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Molecular detection and seroprevalence of leptospirosis among aborted sows in Northern Kerala, India#

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Abstract

Present study envisaged the molecular identification of pathogenic Leptospira spp. from cases of abortions, stillbirths and foetal mummifications in pigs and to confirm the seroprevalence of leptospirosis among pigs from organised and semi-organised farms of Northern Kerala. Whole blood (n=21) from aborted sows and tissue (n=32) samples from aborted foetus tested negative for presence of lipl32 gene of Leptospira by polymerase chain reaction. From the epidemiological data collected, the highest occurrence of swine abortion was found within the age group of one to two years (81.25%) in Large White Yorkshire (56.25%) and during December (40.62%). It is observed that majority of animals with reproductive disorders were housed indoors, housed individually and not fed with additional supplements and hotel wastes. All the sows were regularly dewormed and vaccinated. Haematological studies of aborted sows showed marked anaemia, leucocytosis, granulocytosis and monocytopaenia. Sera samples (n=34) collected from pigs with history of abortion were subjected to Microscopic Agglutination Test (MAT) and it was observed that Leptospira interrogans serovar Icterohaemorrhagia was the most prevalent serovar (32.35 per cent, followed by serovar Tarassovi (23.53 per cent), Pomona (14.71 per cent), Canicola (11.76 per cent), and Grippotyphosa (8.82 per cent). An overall seroprevalence of 52.94 per cent for Leptospirosis among pigs was observed.

Keywords: *Leptospira, lipl32 gene, microscopic agglutination test, swine, abortion*

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Many infectious agents cause reproductive disorders in sows and produce a broad spectrum of sequelae, including abortions, weak neonates, stillbirth, mummification, embryonic death and infertility, which lead to huge economic losses to the farmers (Asdell *et al.,* 1941). The major infectious causes of reproductive failure in pigs include Porcine Respiratory and Reproductive Syndrome (PRRS), porcine parvoviral infection, classical swine fever, porcine circoviral infection, brucellosis and leptospirosis (Holler *et al.,* 1994). Simple and rapid diagnostic techniques for the identification of such diseases are still lacking and hence, in most instances, remain underdiagnosed. The prevalence of some of these swine diseases and the resulting economic impact on swine husbandry are reported from a few parts of the country. It is highly demanding that the occurrence of such infectious causes of abortions in Kerala needs to be explored to understand the magnitude and spectra of pathogens circulating, designing proper control and management strategies and thus ensuring economically viable pig farming in the state.

Leptospirosis is a continuing global crisis, especially in developing countries like India, where the tropical climate and other natural phenomenon favours the growth and survival of the causative organism (Behera *et al.,* 2021). The disease is caused by the Spirochaete, Leptospira, the disease affects a wide variety of mammals including humans, cattle, dogs, pigs, horses, sheep and goats (Faine *et al.,* 1999). Human beings are considered as the dead-end hosts who acquire the infection via contact with water and soil contaminated with urine of infected animals as well as via direct contact (Habus *et al.,* 2017). The studies on swine leptospirosis are critical as far as the pig industry is concerned, since it is one among the infectious etiologies associated with abortions worldwide. *Leptospira interrogans* infection in sows and gilts include abortions or birth of dead or weak pigs often occur without premonitory signs (Ramos *et al*., 2006).

Diagnosis includes culture techniques, serological and molecular methods. Microscopic agglutination test is the gold standard test for the diagnosis of leptospirosis (Mousing *et al.,* 1995). It is a useful herd test which also enables identification of the prevalent serovars in an area, thereby providing an epidemiological database for further studies. There exists serovar-wise variation in the clinical presentation of the disease in pigs. Microscopic agglutination test enables serovar specific and quantitative diagnosis of leptospirosis based on antibody detection (OIE, 2014).

