based on a prior seroprevalence survey conducted in endemic states, viz. Uttar Pradesh, Uttaranchal, and Tamil Nadu (Ellingshausen and McCullough 1965, Ratnam *et al.* 1983, Natarajasreenivasan and Ratnam 1997, Karthikeyan 2004, Nagarajan 2005, Koteeswaran 2006, Mariya *et al.* 2007, Selvaraj *et al.* 2010).

Microscopic agglutination test: Live Leptospira were cultivated in EMJH liquid medium at 29 ± 1°C for 5–8 days, and cultures at a concentration of 1-2 × 10^8 organisms/mL were utilized as live antigens in the MAT.

Counting of leptospires: Counting of the leptospiral organisms was done to fix the number of organisms per ml of culture to be used for MAT. A loopful of overnight formalized culture (10 µl) was taken on a clean glass slide

Table 1. Details of the serum samples used for testing of anti-leptospiral antibodies by MAT

Region	Districts (Number of samples)	Total number of samples
Bengaluru	Bengaluru Urban (30) Bengaluru Rural (31) Tumkur (35) Chikkaballapur (22) Shivamogga (44)	162
Belgaum	Belgaum (88) Uttar Kannada (37) Dharwad (26)	151
Gulbarga	Gulbarga (31) Bidar (37) Bellary (36) Koppal (24)	128
Mysuru	Dakshina Kannada (66) Mandya (40) Udupi (35)	141
Total		582



(a) (b) (c)

Fig. 1. The samples collected from bovines with varied clinical history. (a) Uterine discharge. (b) Retention of placenta. (c) Aborted foetus

and covered with clean cover glass of dimension 22 mm × 22 mm. The number of leptospires was counted in 10 microscopic fields, using 10 × 40 magnification under dark field microscope (Olympus CX31). Counts associated with the same field were avoided. A serial two-fold dilution of the culture was made when counts exceeded 50 or more organisms per field. The average number of organisms per ml was then determined as described by Subramaniam (1984) and Ajitkumar (1991).

Calculation of leptospires per mL of culture

Determination of the area of the microscopic field:

Diameter of microscopic field as determined by the stage micrometer	: 0.44 mm
Radius of the microscopic field	: 0.22 mm
Area of the circle	: 0.1519 mm^2
Area of the cover slip	
Length of the cover slip	: 22.00 mm
Breadth of the cover slip	: 22.00 mm
Area of the cover slip	: 484.00 mm^2

Calculation of the number of microscopic fields which fit cover slip area 484/0.1519 = 3120

Average number of organisms per field : N

Number of organisms in 10 µl (0.01ml) of culture : N × 3120

Total number of organisms in 1.0 ml of culture :N×3120×100

The test adhered to standard procedures (WHO, 2003, WOAH 2018). MAT was executed in 96-well 'U' bottom titration plates employing the eight reference serovar strains. Serum dilutions were prepared in a deep well (96-well) dilution plate (Laxbro-India). 20 µL of serum sample was mixed with 980 µL of phosphate-buffered saline (PBS) to achieve a dilution of 1:50. Diluted serum samples (25 µL) were introduced to each of the eight wells in columns 1 to 11 of the 'U' bottom microplates. In the last column, only 25 µL of PBS was added to all the wells, serving as the antigen control. Hence, each column corresponds to a single sample. Eight antigens (25 µL each) were dispensed to all the wells in the respective rows (antigen 1 in row 1, antigen 2 in row 2, and so forth), including the corresponding antigen control wells, resulting in a final serum dilution of 1:100. The plates were then incubated at 29 ± 1°C for two hours.

A drop (5 µL) of the mixture (final dilution of 1:100) was placed on a grease-free slide, without a coverslip and was scrutinized at 100X and 200X magnification of the dark-field microscope for the presence of agglutination and/or a reduction in the number of organisms compared to the respective antigen control. A 50% reduction in the number of free leptospires in the test sample compared to the respective antigen control was considered positive, irrespective of the presence of agglutination.

