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Research Article

PATHO-MICROBIAL INVESTIGATIONS ON RESPIRATORY DISEASES OF CATTLE CALVES

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ABSTRACT

Patho-microbial investigations on respiratory diseases were carried out in six cattle calves submitted for necropsy at the Department of Veterinary Pathology, COVS, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, from August 2018 to February 2019. The pathological studies revealed pulmonary emphysema, atelectasis and vascular disturbances such as congestion and haemorrhage associated with sero-fibrinous and interstitial pneumonia. Microbiological isolations from lungs and tracheal swabs revealed isolation of *Escherichia coli* belonging to O83, O134 and O149 serotypes. *E. coli* strains were found to be most sensitive to chloramphenicol (94.29 %), gentamicin (85.72 %), ceftriaxone/tazobactum (82.86%), cefoperazone plus sulbactum (74.30 %). Molecular investigations revealed negative results for both *Pasteurella multocida* and *Mycoplasma bovis*. The results of the present study indicated that *E. coli* as the main respiratory affection affecting cattle calves which highlights the role of maintenance of proper hygienic conditions and managemental practices at farm level and use of appropriate antibiotics by employing drug sensitivity test for the treatment and control of such conditions.

Keywords: Cattle calves, *Escherichia coli*, Interstitial pneumonia, *In-vitro* drug sensitivity test, Sero-fibrinous pneumonia

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In cattle, respiratory infections are still a major source of clinical illness, mortality, productivity loss, and poor carcass quality. The term bovine respiratory disease (BRD) encompasses pneumonias in cattle caused by an array of infectious agents and environmental factors, either singly or in combination resulting in a complex range of pulmonary lesions. Many bacterial conditions like colibacillosis, pasteurellosis, mycoplasmosis, *Mannheimia haemolytica* infection, *Histophilus somnus* infection, salmonellosis, tuberculosis and paratuberculosis; viral disease conditions like bovine herpes virus-1 infection (BoHV-1), parainfluenza virus-3 infection (PI-3), bovine respiratory syncytial virus infection (BRSV), adeno virus, infectious bovine rhinotracheitis (IBR) and bovine viral diarrhoea virus infection (BVDV); parasitic diseases primarily lungworm infestation and fungal infection like aspergillosis affects respiratory system of cattle resulting in the heavy mortality and decline in overall production. These pathogens are also responsible for weakening the host immune response thus making the host more susceptible to opportunistic pathogens. Deterioration of the hygienic conditions along with unusual rainfall, floods and improper managemental practices are some of the most important predisposing factors that aggravate and promote respiratory infections in cattle particularly young ones.

Calf mortality is considered as one of the major constraints to herd expansion and genetic improvement in the dairy sector. According to researchers, 20% calf mortality reduces net profit to approximately 40% (Singh *et al.*, 2009). Incidence of calf mortality vary from 2 to 20% in exotic dairy breeds under temperate climate (Radostits *et al.*, 2000). However, in India calf mortality ranges from 12.5 to 30% (Singh *et al.*, 2009), even it may be as high as 81%. Because of the similarities in clinical presentation in different respiratory affections, it is important to develop methods to quickly and accurately differentiate the cause of infection, so that the appropriate treatment regimen can be started and control or prophylactic measures may be initiated for infected animal. The diagnosis of BRD poses a paramount challenge as numerous infectious aetiologies are operating either singly or concomitantly and some of the etiological agents can be isolated from normal tissue of the bovine respiratory tract as well. A specific diagnosis is useful to direct antimicrobial or anthelmintic therapy, vaccination programs, and bio security practices and to satisfy the curiosity and concern of producers and veterinarians (Caswell *et al.*, 2012). Present investigation was carried out to fulfil this aim as post-mortem examinations coupled with pathomicrobial and molecular investigations of respiratory system affections provide confirmatory diagnosis; which in combination with history can help in effective management of the respiratory affections in cattle calves.

MATERIALS AND METHODS

The present study was conducted on six cattle calves carcasses suspected of respiratory disorders brought to the Department of Veterinary Pathology, Lala Lajpat Rai

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University of Veterinary and Animal Sciences (LUVAS), Hisar, Haryana from the month of August, 2018 to February, 2019.