Swine leptospirosis, characterised by anorexia, weakness, icterus and complications with reproductive problems, have been reported from many parts of India. Yet, further epidemiological studies for identification of the prevalent serovars of Leptospira and implementation of proper control strategies are far from priorities. Present study envisaged molecular identification of pathogenic *Leptospira* spp. from cases of abortions, stillbirths and foetal mummifications and to identify the important serovars of Leptospira among pigs from organised and semi-organised farms of Northern Kerala, India as a preliminary study for future research.

Materials and methods

The study was conducted in the Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Science, Pookode, Wayanad during the period of September 2022 to January 2023 to identify leptospiral antigens associated with infectious abortion in domestic pigs by molecular diagnostic techniques. Whole blood (n=21) from aborted sows and tissue samples (n=32) from aborted foetuses were collected, processed and analysed in the present study. Epidemiological data such as the details and source of the animal, deworming and vaccination history, details of farrowing, the date of breeding and abortion and details of feeding and management were collected from registers maintained at the farm. A complete blood count including the following parameters *viz.* total erythrocyte count, total leukocyte count, haemoglobin concentration, platelet count, VPRC, MCV, MCH, MCHC, and differential leukocyte counts such as percentage of granulocyte, lymphocyte, and monocyte, were performed within 12 hours of collection of samples.

Serum samples from 34 sows with a history of abortion and stillbirth in the last one month were collected randomly from organised and semi-organised pig farms of Northern districts of Kerala to study the seroprevalence. Blood samples of two to three mL were collected in plain clot activator vials. The samples were centrifuged for separating serum and stored at -20°C. All the samples were subjected to MAT employing a panel of five live reference serovars of Leptospira, *viz*. *Leptospira interrogans* serovars *Icterohaemmorhagiae, Pomona, Canicola, Grippotyphosa* and *Tarassovi*. A 1:100 serum dilution was prepared in sterile PBS (HiMedia®, India), 25 µL of which was taken in 96 well microtitre plates (Tarsons®) and mixed with 25 µL of each of the five to ten day-old live Leptospira serovars separately. Antigen controls were set with 25 µL sterile PBS and 25 µL of different live Leptospira serovars and the plates were incubated at 37°C for two hours. After incubation, the results were read by examining a drop of the serum-antigen mixture from each well under low power of a dark field microscope for agglutination of leptospires.

Further, quantitative assay was carried out in 96 well microtitre plates against the reacting serovars of Leptospires. All the 96 wells were filled with 25 µL PBS. In the first well of each row, 25 µl of 1 in 100 dilution serum samples were added and mixed. Then, serial double fold dilutions were made up to twelve wells and 25 µL was discarded from the last well. A constant volume of 25 µL of a particular serovar was added in each row and incubated at 37°C for two hours. All the final dilution mixtures (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100 and 1200) were observed under a dark field microscope and the results were recorded. The reciprocal of the highest dilution of the serum which showed 50 per cent agglutination or 50 percent reduction in the number of free Leptospires, in comparison to the control, was considered as the respective titre (Goris *et al.,* 2014).

Molecular detection of Leptospira from tissue and whole blood

To detect infectious etiologies of abortions, total of 32 tissue samples from aborted foetus and 21 whole blood samples from aborted sow were collected and subjected to *lipl32* gene-specific PCR for detection of Leptospira (Amrutha *et al*., 2007). Amplification was done using *lipl32* F (- 5'- CGCGCTGCAGTTACTTAGTCGCGTCAGAAG – 3') and *lipl32 R* (5'-CGCGGTCGACGCTTT CGGTGGTCTGCCAAGC-3') with cycling conditions including and initial denaturation (94°C for 4 min) followed by 25 cycles of denaturation (94°C for 30 sec), annealing (54°C for 30 sec) and extension (72°C for 45 sec) and a final extension of 72°C for 2 min. Agarose gel electrophoresis and visualisation in gel documentation system were done to assess the amplicons.

