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Phylogenetic analysis of Indian isolates of *Pasteurella multocida* based on partial 16S rRNA gene sequences: Association of caprine isolate with lineage B

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ABSTRACT

Pasteurella multocida is responsible for diseases, which are endemic and economically important in India, still comparative investigations on phylogenetic relations of Indian *P. multocida* isolates are scarce. Therefore, present study was undertaken to understand the phylogenetic relationship of several isolates belonging to different host, place of isolation and capsular types based on partial 16S rRNA gene sequencing. In the current study, a 838 bp fragment of 16S rRNA gene of 35 field isolates of *P. multocida* belonging to different capsular types, recovered from cattle, buffalo, sheep, goat, pigs and birds, collected from different states of India sequenced and analyzed. There were 12 unique 16S rRNA types among 35 isolates, which clustered into 2 distinct phylogenetic lineages, viz. A and B. There were strong correlations between the phylogenetic relations and capsular types, with maximum heterogeneity seen among isolates of capsular type A. However, there was no clustering based on the host or place of isolation indicating the potential hazard of interspecies sharing and the possibility of translocation of infected animals across international borders. Moreover, one of the caprine isolates belonged to lineage B. To the best of our knowledge, this is the first report of a caprine isolate in lineage B, since lineage B is reported to be exclusively associated with birds and cats. It may be alarming that the strains of lineage B are becoming adapted to different host species.

Key words: Lineage B, *Pasteurella multocida,* Phylogenetic analysis, 16S rRNA

Pasteurella multocida is a well-known pathogen responsible for a wide range of diseases and severe economic losses in cattle, buffaloes, sheep, goats, pigs and birds throughout the world (Hotchkiss *et al.* 2011, Tahamtan *et al.* 2016). The economic significance of this pathogen in the country is evidenced from the fact that HS is the cause of maximum number of bovine deaths and causes a total economic loss of‘ USD 791.61 million/year for the livestock industry in India (Singh *et al.* 2014). The major diseases caused by *P. multocida* include fowl cholera, haemorrhagic septicaemia (HS), fatal pneumonia, enzoonotic pneumonia, atrophic rhinitis, septicaemia and pneumonic pasteurellosis in different species of animals (Chandrasekaran *et al.* 1991, Watson and Davies 2002, Shivachandra *et al.* 2011, Wilkie *et al.* 2012).

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The genetic diversity of *P. multocida* isolates, either from various hosts or place of isolation, had been investigated separately using numerous molecular techniques, including restriction endonuclease analysis (Rimler 2000), 16S rRNA gene sequencing (Shayegh *et al.* 2010, Gluecks *et al.* 2017), pulsed field gel electrophoresis (Yeh *et al.* 2017), repetitive sequence-based PCR and amplified fragment length polymorphism (Amonsin *et al.* 2002), random amplified polymorphic DNA polymerase chain reaction (Zahoor *et al*. 2014) and multilocus enzyme electrophoresis (Blackall *et al.* 1998). However, the comparative studies on different capsular types of *P. multocida* isolates from various hosts and place of origin are very infrequent, especially in India. Therefore, molecular epidemiology of Indian *P. multocida* isolates was examined by multilocus sequence typing (Sarangi *et al*. 2016) and virulence genotyping (Sarangi *et al.* 2014) in our laboratory. Though these studies had provided innovative epidemiological data on Indian *P. multocida* isolates, underlying phylogenetic relatedness was not well established. Hence, in the present study, we report partial 16S rRNA gene sequence analysis of *P. multocida* isolates belonging to different host, place of isolation and capsular types, and compared with isolates from other parts of the world.

MATERIALS AND METHODS

Bacterial strains: Indian field isolates of *P. multocida* (35) belonging to 4 different capsular types, recovered from birds (poultry and emu) (6), cattle (5), buffalo (2), pigs (7), goats (5) and sheep (10) within 9 different states of India were utilized in this study. These isolates were selected to represent the major variations associated with capsular polysaccharide, host of origin and place of isolation (Table 1). The lyophilized culture of each isolate was streaked onto blood agar and incubated aerobically at 37ºC overnight to cross check for the possible contamination. Molecular confirmation was done by PCR using species specific KMT1SP6 and KMT1T7 primers (Townsend *et al.* 1998). Subsequently, the confirmation of capsular type of each isolate was done by multiplex PCR using specific primers for each capsular type (Townsend *et al.* 2001).

