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Molecular diagnosis and antibiogram of *Pasteurella multocida* type B associated with septicemic pasteurellosis in pigs

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Mortality is an important factor affecting the economic viability and profitability of swine industry. Infectious porcine bronchopneumonia is a widespread disease of major economic significance (Sorensen *et al.* 2006). A frequent finding among the infectious agents incriminated in bronchopneumonia of pigs is the Gram-negative bacterium *Pasteurella multocida* (Hansen *et al.* 2010). *P. multocida* is one of the causative agents of porcine respiratory disease complex (PRDC) (Hansen *et al.* 2010). *P. multocida* is a bacteria found worldwide and is associated with pneumonia and pleuritis in pigs (Register *et al.* 2012). These bacteria are classified based on its capsule into 5 serotypes as types A, B, D, E and F (Carter *et al.* 1955). Serotypes A and D are most commonly associated with pneumonia, rhinitis and pleuritis in pigs, but serotype D is more commonly associated with atrophic rhinitis (Register *et al.* 2012). Although acute septicaemic pasteurellosis is uncommon in pigs, sporadic outbreaks are reported in limited geographical regions of the world (Townsend *et al.* 1998, García *et al.* 2011). An outbreak of septicaemic pasteurellosis in free-range pigs characterised by high morbidity and mortality with peracute or acute disease onset caused by a type B, biovar 13 *P. multocida* strain was reported from Spain (Cardoso-Toset *et al.* 2013).

In India, capsular type A of *P. multocida* was more frequently detected among pigs sampled in Uttar Pradesh, Asom and Mizoram (Sarangi *et al.* 2014, Varte *et al.* 2014) but occasional outbreak resulting in sudden death in young and growing pigs due to septicaemic disease caused by type B has been reported (Verma *et al.* 2014). The same organism, *P. multocida* type B is responsible for outbreaks of hemorrhagic septicaemia in dairy cattle and buffaloes all over India (Kumar *et al.* 2006) and interspecies transmission between bovine and swine species may be happening (Ghosh *et al.* 2011, Kalorey *et al.* 2008). Pigs that recover from the disease may therefore act as a reservoir of *P. multocida* type B:2 not only for other swine, but also for nearby dairy herds (Kumar *et al.* 2007). The virulence of the porcine isolate of *P. multocida* type B to pigs is probably more than that of the bovine isolate P52 (B: 2), as the death time after challenge with the former is significantly shorter than that of the latter (Verma *et al.* 2014). The objective of the present study was to identify the causal agent involved in an outbreak of septicaemic disease among pigs reared in an organized farm in Bareilly, Uttar Pradesh. The clinicopathological observations in affected animals, results of the diagnostic investigation using conventional and molecular tools and antibiotic sensitivity of the isolate are reported here.

The affected animals showed symptoms of pyrexia (41–42°C), lethargy, staggering gait, serous nasal discharge with dyspnoea, anorexia and erythema of skin. Within a week after onset of clinical symptoms, 8 piglets and 3 adult pigs which were housed in adjacent pen died. Systematic necropsy was carried out and gross lesions were recorded. Upon opening of the animal, accumulation of straw coloured fluid was observed in the thoracic cavity. Lungs showed multiple focal haemorrhages with consolidation of apical lobes. Spleen was enlarged and its capsule was thickened. Liver was enlarged and congested. Pin point hemorrhages were found on the surface of both kidneys and heart. Samples of lungs, liver, spleen, lymph nodes and kidney were collected in 10% formalin for histopathological study and also fresh tissue samples were stored at 4°C for bacteriological examinations.

Isolation of bacteria was done on blood agar from specimens of lungs and spleen. The colonies appeared were smooth, convex, glistening translucent greyish colour with circular edges observed after 24 h incubation at 37°C. The pure individual colonies stained with Gram's stain identified the bacteria as Gram-negative coccobacillary organisms. Isolate was positive for catalase, oxidase, indole tests and negative for citrate, Methyl red and Voges-Proskauer tests.

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Shivachandra *et al.* (2011) also reported similar biochemical characteristics for *P. multocida* type B.