Results and discussion

In this study, 81.25 per cent of aborted sows were in their first and second parity and within the age of one to two years. Majority of animals aborted during their younger age, which was in agreement with Canario *et al.* (2006), who mentioned that, sows within their first parity, who can have a relatively high number of stillborn piglets per litter which might be due to a narrow birth canal. According to Borges *et al*. (2005), an increasing number of stillborns with increasing parity might be due to poor uterine muscle tone, less efficient labour and prolonged farrowing in older pigs. The various reproductive disorders of pigs under study are shown in Fig 1 to 5.

Epidemiological data of aborted sows and sero positive animals is shown in Table 1. Present study showed that, 44.44 per cent of sows, seropositive for leptospirosis were in their first parity and within the age group of one to one and a half years followed by 33.33 per cent in their second parity within the age group of one and a half to two years. Upon comparison between aborted and seropositive animals within the age group, highest seropositivity of leptospirosis was observed within two and half to three years (100 %) followed by one to one and half years (57.14 %). Present results are in agreement with Yung *et al.* (2008), who reported a positive correlation between age and seropositivity when compared within the age group between aborted and seropositive

Fig. 1. Early embryonic death

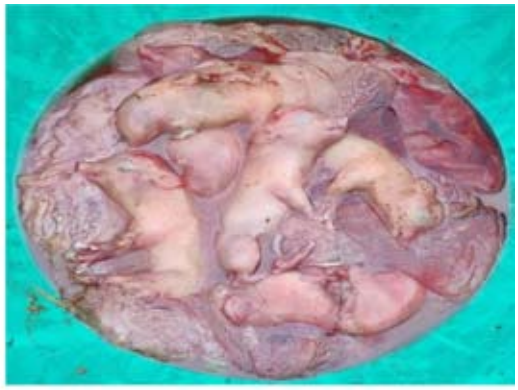

Fig. 2. Abortion at mid term

Fig. 3. Abortion at late term

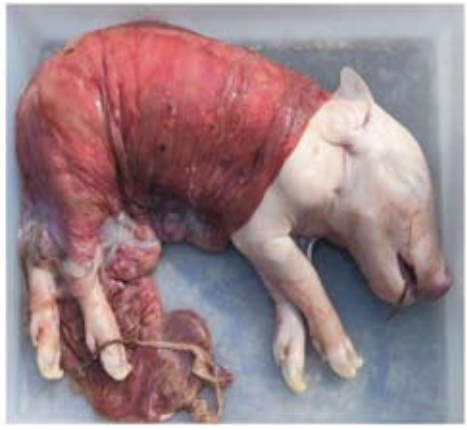

Fig. 4. Still birth piglet

Fig. 5. Mummified foetus

animals. According to Suepaul *et al.* (2011), there was a significant difference observed in the seroprevalence of leptospirosis among different age groups and highest prevalence was observed between one to three years of age group. According to Behera *et al.* (2021), higher seropositivity were observed in adult pigs of greater than two years which is in agreement with the result when compared within the age group between aborted and seropositive animals.

Large White Yorkshire (LWY) showed a high occurrence of (56.25%) abortion followed

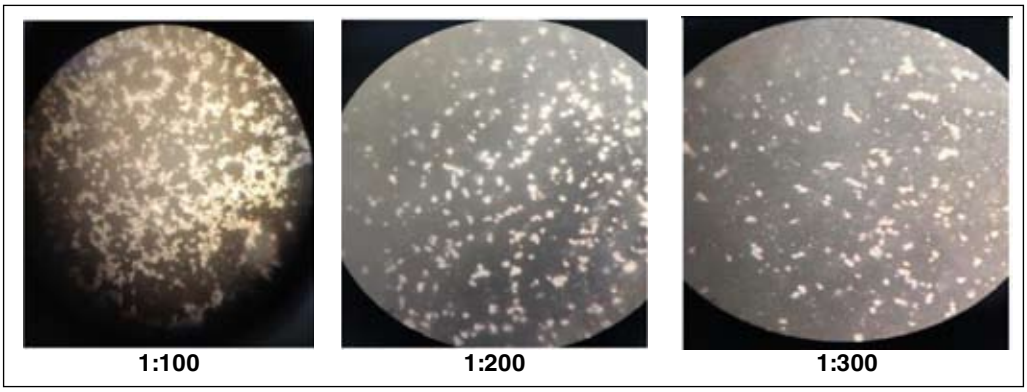

Fig. 6. Microscopic agglutination test by DFM (10X)