Extraction of genomic DNA: Genomic DNA of all the isolates was extracted using CTAB method (Wilson 1997). The concentration and purity of the isolated DNA was estimated spectrophotometrically (Nanodrop®, USA), and the integrity was assessed by electrophoresis on a 0.7% agarose gel.

PCR amplification of 16S rRNA gene: PCR fragments corresponding to the nucleotides 513–1509 of 16S rRNA gene were amplified from the genomic DNA of all isolates using universal primers, F515 (5'-GTGCCAGCMGCC-GCGG-3') and 1492R (5'-TACGYTACCTTGTTACG-ACT-3') as described elsewhere (Lane 1991). The PCR products were then resolved by submarine gel electrophoresis on 1% agarose gel containing 0.5% ethidium bromide and visualized under UV light.

Sequencing and phylogenetic analysis: The amplified PCR products were purified using gel extraction kit (Qiagen) following the manufacturer's instructions. The purified PCR products were then sequenced at a custom DNA sequencing facility (Eurofins Genomics India). The sequences obtained were edited and compiled using Editseq program (DNASTAR Lasergene, USA). Pair alignments and sequence identity generation were performed using MegAlign program (DNASTAR Lasergene, USA). The newly acquired sequences of this study were then submitted to GenBank (NCBI).

The overlapping 838 bp size segment of the gene in the forward and reverse sequencing was used for phylogenetic study to minimize the possibility of PCR errors. Various *P. multocida* isolates were also included in the phylogenetic study (Table 1). The sequences were aligned using ClustalW and the sequence distances were calculated by MegAlign program (DNASTAR Lasergene, USA). Subsequently, neighbor-joining (NJ) tree was constructed by MEGA version 6.06 (Tamura *et al.* 2013) using Jukes-Cantor model (Jukes and Cantor 1969). The confidence in the NJ tree was estimated by 1,000 bootstrap replicates.

RESULTS AND DISCUSSION

The isolates were characterized by the morphological and cultural characteristics followed by molecular confirmation by species-specific PCR (Fig. 1A). Subsequently, capsular types of the isolates were confirmed by multiplex PCR (Townsend *et al.* 1998). The isolates which yielded the product of 1044, 760, 657 and 851 bp size were classified as serotype A, B, D and F, respectively (Fig. 1B). PCR amplification of 16S rRNA gene was done using universal primers in which an amplicon of ~997 bp was obtained with all isolates (Fig. 2), which were then purified, sequenced and used for the phylogenetic analysis.

Nucleotide sequences of 16S rRNA gene of different isolates were submitted to Gen-bank (NCBI) and assigned with accession numbers (KU666518-KU666533; KT222117-KT222131; KT222133-KT222136). The homology of these 35 isolates was 98.2–100% at nucleotide level. There was 100% homology between the isolates of B and F capsular type at the nucleotide level, while that of isolates belonging to A and D capsular type was 98.2–100% and 99.6–100% respectively. The homology of caprine, avian, ovine, bovine and porcine isolates was 98.2–100%, 99.8–100%, 99.4–100%, 99.8–100% and 99.6–100% respectively. There were 12 unique 16S rRNA types within the isolates sequenced in this study (Table 1), in which type 2 followed by type 11 were the most prevalent 16S rRNA types. Among the 35 isolates of the present study, 62.86% were associated with either of these two 16S rRNA types.