The isolated colonies when subjected to 16SrRNA PCR by using 530 Forward primer (GTGCCAGCMGCCGCGG) and 1492 Reverse primer (TACGYTACCTTGTTACG ACT) yielded the expected 994 bp products suggestive for *P. multocida*. Purified PCR product was sequenced and submitted to GenBank (Acession no KY673261) and phylogenetic analysis using the neighbour joining method with Mega 6 software showed highest similarity with the *P. multocida* detected in swine or other domestic animals. Multiplex PCR was done to identify bacteria as well as capsular types which contained following components: Dream Taq. Green PCR Mastermix (Thermo Scientific), 0.4 µM of *P. multocida* species specific primers (KMT1T7, 5'ATC CGC TAT TTA CCC AGT GG 3') and (KMT1SP6, 5' GCT GTA AAC GAA CTC GCC AC 3') (460 bp, Townsend *et al.* 1998) along with Capsular type B *P. multocida* specific primers (Forward 5'CATTTATCCA AGCTCCACC3' and Reverse 5'GCCCGAGAGTTT CAATCC 3', 760 bp) of the specific colony of bacteria. Negative control had no template DNA. The cycling conditions were initial denaturation at 95°C for 5 min followed by 34 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 1 min followed by final elongation for 10 min at 72°C. The PCR product was visualized by agarose gel (1.6% agarose) electrophoresis stained with ethidium bromide. Result of gel electrophoresis showed the presence of two bands corresponding to approximately 460 bp and 760 bp indicating that involved organisms were *P. multocida* serotype B (Fig. 1).

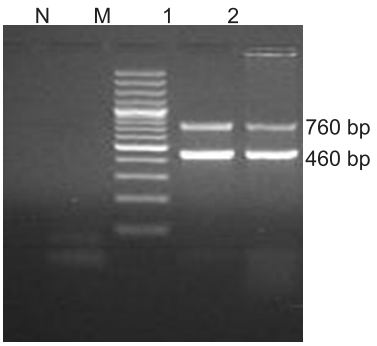
Isolate was tested for susceptibility to 16 different antibiotics using disc diffusion method, which are listed in Table 1. These antimicrobials were recommended in OIE terrestrial manual chapter on haemorrhagic septicemia and/or used by field veterinarians in different parts of India and have proven their clinical efficacy (Shivachandra *et al.* 2011, OIE 2008). The test was performed by disc diffusion method recommended by Clinical and Laboratory Standard Institute (CLSI; formerly the NCCLS) and the interpretations were carried out as per CLSI standards (Performance Standard for Antimicrobials Disk and Dilution Susceptibility test for Bacteria Isolated from Swine Sample).

In the present study, the isolate were most sensitive to norfloxacin, chloroamphenicol and tetracyclin followed by intermediate sensitivity to nalidixic acid and resistant to ciprofloxacin, co-trimaxazole along with trimethoprim-sulphamethoxazole combinations. According to a previous report, enrofloxacin and chloramphenicol were found to be quite effective against pasteurellosis (Portis *et al.* 2012). The isolate in this study showed sensitivity to cephalexin but 100% resistance to cephalexin has been reported (Tigga *et al.* 2014). However, the resistance to sulphonamides, tetracyclines, first-generation quinolones and aminoglycosides was remarkable, and thus the use of these compounds for the treatment of infection caused by *P. multocida* is not recommended (Sellyei *et al.* 2009).

Histopathological findings in the affected piglet revealed severe oedema, haemorrhages, congestion of lung parenchyma and abundant fibrin as well as infiltration of neutrophils, mononuclear cells in intralveolar septum of lungs (Fig. 2). Lung parenchyma was congested, peribronchial, alveolar and interlobular oedema together with intra alveolar infiltrate by monocytes, neutrophils and erythrocytes, abundant fibrin and bacterial micro-thrombi in lymphatic vessels in case of free range pigs associated with *P. multocida* type B (Cardoso-Toset *et al.* 2013) and type A (Ray *et al.* 2016, Jeny *et al.* 2017) was reported earlier. Haemorrhagic foci were found in lymph nodes.