Necropsy examination: The carcasses were thoroughly examined externally for any injuries, markings, unusual secretions and subsequently after opening of the carcass, any gross pathological lesions in the respiratory organs (mainly lungs, trachea, mediastinal lymph nodes) and other associated organs (*viz*. heart, liver, spleen, kidney, intestine etc.) were examined thoroughly. For histopathological analysis, representative tissue samples from organs with lesions were obtained in 10% buffered formalin. The samples were processed by standard paraffin embedding technique using H&E staining method and for special staining procedures as described by Luna (1968).

Microbiological studies

Isolation of bacteria: Samples from the heart blood, lung tissues, and tracheal swabs were collected aseptically in sterile containers under aseptic conditions from all the carcasses for bacteriological isolations. Samples were inoculated on Nutrient agar (NA), blood agar (BA) and/or Mac Conkey's Lactose agar (MLA) plates and were incubated at 37 ºC for 24 h. The plates were examined for the presence and type of growth, hemolysis and sub-cultured whenever required. Pure bacterial cultures were examined morphologically by Gram's staining and biochemical characterization was done by Vitek-2 system (BioMerieux, Inc. Hazelwood, MO, USA). Positive isolates of *E. coli* were sent to the National Salmonella and Escherichia Centre (NSEC), Central Research Institute, Kasauli, Himachal Pradesh for serotyping.

***In-vitro* drug sensitivity assay:** Bacterial isolates were subjected to antimicrobial sensitivity testing by using disc diffusion method as described by Bauer *et al.* (1966). Briefly, test culture was inoculated into tryptic soya broth using a sterile platinum loop and incubated at 35 ºC for 2-5 hr till development of turbidity. The broth culture was evenly spread by smearing over Mueller Hinton agar plates and the discs of standard concentrations were placed and pressed on the agar gently using a sterile forceps at a distance of 24 mm (centre to centre) to have a close contact with the medium. The plates were incubated at 37 ºC for 24 h and the sensitivity was recorded as sensitive (S) and resistant (R) using zone size interpretation chart provided by the manufacturer.

Molecular studies on *Mycoplasma bovis* and *Pasteurella multocida*: During post-mortem examination, representative tissue samples of affected lung tissues and heart blood were collected in separate sterile vials and stored at -20 ºC for molecular studies by using appropriate polymerase chain reaction (PCR). Samples were screened for *Mycoplasma bovis* (causing Mycoplasmosis) and *Pasteurella multocida* (causing Haemorrhagic Septicaemia) by conventional PCR. Total DNA was extracted from lung tissue and heart blood samples using the commercial kit (PureLink Genomic DNA mini kit, Invitrogen) as per manufacturer's tissue protocol. DNA quantity was determined using A_{260} values in spectrophotometer and the purity was judged using $A_{260/280}$ ratio >1.5-1.8. Species specific forward and reverse primers were used for *Mycoplasma bovis* and *Pasteurella multocida* as suggested by Pranay *et al.* (2018) and Ullah *et al.* (2009), respectively. The PCR amplification of DNA using primers specific for *Mycoplasma bovis* and *Pasteurella multocida* (Table 1) were standardized by varying the concentration of the reaction mix and cycling conditions. Reaction mixture composition of PCR for *Mycoplasma bovis* consisted of 6.25 µl PCR master mix (2X); 0.5 µl Forward primer (50 pmol/µl); 0.5 µl Reverse primer (50 pmol/µl); 3.0 µl Template DNA; 2.25 µl Nuclease free water and for *Pasteurella multocida* consisted of 2.75 µl PCR master mix (2X); 0.5 µl Forward primer (50 pmol/µl); 0.5 µl Reverse primer (50 pmol/µl; 3.0 µl Template DNA; 2.25 µl Nuclease free water. Thermal profile of the PCR for *Mycoplasma bovis* and *Pasteurella multocida* is given in table 2. Positive controls for *Mycoplasma bovis* and *Pasteurella multocida* were kept in the PCR. PCR products were analyzed using conventional agarose gel electrophoresis in 1% w/v agarose. The amplified products were run in agarose gel in 1 × TBE buffer containing ethidium bromide at 0.1 µg/µl. Quantitative Gene ruler DNA ladder were used as molecular size ladder. The DNA bands were visualized and imaged using the Molecular Imager®ChemiDocTM XRS-imaging system (Bio-Rad).