by crossbred (31.25 %). Although the between-breed variation in the proportion of stillborn piglets is rather limited, this statement is in agreement with Leenhouwers *et al*. (1999), who mentioned that purebred lines had significantly more stillbirths per litter than crossbred lines. Crossbreeding has a favourable effect on neonatal survival (Blasco *et al*., 1995). In this study, Large White Yorkshire showed a high occurrence of 54.16 per cent followed by crossbred (29.16%).

Large White Yorkshire showed high seropositivity of 55.55 per cent for leptospirosis followed by crossbred (33.33%). Upon comparison between aborted and seropositive animals within the breed, highest seropositivity of leptospirosis was observed in crossbred (60.00%) followed by LWY (55.55%). According to Behera *et al.* (2021), higher seropositivity for leptospirosis were observed in cross bred pigs when compared to indigenous pigs which is in agreement with result when compared within the breed between aborted and seropositive animals.

Highest occurrence of abortion was observed during December (40.62%) followed by November, January and October. This result is in agreement with the findings of Asdell *et al.* (1941), who stated that more stillborn piglets were born during the winter months, it has been suggested that dietary and management changes in the winter months might increase the stillbirth rate. Braude *et al.* (1954) could find no seasonal influence in stillbirth. Ravindranath *et al.* (2011) reported that Indian climate is categorised into hot-dry, warm-humid, temperate and cold and subgroup composite and a greater part of the country falls under the composite followed by warm-humid zone, so we can partially correlate the season with results.

The highest occurrence of leptospirosis was observed during December (44.44 per cent), followed by November and September with the least occurrence was observed during January and October. Upon comparison between aborted and seropositive animals within the months, highest seropositivity of leptospirosis was observed in September (100%) followed by November (85.71%) and December (61.53%). Results of present study are in agreement with Suepaul *et al.* (2011) who reported that prevalent serovars causing porcine abortion changed with seasons, where Canicola during warmer months and Icterohaemorrhagiae in the colder months due to the serovars specific temperature requirements for survival. However, Habus *et al.* (2017) recorded highest incidence of leptospirosis in late summer and autumn months. Tangkanakul *et al.* (2005) stated that peak incidence was reported in September and October months with the rainy season which is in agreement with present result.

Seventy-five per cent of sows were housed in indoor pens, whereas 25 per cent were housed indoor with outdoor access, twenty-five per cent with other sows in their pen, and 75 percent housed individually. Additional supplements and hotel waste were fed to 12.50 per cent of sows. All the 32 sows were regularly dewormed and vaccinated. Data on