Additionally, end-point titration was conducted against the reactive serovars, representing antibodies specific to leptospiral serogroups. Initially, the sera were diluted to 1:50 in PBS, followed by doubling dilutions (25 µL each) in rows containing 25 µL of PBS in 'U' bottom microtitration plates. An equal volume (25 µL) of the corresponding antigens was added to all serum dilutions, while the last column served as the antigen control without the addition of serum. All final dilution mixtures (ranging from 100 to 12,800) were examined under a dark-field microscope (Olympus), and the results were documented as previously described, as the reciprocal of the highest dilution was recorded as the corresponding titters.

For statistical analysis, the current study employed chi-square test through GraphPad Prism V, and analysed at 95% significance level. This approach aimed to comprehend the association of *Leptospira* antibodies in bovines, both between species and across the studied administrative divisions.

RESULTS AND DISCUSSION

The overall prevalence rate of *Leptospira* antibodies was found to be 28% (163 out of 582 animals tested), indicating the presence of antibodies against six specific serogroups, viz. Hardjo (34.35%), Pomona (16.56%), Canicola (11.66%), Icterohaemorrhagie (10.43%), Hebdomadis (10.43%), and Autumnalis (9.81%). No serological evidence was found for the presence of Pyrogenes and Grippotyphosa serovars in the sera tested. Among the 163 reactive seropositive samples identified through MAT, 62.58% (102 samples) were linked to a history of abortion, 28.22% (46 samples) were associated with repeat breeding, and 9.2% (15 samples) to various other reproductive issues of animals.

The chi-square test analysis (Table 2) revealed a significant difference ($p<0.05$) between the clinical history of abortion, repeat breeders and other reproductive clinical disorders. However, when we considered division wise in Bengaluru, Gulbarga and Mysuru or interspecies within a division there is no significant difference ($p>0.05$) between the clinical history of repeat breeders and that of other reproductive clinical disorders.

Similarly in Table 4 it's clearly found that when we compared between the species, in the overall total MAT positive cases the chi-square test analysis revealed a significant difference ($p<0.05$) between the clinical history of abortion, repeat breeders and other reproductive clinical

Table 2. Microscopic agglutination test (MAT) positive cases of bovine with different clinical history

Division	Abortion (more than 5 months)	Repeat Breeder	Others	Total
Bengaluru	$^{x}30^{a}$	$^{y}17^{a}$	$^{y}7^{d}$	54 (33.13)
Belgaum	$^{x}23^{a}$	$^{y}17^{a}$	-	40 (24.54)
Gulbarga	$^{x}20^{a}$	$^{y}4^{b}$	$^{y}3^{c}$	27 (16.56)
Mysuru	$^{x}29^{a}$	$^{y}8^{b}$	$^{y}5^{c}$	42 (25.77)
Total	102 ^{x}(62.58)	46 ^{y}(28.22)	15 ^{z}(9.2)	163

Different superscripts indicate significant difference (p<0.05) i.e. Single common superscript indicates no significant difference.

Table 3. Frequency distribution of various reacted serovars with their titre range

Serovar/Titre	100	200	400	800	1600	3200	6400	Total	Percentage	Titre range
Hardjo	23	12	16	2	2	1	-	56	34.35	100-3200
Pomona	14	10	2	1	-	-	-	27	16.56	100-800
Canicola	11	2	1	1	2	1	1	19	11.66	100-6400
Hebdomadis	12	1	2	2	-	-	-	17	10.43	100-800
Icterohaemorrhagie	10	2	1	-	2	2	-	17	10.43	100-3200
Autumnalis	8	4	2	2	-	-	-	16	9.81	100-800
Pyrogens	-	-	-	-	-	-	-	-	-	-
Grippotyphosa	-	-	-	-	-	-	-	-	-	-
More than one serovar	7	4	-	-	-	-	-	11	6.75	100-200
Total (%)	87 (53.37)	35 (21.47)	24 (14.72)	8 (4.90)	4 (2.45)	4 (2.45)	1 (.61)	163		

disorders. However, for the same type of clinical history there is no significant difference (p>0.05) between the species.