RESULTSAND DISCUSSION

The present study included various pathological, microbiological and molecular techniques to diagnose the respiratory affections of cattle calves. Main pathological changes observed in lungs in cattle calves were congestion, haemorrhages and consolidation due to pneumonic changes. Cattle calves had the history of respiratory distress, anorexia and revealed weak body conditions in most of the cases. Subcutaneous tissues and musculature also revealed pale yellowish discolouration. The gross pathological changes observed in lungs were congestion, pale anaemic appearance with focal area of consolidation in apical lobe (Fig. 1) and presence of ecchymotic haemorrhages (Fig. 2). Other associated organs revealed congested tracheal mucosa, necrotic foci in liver and heart, enlargement of mesenteric lymph nodes and spleen, redness of mucosa and presence of catarrhal

exudate in intestinal lumen.

Histopathologically lungs revealed pulmonary emphysema, atelectasis, vascular changes associated with serous, sero-fibrinous, interstitial type pneumonic changes. Interstitial pneumonia was observed in one case and was characterized by grey hepatization, thickened inter alveolar septa, presence of exudates in bronchial lumen and peribronchial infiltration of mononuclear cells (Fig. 3, 4). Organism isolated in this case was *E. coli* (serotypes O83). Serous or sero-fibrinous bronchopneumonia was observed in four cases which were characterized by presence of congestion, haemorrhage, atelectasis and sero-fibrinous fluid along with leucocytic cells infiltration in alveolar lumen (Fig. 5). There was presence of thrombosed mass in lumen along with thickening of endothelial walls of pulmonary blood vessel (Fig. 6). Peribronchiolar lymphoid aggregates were also present. Severe haemorrhages in alveolar lumen and pleura along with mild infiltration of mononuclear cells were also noticed (Fig. 7). Trachea also revealed inflammatory lesions as congestion and infiltration of mononuclear cells mainly lymphocytes in mucosal epithelium. Organisms isolated from these cases were *E. coli* (serotypes O83, O134). Other organs as heart revealed myocarditis characterized by necrosis and fragmentation of myocardial muscle fibres along with infiltration of mono nuclear cells and fatty change. In spleen, prominent changes noticed were depletion of lymphocytes, necrosis in white pulp and haemosiderosis. Liver revealed presence of vacuolar degenerative changes, centrilobular necrosis along with infiltration of mono nuclear cells and telengiectasis. Pathological findings of Radaelli *et al*. (2008) support our present gross pathological observation seen in present study in cattle calves. Histopathological findings in respiratory organs in cattle calves were mainly vascular in nature which were in corroboration with the observations of Nicholas *et al.* (2002) and Hewicker-Trautwein *et al*. (2002). The pathology of different pneumonic changes observed in present case were similar to that described by Akbor *et al.* (2007) and McGavin and Zachary (2007).

Pneumonia usually results from aerogenous injury to the alveolar epithelium or from haematogenous injury to the alveolar capillary endothelium or alveolar basement membrane (McGavin and Zachary, 2007) due to multiple reasons. Results of the present study revealed prevalence of maximum cases of serous pneumonia or serofibrinous type pneumonia characterized by presence of congestion, haemorrhage, atelectasis and thickening of inter-alveolar septa and sero-fibrinous fluid along with leucocytic cells infiltration in alveolar lumen. Gogolewski *et al.* (1987) also observed similar findings in their study. Apart from respiratory system histopathological changes were also observed in other associated organs as myocarditis, necrotic hepatitis, catarrhal enteritis and splenic necrosis.

Table 1

Sequence of primers used for *Mycoplasma bovis* and *Pasteurella multocida*

Organism	Sequence (5'-3')	Size of Amplicon (Product bp)	Reference (s)
Mycoplasma bovis	F (5'- CCTTTTAGATTGGGATAGCGGATG -3')	278bp	Pranay *et al.* (2018)
	R (5'- CCGTCAAGGTAGCGTCATTTCCTAC - 3')		
Pasteurella multocida	F KMT1SP6 (5' - GCTGTAAACGAACTCGCCAC - 3')	560bp	Ullah *et al.* (2009)
	R KMT1T7 (5'- ATCCGCTATTTACCCAGTGG - 3')		

Table 2

Thermal profile of the PCR for *Mycoplasma bovis* and *Pasteurella multocida*

Organism	Temperature	Time	No of cycle	Remarks
Mycoplasma bovis	94 ºC	3 min	1	Initial denaturation
	94 ºC	30 sec	35 cycles	Denaturation
	60 ºC	30 sec		Annealing
	72 ºC	30 sec		Extension
	72 ºC	5 min	1	Final extension
Pasteurella multocida	95 ºC	1 min	1	Initial denaturation
	95 ºC	15 sec	35 cycles	Denaturation
	60 ºC	15 sec		Annealing
	72 ºC	30 sec		Extension
	72 ºC	7 min	1	Final extension