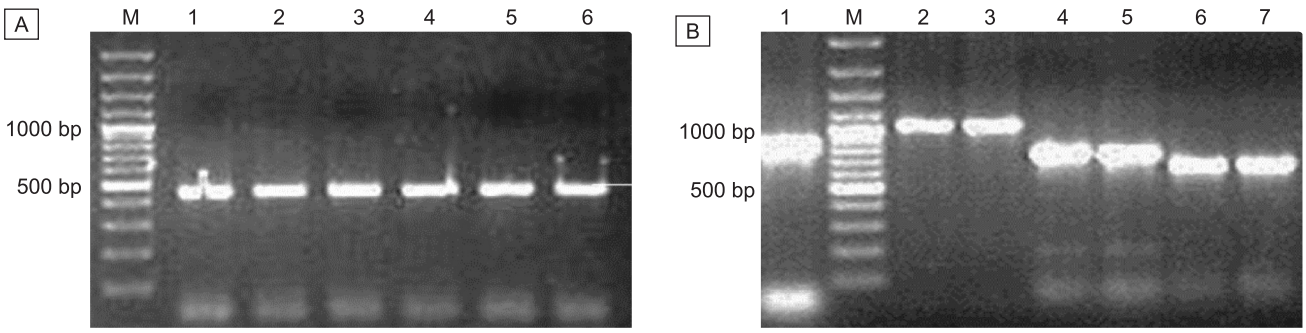

Fig. 1. Characterization of *P. multocida* isolates. **A**. Species specific PM-PCR of representative isolates showing band of 460 bp. **B**. Mutliplex PCR of representative isolates showing amplicons of 851 bp size (CapF positive; Lane 1), 1044 bp (CapA positive; Lanes 2 and 3), 760 bp (CapB positive; Lanes 4 and 5) and 657 bp (CapD positive; Lanes 6 and 7).

More interestingly, 16S rRNA type 11 contained only isolates of B capsular type.

The sequences were then compared with the published gene sequences of a diverse collection of *P. multocida* isolates with regard to host, place of collection and capsular types. There were a total of 72 isolates including the 35 isolates sequenced in the present study. The homology of these 72 isolates was 97.9–100% at nucleotide level. The homology of the isolates belonging to capsular type A, B, D and F was 97.9–100%, 99.6–100%, 99.5–100% and 100% respectively. Therefore, the homology of the isolates belonging to various capsular types at nucleotide level were in the order of F>B>D>A. At the same time, isolates belonging to B capsular type shared a homology of 99.6–

Table 1. *P. multocida* isolates used in phylogenetic analysis

Isolate ID	Cap. Type	Host	Place of Isolation	Accession number	16S rRNA type
464*	A	Avian (Poultry)	Chennai, Tamil Nadu	KU666521	2
647*	A	Ovine	Chennai, Tamil Nadu	KU666524	2
746*	A	Bovine (Cattle)	Palampur, Himachal Pradesh	KU666527	2
876*	A	Porcine	Thrissur, Kerala	KU666531	2
890*	A	Avian (Emu)	Chennai, Tamil Nadu	KU666532	2
920*	A	Avian (Emu)	Anand, Gujarat	KU666533	2
366*	B	Bovine (Cattle)	Palampur, Himachal Pradesh	KU666519	11
400*	B	Bovine (Buffalo)	Ludhiana, Punjab	KU666520	11
633*	B	Avian (Poultry)	Bengaluru, Karnataka	KU666523	11
699*	B	Caprine	Palampur, Himachal Pradesh	KU666525	11
860*	B	Porcine	Guwahati, Asom	KU666529	11
543*	D	Porcine	Bareilly, Uttar Pradesh	KU666522	2
733*	D	Porcine	Guwahati, Asom	KU666526	3
797*	D	Bovine (Buffalo)	Thrissur, Kerala	KT222136	2
863*	D	Ovine	Chennai, Tamil Nadu	KU666530	4
9*	F	Bovine (Cattle)	Pune, Maharashtra	KU666518	2
825*	F	Ovine	Chennai, Tamil Nadu	KU666528	2
212*	A	Ovine	Chennai, Tamil Nadu	KT222117	14
216*	A	Ovine	Srinagar, Jammu and Kashmir	KT222118	16
359*	A	Caprine	Palampur, Himachal Pradesh	KT222119	20
376*	B	Ovine	Chennai, Tamil Nadu	KT222120	2
433*	A	Caprine	Chennai, Tamil Nadu	KT222121	17
534*	A	Caprine	Chennai, Tamil Nadu	KT222122	15
575*	A	Ovine	Chennai, Tamil Nadu	KT222123	18
585*	A	Porcine	Guwahati, Asom	KT222124	14
625*	A	Ovine	Chennai, Tamil Nadu	KT222125	14
675*	A	Ovine	Chennai, Tamil Nadu	KT222126	14
676*	A	Caprine	Chennai, Tamil Nadu	KT222127	7
879*	A	Porcine	Thrissur, Kerala	KT222128	5
942*	A	Avian (Poultry)	Anand	KT222129	2
985*	A	Avian (Poultry)	Gujarat	KT222130	2
1027*	A	Porcine	Guwahati, Asom	KT222131	2
419*	B	Ovine	Chennai, Tamil Nadu	KT222133	11
563*	B	Bovine (Cattle)	Anand, Gujarat	KT222134	11
593*	B	Bovine (Cattle)	Guwahati, Asom	KT222135	11
5	A	Bovine	Germany	AY316314	8
734	A	Porcine	Scotland	AY299305	2
W819	A	Bovine	United Kingdom	AY683485	1
564	A	Bovine	Scotland	AY299306	2
386	A	Bovine	Scotland	AY299304	2
70	A	Avian (Poultry)	USA	AE004439	2
548	A	Bovine	Scotland	AY299309	2
104	A	Avian (Poultry)	Scotland	AY299311	9
338	A	Bovine	Scotland	AY299308	14
152	A	Avian (Poultry)	Scotland	AY299314	21
NCTC11995	A	Feline	France	AY079000	21
82	A	Avian (Poultry)	Scotland	AY299319	21
64	A	Avian (Poultry)	Scotland	AY299315	20
80	A	Avian (Poultry)	Scotland	AY299318	19