Table 1. Epidemiological data of aborted sows and seropositive animals

Variables	Variable Categories	Aborted sows N=32 (%)	Seropositive animals N=18 (%)
Age	*1 to 1 ½ years* *1 ½ to 2 years* *2 to 2 ½ years* *2 ½ to 3 years*	*14 (43.75)* *12 (37.50)* *3 (18.75)* *3 (18.75)*	*8 (44.44)* *6(33.33)* *1(5.55)* *3 (16.66)*
Breed	*Large White Yorkshire* *Crossbred* *Landrace* *Duroc*	*18 (56.25)* *10 (31.25)* *2 (6.25)* *2 (6.25)*	*10 (55.55)* *6(33.33)* *1 (5.55)* *1 (5.55)*
Month	*September* *October* *November* *December* *January*	*2 (6.25)* *4 (12.50)* *7 (21.88)* *13 (40.62)* *6 (18.75)*	*2 (11.11)* *1 (5.55)* *6 (33.33)* *8 (44.44)* *1 (5.55)*
House environment	*Indoor* *Indoor with outdoor access*	*24 (75.00)* *8 (25.00)*	*18 (100)* *0*
Source of Water	*River/Tap Water* *Wallowing pond*	*28 (87.50)* *4 (12.50)*	*16 (88.88)* *2(11.11)*
Source of Feed	*Scavenging/Hotel/kitchen waste* *Concentrate Feed/Corn by products*	*4 (12.50)* *28 (87.50)*	*2 (11.11)* *16 (88.88)*
No. of Animals in pen	*Housed individually* *Housed in group*	*24 (75.00)* *8 (25.00)*	*18 (100)* *0*

Table 2. Data of association between seropositivity and managemental factors

Variables	Variable categories	Seropositive (n = 18)	Seronegative (n = 14)	Chi square statistic	p value
Age	1 - 1.5 years	8	6	3.169[a]	0.366[ns]
	1.5 - 2 years	6	6		
	2 - 2.5 years	1	2		
	2.5 - 3 years	3	0		
Breed	LWY	10	8	0.124[a]	0.989[ns]
	Crossbred	6	4		
	Landrace	1	1		
	Duroc	1	1		
Month	September	2	0	9.58[a]	0.048[*]
	October	1	3		
	November	6	1		
	December	8	5		
House environment	Indoor	18	6	13.714[a]	0[ns]
	Indoor with outdoor access	0	8		
Source of water	River/tap water	16	12	0.073[a]	0.788[ns]
	Wallowing pond	2	2		
Source of feed	Scavenging/hotel waste	2	2	0.073[a]	0.788[ns]
	Concentrate feed	16	12		

Test statistic of Chi square test

ns – non significant, [*]significant at ≤ 0.05 level, [**]significant at ≤ 0.01 level.

Table 3. Comparison of haematological parameters of aborted sows and control group

Haematological parameters	Aborted sows Mean ± SEM	Control group Mean ± SEM	Test statistic	p Value
TEC (10^6/cmm)	6.10 ± 0.51	7.08 ± 0.15	88.00†	0.001**
TLC (10^3/cmm)	18.81 ± 1.47	14.72 ± 0.82	2.42ⁱ	0.02*
Hb (g%)	12.79 ± 1.00	13.77 ± 0.32	102.50†	0.003**
VPRC (%)	0.32 ± 0.03	0.43 ± 0.01	3.52ⁱ	0.001**
Platelets(x10m^3/cmm)	164.81 ± 17.10	160.29 ± 18.39	0.18ⁱ	0.858ns
MCV (fL)	58.37 ± 2.66	60.53 ± 1.75	169.50†	0.199ns
MCH (pg)	21.15 ± 0.46	19.55 ± 0.48	2.41ⁱ	0.02*
MCHC(g%)	37.31 ± 1.34	32.69 ± 1.01	103.00†	0.003**
DLC(G%)	57.34 ± 2.38	41.20+ ± 2.49	4.69ⁱ	<0.001**
DLC(L%)	37.29 ± 2.00	44.80 ± 1.81	2.79ⁱ	0.008**
DLC(M%)	5.37 ± 0.64	14.00 ± 1.45	45.00†	<0.001**

the impact of management related factors on stillborn piglets are scarce and mainly focused on the supervision of farrowing, pen design, ambient temperature or dietary fibre (Lucia *et al.,* 2002).

