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Short Communication

Molecular Detection and Pathological Investigation of Contagious Bovine Pleuropneumonia in Selected Districts of Punjab, Pakistan

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Authors' Contribution
A Anjum conducted the research. A Aslam supervised the research. RA, TY and MRK were members of supervisory committee. RS was research associate in the project and funded this research. SU helped in laboratory work. AZD and MU helped in write up of the manuscript.

Key words
Contagious bovine pleuropneumonia, *Mycoplasma mycoides* subsp. *Mycoides*, PCR, Pathological lesions, Histopathology

ABSTRACT

Contagious Bovine Pleuropneumonia (CBPP) is an infectious respiratory disease of cattle, characterized by anorexia, fever, dyspnea, polypnea, cough, and nasal discharges and caused by *Mycoplasma mycoides* subsp. *mycoides*. This study was carried out in abattoirs of Lahore, Kasur and Jhang districts of Punjab, Pakistan. Different tissue samples were collected from 560 cattle showing respiratory signs at slaughter. Lung samples were used for detection of CBPP through PCR targeting 16S ribosomal RNA. Furthermore, lungs, liver, kidney and lymph node were collected for histopathological examination. Of the 560 samples, 49 (8.25%) were found positive for CBPP. Maximum pathological lesions (50-75% of surface area) were seen in 34.69 percent lung samples, chronic type pleurisy in 97.96 percent, maximum aggregate pathological lesion score in 65.31%, while 48.98% lung samples had 6-10 numbers of sequestra. Besides, infiltration of inflammatory cells, congestion, hemorrhages, necrosis and other degenerative changes were observed in tissue samples of infected cattle. Significantly higher ($P<0.05$) micro-pathological lesion scores were given to lungs, kidney and lymph node samples from infected cattle as compared to those of non-infected ones. In conclusion, CBPP, being a major factor affecting cattle production and associated economics, warrants the implementation of control measures to mitigate the economic losses associated with the disease.

Contagious bovine pleuropneumonia (CBPP) is a disease of the respiratory tract of cattle caused by *Mycoplasma mycoides* subsp. *mycoides*. This disease was first described in 1564 by Gallo, while first explicit description of the disease was documented in Switzerland in 1773. In the 19th century, the disease reached its worldwide distribution but was eradicated from North America and Australia by adapting a stamping-out policy (Iles *et al*., 2019). The disease is prevalent mostly in Africa, where it is responsible for high economic losses, but was sporadically reported from Southern Europe until 1999. The disease is still endemic in the Middle East and many Asian countries (Nicholas and Churchward, 2012).

The disease is characterized by a long incubation period, i.e., up to six months and is manifested by anorexia, fever, dyspnea, polypnea, cough, and nasal discharges. Mostly infection is limited to the respiratory tract, although arthritis occurs in young calves usually less than 6 months of age (Grieco *et al*., 2001). Recovered cattle persist to be chronic carriers and usually suffer from a persistent low-grade fever and the loss of condition along with respiratory signs manifested upon exercise, these animals also become

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a source of infection for the uninfected herds (Scacchia *et al*., 2011). It induces lesions of pleuropneumonia in acute cases and the formation of pulmonary "sequestra" in chronic cases (Di Provvido *et al*., 2018).

The clinical diagnosis of CBPP is difficult as early signs of the disease may be slight or non-existent and are difficult to differentiate from any form of severe pneumonia with pleuritis. Confirm diagnosis of the disease is accomplished by demonstration of characteristic pathological lesions and presence of *Mycoplasma* after postmortem examination, isolated from pneumonic lesions, characteristic of CBPP along with the other diagnostic tests. World Organization for Animal Health (OIE) has approved two serological tests; the complement fixation test and a competitive enzyme-linked immunosorbent assay for the diagnosis of CBPP, but these tests can only be performed at the herd level (Muuka *et al*., 2011). Polymerase chain reaction (PCR) has higher sensitivity than the isolation technique; hence, the use of PCR is highly recommended in CBPP surveillance especially in test and slaughter procedure for the disease eradication program (Musa *et al*., 2016).

The purpose of this study was to evaluate the macro and micro pathological changes induced by CBPP in cattle. The study was restricted to identifying CBPP characteristics lesions in lung samples, collected from three districts of Punjab-Pakistan, which were further confirmed by PCR.