Amongst the 163 MAT-reactive positive samples, 53.37% exhibited an antibody titre of 1:100, 21.47 % had 1:200, 14.72% showed a titre of 1:400, 4.90% had 1:800, 2.45% had titre of 1:1600 and 2.45% had 1:3200. Only one sample (0.61%) showed a titre of 1:6400. (Table 3)

The highest distribution of serovars among the MAT-positive samples was recorded for Hardjo (34.35%), followed by Pomona (16.56%), Canicola (11.66%), Icterohaemorrhagie and Hebdomadis each (10.43%), and Autumnalis (9.81%), with more than one serovar reactivity in 6.75% (Fig. 2 and Table 3 and 5). The division-wise, prevalence of serovars was reported in Figure 3a to 3d.

Surveys conducted in various Indian states have reported the prevalence of anti-leptospiral antibodies against different serovars in diverse animal species, including humans. Additionally, studies on leptospirosis in cattle and buffaloes over the have revealed varying percentages of prevalence, along with different reactive serovars years in different states. These findings underscore the endemic nature of the disease in these regions. In the present study, the overall seroprevalence of leptospirosis was higher in buffaloes compared to cows, (Table 4a). This was reported earlier in buffaloes in unorganized farms in southern

Table 4. Species-wise MAT-reactive positive cases with different clinical histories

Region	Animals							
	Cows				Buffaloes			
	Abortion (> 5 months)	Repeat breeder	Others	Total	Abortion (> 5 months)	Repeat Breeder	Others	Total
Bengaluru	28	13	05	46	02	04	02	08
Belgaum	02	01	--	03	21	16	--	37
Gulbarga	01	01	--	02	19	03	03	25
Mysuru	26	04	04	34	03	04	01	08
Total	^{x}57	^{y}19	^{z}09	85	^{x}45	^{y}27	^{z}06	78

Different superscripts within species indicate a significant difference (P<0.05)

Table 4(a). Division wise seroprevalence by MAT in Cows and Buffaloes (Species wise)

Division	Animal					
	Cow			Buffaloe		
	No. sera screened	Positive	Percentage	No. sera screened	Positive	Percentage
Bangaluru	131	46	^{x}35.11^{a}	31	08	^{x}25.81^{a}
Belgaum	48	03	^{x}6.25^{b}	103	37	^{y}35.92^{b}
Gulbarga	28	02	^{x}7.14^{b}	100	25	^{y}25.00^{a}
Mysore	107	34	^{x}31.78^{a}	34	08	^{x}23.53^{a}
Total	314	85	27.07	268	78	29.10

Different superscripts between rows (a,b) indicate significant difference (p<0.05) and different superscripts between columns (x,y) indicate significant difference (p<0.05)

Table 5. Distribution of various *Leptospira* serovars in four divisions of Karnataka state

Region	No. Tested	No. Positive by MAT (%)	Hardjo	Pomona	Canicola	Icterohaemorrhagie	Hebdomadis	Autumnalis	More than one serovar
Bangaluru	162	54 (33.34)	9 (16.67)	8 (14.81)	12(22.22)	12 (22.22)	10 (18.52)	--	3 (5.55)
Belgaum	151	40 (26.49)	16 (40)	5 (12.5)	1 (2.5)	2 (5)	--	12 (30)	4 (10)
Gulbarga	127	27 (21.09)	13(48.15)	3 (11.11)	--	3 (11.11)	5 (18.52)	--	3 (11.11)
Mysore	141	42 (29.79)	18(42.86)	11(26.19)	6 (14.28)	--	2 (4.76)	4 (9.52)	1 (2.39)
Total (%)	582	163(28.00)	56(34.35)	27(16.56)	19 (11.66)	17 (10.43)	17 (10.43)	16 (9.81)	11 (6.74)
Overall (%)			9.62	4.64	3.26	2.92	2.92	2.75	1.90

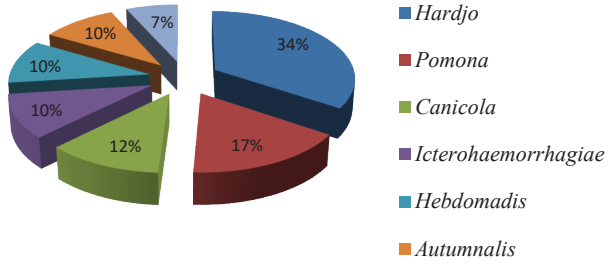


Fig. 2. Distribution of reactive *Leptospira* serovars in bovines in the studied region of Karnataka

peninsular India (Ramani and Punya, 2005), Ratnam *et al.* 1983 and Selvaraj *et al.* 2005, 2010). Similar results were also reported in an organized mixed dairy farm in Gujarat (Balakrishnan *et al.* 2011; Kader *et al.* 2021 and Prapong *et al.* 2023). Generally, the elevated seropositivity for leptospirosis in buffaloes compared to cows can be attributed to wallowing of buffaloes in stagnant water contaminated with drainage water, exposing them to leptospires.