All the 18 sows seropositive for leptospirosis were in indoor pens and housed individually. Hotel waste was fed to 11.11 per cent of animals. Behera *et al.* (2021) reported a higher frequency of seropositivity in pigs reared in farms where rodents had access to feeders and waterers than in farms with limited rodent access. Rodents were the reservoir host of serovar Icterrohaemorrhagiae and it might be one of the reasons for the prevalence of serovar Icterohaemorhagiae in the present study. Factors such as the size of flock, topography, the type of animal housing and the presence of silos did not significantly affect seroprevalence (Ramos *et al.,* 2006).

Association between seropositivity and managemental factors were studied. Statistical analysis of data was done using IBM SPSS software and the results are shown in Table 2. There was significant difference in month ($p \leq 0.05$) when compared seropositive group with seronegative group. There was no significant difference in age, breed, housing, source of water and feed when seropositive group was compared with seronegative group.

Haematology

The complete blood count was estimated and compared haematological parameters of the aborted sows (n=21) with the values obtained from those of healthy control group (n=21). Statistical analysis was done using IBM SPSS software and the results are shown in Table 3. There was significant anaemia, leukocytosis, monocytopenia, lymphopenia and granulocytosis. Haematological parameters are affected by a variety of factors including age, sex, health status, breed and season (Cooper *et al*., 2014). An association has been recorded between stillbirths and haematological values in the sows and which might be related to oxygen supply during farrowing (Bhattarai *et al*., 2018). Increased WBC count could be associated with an inflammatory response to uterine involution or responses to infections (Oven *et al.,* 2018).

The complete blood count was estimated and compared with the haematological parameters of the sows, seropositive for leptospirosis (n=15) with the values obtained from apparently healthy control animals (n=15). Statistical analysis was done using IBM SPSS software and the results are shown in Table 4. The results of the present study showed significant monocytopaenia and granulocytosis. Statistically significant alterations ($p \leq 0.05$) were not observed in average erythrocyte count, VPRC and haemoglobin concentration, which might be due to the relative resistance of porcine erythrocytes to the haemolytic effects of serovar Pomona (Alexander *et al.,* 1956). In experimental work with haemolysins, the ovine and bovine erythrocytes were highly susceptible to haemolysins, while the pig erythrocytes were relatively resistant (Bauer *et al.,* 1958). Haemoglobinuria, icterus and anaemia were the common findings in experimental and natural

Table 4. Comparison of haematological parameters of sows seropositive for leptospirosis and control group

Haematological parameters	Sows seropositive for leptospirosis (n=15)	Control (n=15)	Test statistic	p Value
	Mean ± SEM	Mean ± SEM		
TEC(10°/cmm)	6.48 ± 0.66	6.99 ± 0.20	55†	0.016*
TLC(10^3/cmm)	19.17 ± 1.69	14.64 ± 0.94	2.340ⁱ	0.027*
Hb(g%)	13.61 ± 1.27	13.82 ± 0.41	67.50†	0.062ns
VPRC(%)	0.34 ± 0.03	0.42 ± 0.02	2.348ⁱ	0.029*
Platelets(x10m^3/cmm)	162.27 ± 20.04	190.4 ± 20.55	0.980ⁱ	0.335ns
MCV(fL)	56.94 ± 3	60.84 ± 2.21	73.50†	0.106ns
MCH(pg)	21.28 ± 0.56	19.89 ± 0.62	1.675ⁱ	0.105ns
MCHC(g%)	38.25 ± 1.39	33.09 ± 1.21	2.797ⁱ	0.009**
DLC(G%)	55.48 ± 2.88	41.37 ± 3.28	3.236ⁱ	0.003**
DLC(L%)	38.93 ± 2.38	45.54 ± 2.51	1.908ⁱ	0.067ns
DLC(M%)	5.59 ± 0.86	13.09 ± 1.63	4.063ⁱ	0.001**

SEM; Standard Error of Mean, † Test statistic of Mann Whitney U test,

ⁱ Test statistic of Independent t-test, ns – non significant, ˙significant at ≤ 0.05 level, ¨significant at ≤ 0.01 level.