Table 1. Antibiotic sensitivity test of *P. multocida* type-B

Antibiotic	Standardized interpretation criteria			Results	
	Sensitivity (≥)	Intermediately sensitivity	Resistant (≤)	Zone of inhibition in mm	Interpretation
Kanamycin	18	14-17	13	18	Sensitivity
Nitrofurantoin	17	15-16	14	22	Sensitivity
Ciprofloxacin	21	17-20	16	16	Resistant
Co-Trimaxazole	16	11-15	10	10	Resistant
Colistin	11	-	10	17	Sensitivity
Norfloxacin	17	13-16	12	27	Sensitivity
Streptomycin	15	12-14	11	17	Sensitivity
Tetracycline	19	15-18	14	24	Sensitivity
Ampicillin	17	14-16	13	20	Sensitivity
Chloramphenicol	18	13-17	12	24	Sensitivity
Cefotaxime	21	18-20	17	21	Sensitivity
Nalidixic acid	19	14-18	13	18	Intermediate
Gentamycin	15	13-14	12	16	Sensitivity
Cephalexin (CN)	18	15-17	14	22	Sensitivity
Ofloxacin	21	17-20	16	22	Sensitivity
Trimethoprim-sulphamethoxazole	16	11-15	11	10	Resistant



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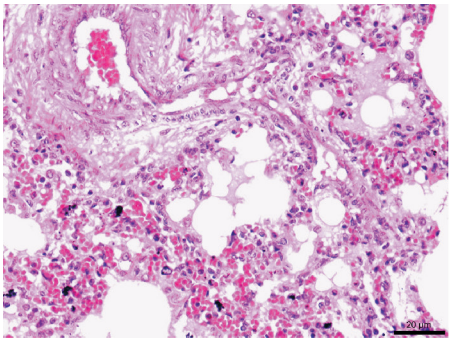

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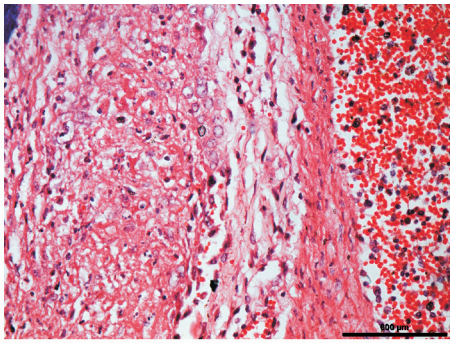

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Figs 1–3. **1.** Multiplex PCR for simultaneous detection of *Pasteurella multocida* and serotype B. Lane N, No template control; lane M, 100 bp ladder; lane 1, Positive control for Capsular type B *P. multocida*; lane 2, Test sample positive for Capsular type B *P. multocida*. **2.** Lungs showing severe oedema, haemorrhagic changes accompanied with infiltration of neutrophils and mononuclear cells in the interalveolar septum. H&E, 400×. **3**. Fibrinous thickening of splenic capsule and haemorrhages, H&E, 400×.

Severe congestion and haemorrhages were observed around glomerular area and cortical tubules of kidney. Spleen was severely haemorrhagic with thickening of capsule (Fig. 3). There was fibrinous exudation and congestion along with infiltration of macrophages and mononuclear cells were found in the liver parenchyma and perivascular infiltration with haemorrhages was also found in myocardium. Severe haemorrhages were found in the spleen, lungs, kidneys and liver of systemic pasteurellosis by *Pasteurella* Type B (Ujvári *et al.* 2015).

SUMMARY

An outbreak of septicaemic pasteurellosis caused by *P. multocida* type B in an organized swine herd resulted in sudden mortality among young and adult pigs. As disease is associated with sudden death without any significant clinical signs, quick and precise diagnosis multiplex PCR and use of appropriate antibiotics is very crucial to prevent mortality. In the present study, the isolate was found most sensitivite to norfloxacin and resistant to co-trimaxazole, trimethoprim sulphamethoxazole combination and ciprofloxacin. Phylogenetic analysis based on 16SrRNA sequence showed that isolate showed highest similarity with the *P.multocida* type B originating in swine or other domestic animals.

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