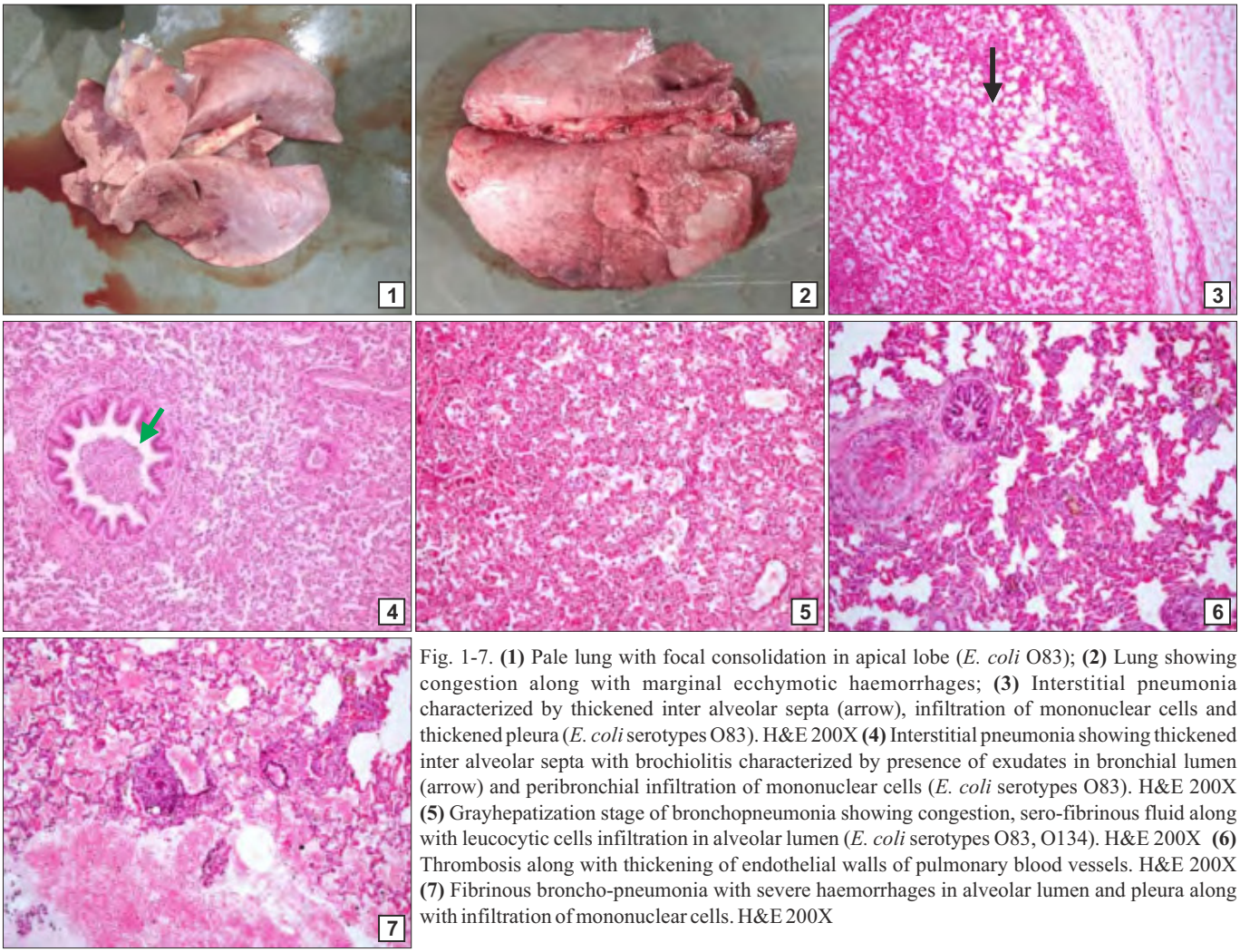

Fig. 1-7. **(1)** Pale lung with focal consolidation in apical lobe (*E. coli* O83); **(2)** Lung showing congestion along with marginal ecchymotic haemorrhages; **(3)** Interstitial pneumonia characterized by thickened inter alveolar septa (arrow), infiltration of mononuclear cells and thickened pleura (*E. coli* serotypes O83). H&E 200X **(4)** Interstitial pneumonia showing thickened inter alveolar septa with brochiolitis characterized by presence of exudates in bronchial lumen (arrow) and peribronchial infiltration of mononuclear cells (*E. coli* serotypes O83). H&E 200X **(5)** Grayhepatization stage of bronchopneumonia showing congestion, sero-fibrinous fluid along with leucocytic cells infiltration in alveolar lumen (*E. coli* serotypes O83, O134). H&E 200X **(6)** Thrombosis along with thickening of endothelial walls of pulmonary blood vessels. H&E 200X **(7)** Fibrinous broncho-pneumonia with severe haemorrhages in alveolar lumen and pleura along with infiltration of mononuclear cells. H&E 200X

These above observations are well supported by the studies of Bryson *et al.* (1983) and Sushma *et al.* (2016). The observations are indicative of systemic spread of the infection leading to lesions in the other associated organs.

Microbiological isolation studies revealed that out of 18 representative samples from heart blood, lung and tracheal swabs of each case; six bacterial isolates from the lungs and tracheal swabs were isolated. The isolates were showing lactose fermenting pink coloured colonies on MLA agar plates and greenish metallic sheen on EMB agar plates. The isolates were further confirmed by standard biochemical tests with Vitek-2 system as *Escherichia coli* (*E. coli*). Serotyping result of the isolates revealed that *Escherichia coli* belong to O83, O134 and O149 serotypes. In literature, it is a well-established fact that serotype O83 of *E. coli* acts as an adherent invasive *Escherichia coli* (AIEC) (Nash *et al.*, 2016) and serotype O149 of *E. coli* acts as enterotoxigenic *Escherichia coli* (ETEC) (Jamalludeen *et al.*, 2009) and they are primarily responsible for gastrointestinal tract pathology. However, O134 have not been reported to be a pathogenic serotype anywhere in literature. Oliveira *et al.