Table 5. MAT titres to different serovars

Serovars	Leptospira titres		
	1:100	1:200	1:300
Canicola	5	-	-
Grippotyphosa	2	-	-
Pomona	5	-	-
Icterrhaemarrhagia	8	2	1
Tarassovi	8	-	-

Table 6. Seroprevalence of leptospirosis by MAT

Serovars	No. of positive samples	Percent positivity (%)
Canicola	4	11.76
Grippotyphosa	3	8.82
Icterohaemorrhagiae	11	32.35
Pomona	5	14.71
Tarassovi	8	23.53

serovar Pomona infections in cattle and sheep. Such manifestations were rarely observed in swine (Bauer *et al.,* 1961).

A total of 34 serum samples from sows with history of recent abortion or stillbirth, were collected from different areas of the Wayanad and Kozhikode districts and the serum samples were subjected to MAT. A titer of 1:100 or above was regarded as positive and the serovar reacting to the highest titer corresponded to the infecting serovar. Out of the 34 porcine serum samples subjected to MAT, 18 samples were positive (52.94 percent). Among positive samples, five had mixed infection with two serovars, one sample had mixed infection with three serovars and two samples had mixed infection with four serovars. Six out of eighteen (33.33 per cent) samples had mixed infection of Leptospira serovars Icterohaemorrhagiae and Tarassovi, four (22.22 per cent) had Pomona, three (16.66 per cent) had Canicola and two (11.11 per cent) had Grippotyphosa. The most predominant serovar was found to be Icterohaemorrhagiae (32.35 per cent), followed by Tarassovi (23.53 per cent), Pomona (14.71 per cent), Canicola (11.76 per cent) and Grippotyphosa (8.82 per cent). The titres ranged from 1: 100 to 1:300 with highest titre of 1:300 for serovar Icterohaemmorhagiae.

Pomona was the most prevalent serovar in studies conducted in past decades, while Icterohaemorrhagiae became

predominant after 1980s. The most probable explanation for this could be increase in pig farming, making more difficult for pig to pig contact and direct transmission of Pomona strains (Delbem *et al.,* 2004). Conversely, the prevalence of Icterohaemorrhagiae indicates direct or indirect contact with rodents as the source of infection of the animals and its occurrence suggests a failure in rodent control in and around pig farms (Delbem *et al.,* 2002). Serovars Icterohaemorrhagiae and Canicola were found to be associated with stillbirths and weak newborn piglets (Ramos *et al*., 2006). The results of MAT have been summarised in Table 5 and Fig 6 and seroprevalence of leptospirosis by MAT as shown in Table 6.

Molecular detection

To identify major infectious etiologies of abortions, all the samples were subjected to PCR for the detection of Leptospira. Gel electrophoresis was performed and none of the tissue and blood samples were found to be positive in PCR for Leptospira even those which tested positive in MAT.

Conclusion

From the epidemiological data collected, the highest occurrence of swine abortion was found within the age group of one to two years in Large White Yorkshire during December. Blood parameters of aborted sows were analysed and found that there was marked anaemia, leucocytosis, granulocytosis and monocytopenia. Several environmental factors and stress factors *viz.* housing system, overcrowding, feeding habits, vaccination and deworming schedule were studied in which majority of animals were housed indoor without access to outside, housed individually and not fed with additional supplements and hotel waste. All the sows were regularly dewormed and vaccinated. The present study also reports the prevalence of Leptospira serovars among pigs in northern Kerala which are associated with abortions in pigs. The animals having antibodies against leptospirosis might be exposed to infection and presently serve as serologically positive carrier animals. Most studies on porcine leptospirosis so far in Kerala are concentrated to discrete regions. Hence, more elaborate studies including isolation of Leptospira from the environment and animal reservoirs must be undertaken in addition to serology for a better understanding of the epidemiology of the infection in the area.

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Conflict of interest

The authors declare that they have no conflict of interest.

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