(*Contd...*)

(*Table 1 contd...*)

Isolate ID	Cap. Type	Host	Place of Isolation	Accession number	16S rRNA type
214	A	Bovine	Germany	AY316315	2
NCTC10322	A	Porcine	Canada	AY078999	2
Tabriz1	A	Bovine	Azerbaijan	FJ231205	2
NCTC10204	A	Bovine	United Kingdom	AY078998	2
P2191	A	Avian (Poultry)	Denmark	JX569198	11
B847-1-98	A	Avian (Poultry)	UK	JX569201	21
HIM746	A	Feline	France	AF326325	21
1800S189	A	Avian(Blackbird)	Germany	JX569203	20
P356-2007	A	Avian (Poultry)	Norway	JX569199	20
Tabriz98	A	Ovine	Azerbaijan	FJ231209	10
86	B	Caprine	India	DQ288146	13
P52	B	Bovine (Buffalo)	India	DQ286927	12
49	B	Porcine	India	DQ286929	11
B	Ovine	India	DQ288145	11	
75	B	Bovine	India	DQ286928	11
GD2Q201401	B	Bovine	China	KP083466	11
NCTC10323	B	Bovine	Scotland	AY299312	11
B	Avian	Scotland	AY299310	9	
714	D	Porcine	Scotland	AY299307	3
Tabriz40	D	Bovine	Azerbaijan	FJ231207	6
Tabriz31	D	Bovine (Buffalo)	Azerbaijan	FJ231206	11
NCTC10326	E	Bovine	Africa	AY324032	9
2	F	Ovine	Scotland	AY078996	2

*Isolates sequenced in this study.

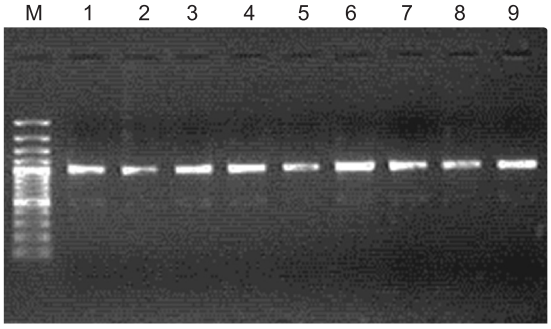

Fig. 2. 16S rRNA specific PCR of representative isolates showing 997 bp amplicon.

100% with reference strain of E capsular type. The homology of avian, caprine, ovine, bovine and porcine isolates was 97.7–100%, 98–100%, 99.4–100%, 99.4–100% and 99.6–100% respectively. Thus, the homology of the isolates belonging to various hosts at nucleotide level were in the order of Porcine>Bovine/Ovine>Caprine> Avian.

Altogether, a total of 21 unique sequences designated 16S types 1–21, were identified (Table 1). The type 2 followed by type 11 were the most prevalent 16S rRNA types in which 54.17% of the isolates were associated. However, visual judgment of the polymorphic nucleotide sites noticeably indicated that partial 16S rRNA sequences of *P. multocida* isolates consist of two separate sets, designated as A and B, represented by 16S types 1–18 and 19–21, respectively. One of the caprine isolate sequenced in the present study was included in B set (Type 20).