Geographic-wise comparison between cows and buffaloes (Table 4) in the Bengaluru and Mysore divisions revealed higher seropositivity in cows compared to buffaloes. This could be attributed mainly to the large population of crossbred cows and the higher number of samples collected from cows compared to buffaloes. Similarly, in the Gulbarga and Belgaum divisions, seropositivity was higher in buffaloes compared to cows, mainly due to a large buffalo population in these two divisions and the collection of a higher number of samples from buffaloes than cows.

Regarding clinical conditions in both the species, the findings indicate (Table 4) that a history of abortion becomes a crucial criterion when collecting samples for diagnosing leptospirosis. More emphasis should be given to abortion compared to other reproductive disorders as stated earlier (Loureiro & Lilenbaum, 2020). Interestingly, Helio *et al.* (1999) also observed similar findings, with abortion being the major clinical observation compared to other reproductive disorders.

In overall, the seroprevalence study, conducted by employing eight reference serovars, showed the prevalence of six serovars representing serogroup-specific *Leptospira* antibodies in animal cases associated with reproductive disorders. The prevalence of Hardjo serogroup specific

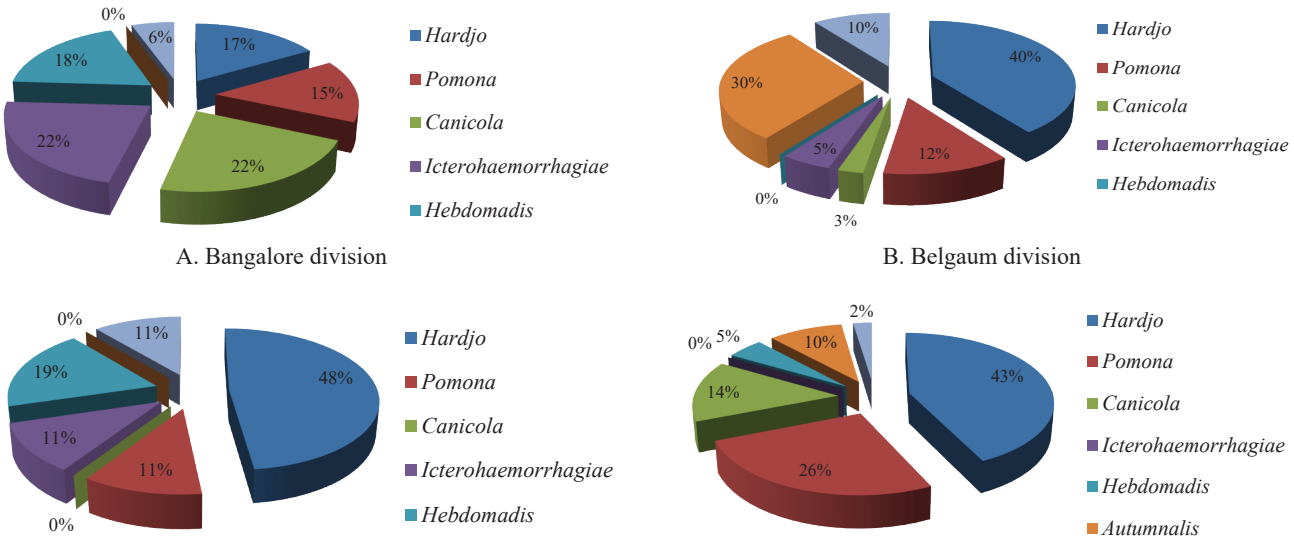


Fig. 3. Distribution of reactive *Leptospira* serovars in bovines in Different division of Karnataka a) Bangaluru b) Belgaum c) Gulbarga and d) Mysore

antibodies was the highest (9.62%), followed by Pomona (4.64%), Canicola (3.26%), Icterohaemorrhagie and Hebdomadis, (each 2.92%), Autumnalis (2.75%), and more than one serovar of 1.90%. These findings correlate with the report of Senthilnathan (2009) in Mandya district of Karnataka with a 6 % prevalence of Pomona serovar antibodies in bovines.