Materials and methods

The current study was carried out in abattoirs of Lahore, Kasur and Jhang districts of Punjab, Pakistan. A total of 560 liver, kidney, lymph node and lung samples of cattle were collected. For histopathological examinations, tissue samples of these organs were also collected in 10% neutral buffered formalin. The samples were processed in the Department of Pathology, University of Veterinary and Animal Sciences, Lahore.

All animals were inspected before slaughter for any obvious sign of disease. Special attentions were given to respiratory issues such as breathing pattern (labored/distressed), coughing (dry/moist), standing posture (nostrils dilated, neck extended).

Post-mortem examination of animals was also done as described by (Phiri, 2006). Characteristic gross pathological lesions of lung sample including pleurisy (acute or chronic), consolidation, marbling or thickening of interlobular septa, red or grey hepatization, necrosis, sequestra formation and fibrosis (Di Provvido *et al*., 2018). If a lung sample had lesions covering <25% of its surface area it was scored as 1; samples with lesions on 25-50% of surface were scored as 2; lungs with 50-75% lesions were scored 3 and the lung samples where lesions were observed on more than 75% of surface area, were scored as 4.

Lungs samples were used for DNA extraction using the DNeasy Blood and Tissue Kit (Qiagen). DNA was suspended in 25 µL double distilled water to use it as a template for PCR.

For confirmation of *Mycoplasma mycoides* subsp. *mycoides* in lung samples, PCR was performed using specific primer set (Table I), targeting the 16S ribosomal RNA (rRNA) gene. Amplification conditions were as: initial denaturation 95℃ (5 min), with 35 cycles of denaturation 95℃ (30 sec); annealing 55℃ (30 sec); extension 72℃ (50 sec); and final extension 72℃ (7 min). PCR product was run on 1% agarose in TAE buffer using gel electrophoresis apparatus keeping 120 Volts and 300 Ampere for 35 minutes. Agarose gel was seen using UV transilluminator for confirmation of results.

For histopathological examination, samples were processed, embedded with paraffin, sliced with microtome and stained with hematoxylin and eosin stain as described by Bancroft and Layton (2018), Spencer (2018) and Wolfe (2018). Lesion scoring of tissue samples were performed as documented by (Eveillard *et al*., 2010; Kleiner *et al*., 2005; Klopfleisch, 2013; Park *et al*., 2009).

Unpaired t-test was used to analyze the differences in lesions scoring in infected and non-infected animals. Significant differences between the groups were reported at value of $P < 0.05$ using SPSS for Windows (version 23.0)

Table I. Primer sequences used in PCR targeting 16S rRNA gene.

Primer	Sequence (5'-------3')	Product size
Forward	AAAATGAGAGTTTGATCCTGG	1525 bp
Reverse	AGAAAGGAGGTGATCCATCCG	

Results and discussion

Out of 560, 49 (8.25%) samples were found positive for CBPP as confirmed through PCR with amplicon size of 1525 (Supplementary Fig. 1), using specific primers targeting 16S rRNA gene. Nucleotide sequence results of these isolates are available on NCBI GenBank Database with accession no. (MK692950-54).

Table II shows pleurisy score, lesion score on the basis of % surface area, lesion score on the basis of number of sequestra and aggregate lesion score of lung samples of cattle infected with CBPP. Histopathological lesion score of lungs, liver, kidney and lymph node samples of infected and non-infected cattle were described in Table III.

Outcomes of chi-square statistical assessment revealed that a significant difference (p= 0.016) was seen

Table II. Macro-pathological lesion scoring of lung samples of cattle infected with CBPP.

Parameters	Negative	Positive		Chi-square
		n	%	
Pathological lesions on surface area				
<25%	43	6	12.24%	p= 0.016
25-50%	38	11	22.45%	
50-75%	32	17	34.69%	
>75%	34	15	30.61%	
Number of sequestra				
None	49	00	0.00%	p=0.000
1-5	43	6	12.24%	
6-10	25	24	48.98%	
11-15	30	19	38.78%	
Type of pleurisy				
Acute	48	1	2.04%	p=0.000
Chronic	1	48	97.96%	
Pleurisy score				
0	49	0	0.00%	p=0.016
1	48	1	2.04%	
2	48	1	2.04%	
3	45	4	8.16%	
4	46	3	6.12%	
5	46	3	6.12%	
6	44	5	10.20%	
7	45	4	8.16%	
8	42	7	14.29%	
9	39	10	20.41%	
10	43	6	12.24%	
11	44	5	10.20%	
Aggregate pathological lesion score				p=0.000
<25	43	6	12.24%	
25-50	17	32	65.31%	
>50	38	11	22.45%	

Table III. Microscopic lesion score (Mean ± SD) in various tissue samples.