The neighbour-joining dendrogram demonstrating the phylogenetic relationships of the various *P. multocida* isolates based on 838 bp fragment of 16S rRNA gene was constructed (Fig. 3). There were 2 major lineages, A and B corresponding to the 2 separate sets of 16S rRNA types as already described. The branching of lineages A and B was exceptionally strong, as indicated by the high bootstrap value of 99%. In contrast, bootstrap values for other branches were relatively less due to the high similarity between the sequences (less than 70%). With the exception of one caprine isolate (PM-359), all the 34 our isolates had their place in lineage A. Lineage A itself consisted of 3 clusters (A1-A3) and 18 out of 35 isolates belonged to cluster A1. Cluster A2 and A3 contained 8 isolates in each. More importantly, all isolates of B capsular type belonged to cluster A2. Cluster A1 contained isolates of other 3 capsular types (A, D and F) and A3 contained only isolates of A capsular type. Caprine isolates were present in all the 3 clusters of lineage A. Cluster A3 contained only isolates of mammalian origin.

Two distinct lineages (A and B) of *P. multocida* had already been reported based upon phylogenetic analyses by MLST and sequence analysis of *16S rRNA*, *rpoB* and *atpD* genes (Korczak *et al.* 2004, Korczak and Kuhnert 2008, Shayegh *et al.* 2010, Bisgaard *et al.* 2013). However, all these reports mentioned that the lineage B included isolates mainly from birds and some isolates from Felidae, whereas group A included isolates from all types of hosts. It is important to note that none of these studies had included caprine isolates. It has been concluded from earlier studies that the isolates representing lineage B of *P. multocida* are associated almost exclusively with birds and cats and

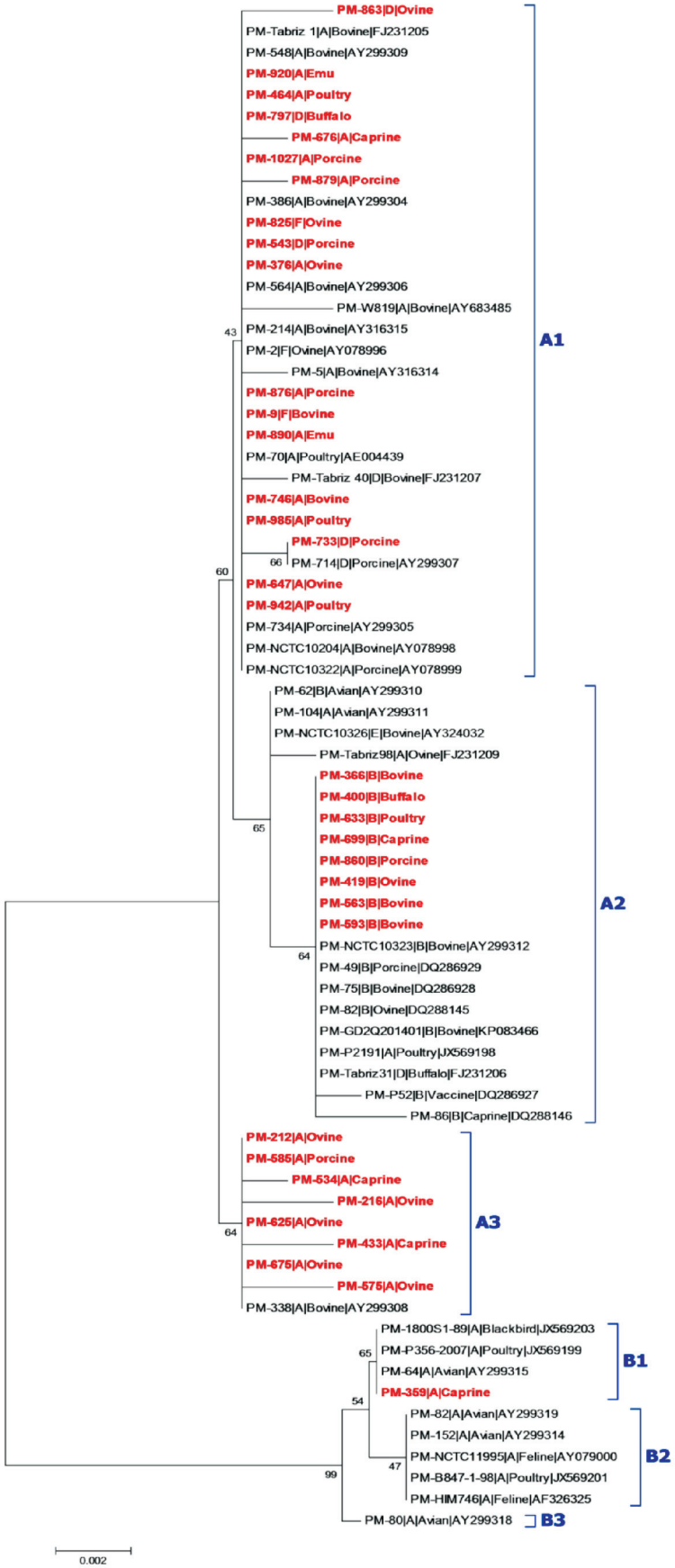

Fig. 3. Phylogenetic analysis based on 16S rRNA gene sequence. Name of the isolates sequenced in our laboratory are given in red.