* (2016) also detected *E. coli* along with other bacterial speciesfrom tracheobronchial lavage of the bovine, but in lower prevalence. In other countries, Elshafee (2003) detected *Escherichia* spp. along with other bacterial species from bovine pneumonic lungs. Many of mentioned microorganisms are present in the environment and could be inhaled by calves and detected in both upper and lower respiratory tracts. The isolates were found to be most sensitive to chloramphenicol (94.29%), gentamicin (85.72%), ceftriaxone/tazobactum (82.86%), cefoperazone/sulbactum (74.30%), streptomycin and co-trimoxazole (42.86%), ciprofloxacin (25.72%), tetracycline and enrofloxacin (22.86%), amoxyclav and ofloxacin (20.00%), moxifloxacin (17.20%), cloxacillin (14.30%), cefixime and erythromycin (8.60%). No resistance to any antibiotic was found in these *E. coli* strains. *In-vitro* drug sensitivity testing against different *E. coli* strains was most sensitive to chloramphenicol, gentamicin, ceftriaxone/tazobactum, cefoperazone/sulbactum. More or less similar results with respect to antimicrobial susceptibility resistance patterns

have been reported previously by Elshafee (2003).

Conventional PCR assay was also carried out using lung and heart blood samples from all the cases for the detection of *Pasteurella multocida* and *Mycoplasma bovis* which are important bacterial agents involved in the respiratory affections. All the samples were found negative for both *Pasteurella multocida* and *Mycoplasma bovis* as PCR products of expected size did not appear in Agarose Gel Electrophoresis (AGE). Earlier, Autio *et al.* (2007) has also not detected *Mycoplasma bovis* in cattle calves in respiratory disorder studies. Whereas, Gabinaitiene *et al.* (2011) reported that out of 35 male cattle calves *Mycoplasma bovis* was found in 5.7% of calves younger than three months of age in combination with *Pasteurella* spp. *Mycoplasma bovis* in combination with *Pasteurella multocida* and *Mannheimia haemolytica* was isolated from 5.7% and 2.9% of cattle at 17 months through PCR test.

CONCLUSION

The results of the present study indicated *E. coli* infection as the main respiratory affection affecting cattle calves which highlight the role of maintenance of proper hygienic conditions and managemental practices at farm level and give importance to the use of appropriate antibiotics by employing drug sensitivity tests for the treatment and control of such conditions.

CONFLICT OF INTEREST: Author(s) declares that there is no conflict of interest regarding this article.

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