Tissue sample type	Microscopic lesion scores		P value
	Infected animals	Non-infected animals	
Lungs	3.35±0.75[a]	0.53±0.49[b]	0.000
Kidney	1.00±0.57[a]	0.46±0.29[b]	0.000
Liver	0.53±0.04	0.50±0.03	0.199
Lymph node	1.92±0.81[a]	0.76±0.43[b]	0.000

[a,b] within a row, values having different superscripts are different significantly (p<0.05)

in terms of pathological lesions on percent surface area of lungs. The maximum number of lung samples (34.69%) had lesions on 50-75% while least (12.24%) had lesions on < 25% surface area (Table II). A significant difference (p=0.000) was also observed regarding the presence of number of sequestra, as maximum lung samples (48.98%) had 6-10, followed by 38.78% lung samples 11-15 and least 12.24% lung samples had 1-5 sequestra formation with encapsulated necrotic debris (Table II). Acute type of pleurisy was seen in only 2.04% lung samples while chronic type of pleurisy was seen in 97.96% lung samples with p=0.000 (Table II). Our results are in line with the observations of Francis *et al*. (2018) who reported that animals infected with CBPP had thick layer of fibrous connective tissue in visceral and parietal layer of pleural, hence chronic type of pleurisy was more common in CBPP infected cattle as compared to acute type.

While taking into account the pleurisy score, statistical analysis revealed a highly significant difference (p=0.016) in lung samples. In addition to this, data analysis also showed a high significance difference (p=0.000) in lung samples with respect to aggregate pathological lesion score: 12.24%, 65.31% and 22.45% lung samples had aggregate pathological lesion score of <25, 25-50 and >50, respectively (Table II). These results are compatible with the findings of Di Provvido *et al*. (2018) who reported that maximum number of lung sample had aggregate pathological lesion of 25-50, which can be attributed due to cranio-ventral pattern of disease, hence 7-8 lobes of lungs are mostly infected instead of 11 lobes.

Moreover, unpaired t-test statistical assessment revealed a significant difference (p<0.05) in terms of microscopic lesions in lung samples. Non-infected animals have normal structure of lung samples with normal architecture of alveoli while infected animals had pulmonary hemorrhages, thickening of interlobular septa, pulmonary edema and bronchiolitis and alveolitis. Our results are similar to findings of Li *et al*. (2016) who reported the accumulation of inflammatory cells in alveoli, bronchioles, necrotic debris in bronchiolar lumen, disruption of ciliated epithelium of bronchioles and pulmonary congestion in lung samples of CBPP infected cattle. Likewise, a significant difference was observed (p<0.05) in relation to microscopic lesions in kidney samples, as non-infected animals have normal kidney with no observable changes in renal tubules, renal corpuscles and interstitium while infected animals had mild tubulo-interstitial nephritis with renal hemorrhages and mild glomerulopathy. These findings are similar to those of Grieco *et al*. (2001) who reported interstitial hemorrhages and nephritis in kidney samples of CBPP infected cattle. There was no significant difference (p>0.05) in liver

samples, as no observable microscopic lesions were seen in infected and non-infected animals, though mild hepatic congestion was noticed in infected animals. A significant difference ($p<0.05$) was also observed in histopathological examination of lymph nodes. Non-infected animals had normal lymphoid follicle containing immune cells including lymphocytes and macrophages whereas infected animals had hyperplastic lymphoid follicle with increased number of reactive immune cells. Our results are consistent with the findings of Muuka *et al*. (2017) who documented increased number of lymphocytes with mild coagulative necrosis in lymph nodes of CBPP infected cattle. This can be attributed due to enhanced reactivity of immune cells in disease conditions.

Conclusion

Conclusions of the current research provide evidence about molecular detection (8.25% positive samples) and significantly high ($P<0.05$) micro-pathological lesions induced by CBPP in lung, kidney and lymph node sample of cattle in abattoirs of three districts of Punjab.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20190706160736

Statement of conflict of interest

All authors declared no conflict of interest.

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