Earlier random survey conducted in animals with history of non-abortion or abortion cases in a few southern districts of Karnataka also reported an overall seroprevalence ranging from 4 to 12% with MAT employing only two serovars. In the present study, a high prevalence of 28% was observed in animals associated with a history of reproductive disorders in MAT using eight serovars. If the study had been restricted to only two serovars, (Pomona and Canicola), the prevalence rate could come down to 7.9%, similar to the observation of an earlier study (Upadhye *et al.* 1981). When comparing seroprevalence with other studies conducted globally as well as in India, the observation of high prevalence of Hardjo followed by Pomona was common in many studies (Leahy *et al.* 2021). Furthermore, studies in Tamil Nadu, Gujarat, Orissa, Maharashtra, and Andaman & Nicobar have also shown high seropositivity compared to the present study in Karnataka, aligning with the observations of earlier researchers. However, if the study was conducted with the addition of more serovars, the overall seroprevalence rate could vary as reported earlier (Amitabha *et al.* 2019; Kader *et al.* 2021; Murugavelu *et al.* 2022).

Moreover, the comparison of seroprevalence across different divisions showed a notably high occurrence of Canicola and Icterohaemorrhagiae serovars in the Bangaluru division. This could be primarily associated with the substantial population of dogs in both the urban and rural parts of Bengaluru and bordering the districts of neighbouring states, which have reported the prevalence of leptospirosis, which might have led to high seropositivity. The transmission rate from the host to the bovines seemed to be high due to close interaction between these species as well. These results coincide with earlier studies (Bahari *et al.* 2011; Ebrahimi *et al.* 2004) that suggested a correlation between the prevalence of Canicola serovar and the number of dogs present in dairy farms in the Sharekord region and Hamedan suburb of Iran. Interestingly, a study by Jafari *et al.* (2011) also indicated a high occurrence of Icterohaemorrhagiae in cattle herds in the aforementioned region, linking it to environmental factors and interactions with other carrier species. Conversely, Hardjo serogroup-specific antibodies were predominantly observed in Mysore, Gulbarga, and Belgaum divisions, compared to the Bangaluru division, suggesting the spread of this serovar across the entirety of Karnataka state.

In the present study, relatively high overall seropositivity with the prevalence of more serovars was noticed in Bangaluru, Mysore, and Belgaum divisions compared to the Gulbarga division, mainly due to the presence of more risk factors in these divisions. As like Bengaluru division, in the Belgaum division, risk factors like the coastal belt, high rainfall, and bordering with the districts of neighbouring states, which have reported the prevalence of leptospirosis, might have led to high seropositivity. In the Mysore division, risk factors like the presence of high paddy cultivation, districts with the coastal belt, and bordering the districts of leptospirosis prevalent in neighbouring states, might have led to high seropositivity. The above findings were in agreement with the earlier reported findings of researchers (Shivakumar, 2008; Veronica *et al.* 2011; Premdas *et al.* 2019), who also reported that risk factors like rainfall, coastal region (humidity), and movement of animals from one area to the other are the major risk factors for high seropositivity.

This study highlights the significant seroprevalence of leptospirosis in bovines in Karnataka, as determined by detecting specific antibodies through the MAT. Further, the research delineates the prevalence of six serovars, indicative of serogroup antibodies, within specific areas of the studied regions. Recognizing these predominant serovars representing specific antibodies holds the potential to enhance the composition of antigen reference panels utilized in MAT for diagnostic laboratories addressing both human and animal diseases. This, in turn, could facilitate more accurate, efficient, and timely diagnosis of leptospirosis, ultimately contributing to the management of public health and 'One Health' concerns. Additionally, understanding the prevalence of these serovars can aid in the development of vaccines, thereby contributing to strategic planning and reducing the impact of the disease in these areas.

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