presumably, cats had acquired avian-adapted strains of *P. multocida* as a consequence of their predator-prey relationship (Davies 2004). Another study has hinted the evolutionary implications of the association of lineage B with Aves and Felidae (Bisgaard *et al.* 2013). It has also been suggested that isolates of lineage A have evolved from a common ancestor and have become adapted to different host species, whereas avian strains of lineage B have diverged from a different common avian ancestor and have remained highly adapted to birds (Davies 2004). However, in the present study, we have shown that one of the caprine isolates belonged to lineage B, the biological significance of which has to be elucidated in future. This may be an alarming signal that the strains of *P. multocida* lineage B are becoming adapted to different hosts as a predator-prey relationship cannot be applied in this case.

Out of 72 isolates analyzed in the current study, 62 isolates from various hosts including cattle, buffaloes, pigs, sheep, goats and birds (chicken and emu) were present in lineage A, whereas lineage B (10 isolates) was represented by 7 isolates of avian origin, 1 isolate of caprine origin and 2 isolates of feline origin. A noteworthy observation in the present study was a strong correlation observed between the phylogenic relations and capsular types of *P. multocida* which proves that isolates of the same capsule are genetically related and represents distinct clones as suggested earlier (Davies 2004). The homologies of the isolates belonging to various capsular types at nucleotide level were in the order of F>B>D>A. Moreover, all the F isolates which were isolated from different hosts shared 100% homology at the nucleotide level and all belonged to A2 cluster which suggest that isolates of F capsular type belonging to different host species have a common evolutionary origins as already proposed (Davies 2004). Similarly, all the isolates of B capsular type belonged to cluster A2 and majority of D capsular type (with exception of one) isolates belonged to A1 cluster. The B isolates (total 8) of various hosts sequenced in the present study showed 100% homology and all the B isolates included in phylogenetic analysis (16) represented a distinct branch within lineage A (Cluster A2) with a homology of 99.6–100% at the nucleotide level. Such significant relation between B capsular type isolates belonging to different hosts had already been reported (Dey *et al*. 2007). More importantly, the phylogenetic tree indicated that all the capsular type B isolates with the exception of one avian B isolate from England formed a distinct branch within cluster A2 away from the African HS causing type E strain NCTC 10326. The neighbouring position of capsular type B isolates with capsular type E of *P. multocida* in the phylogenetic tree had also been reported earlier (Davies 2004).

Of the total 72 isolates analyzed in the study, maximum heterogeneity was seen in avian isolates which was similar to the earlier reports (Gunawardana *et al.* 2000, Davies *et al.* 2003, Shivachandra *et al.* 2013) immediately followed by caprine isolates. The heterogeneity of caprine *P. multocida* isolates had not been reported earlier which may be due to the scarcity of such studies on caprine isolates. The evolutionary implications and the biological significance of the higher heterogeneity observed among caprine isolates need further investigation. It was also observed that there was no clustering of *P. multocida* isolates based on their host in the phylogram indicating the potential hazard of interspecies sharing of these organisms, which deserves due attention in the epidemiology of various *P. multocida* causing diseases. Similarly, there was no clustering of the isolates based on their place of isolation which points to the possibility of translocation of infected animals across the international borders.

To the best of our knowledge, this is the first report of a caprine *P. multocida* isolate in the lineage B which may be an alarming signal suggesting that the strains of *P. multocida* lineage B are becoming adapted to different hosts. There were strong correlations between the phylogenic relations and capsular types, which suggest that isolates of the similar capsular type are genetically related and represent distinct clones. Nevertheless, the capsular type A isolates were highly heterogeneous. There was no clustering of the isolates based on their host and place of origin indicating the potential hazard of interspecies sharing of these organisms and the possibility of translocation of infected animals across international borders both of which deserves due attention in the epidemiology of various *P. multocida* mediated diseases. Moreover, to the best of our knowledge, this is the first report on the heterogeneity of caprine *P. multocida* isolates the biological significance of which has to be explored in future.

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