Indian Journal of Animal Sciences **91** (10): 819–825, October 2021/Article
https://doi.org/10.56093/ijans.v91i10.117212

Conservation of *ptfA* gene encoded Type IV fimbrial protein among circulating *Pasteurella multocida* serogroup A strains causing pneumonia in sheep

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Received: 31 August 2020; Accepted: 17 August 2021

ABSTRACT

Respiratory infections are most common in small ruminants. Differentiation of homogenous bacterial strains originated from similar clinical forms (acute or chronic) of infections in a particular or diverse host origin and regions posses a greater challenge for rapid epidemiological studies. *Pasteurella multocida,* being a multi-host pathogen with wide range of infections among small ruminants especially sheep, is of greater economical concern among small and marginal farmers. In our study, we report *ptfA* gene sequence based analysis of circulating *P. multocida* strains recovered from clinically ailing sheep either with pneumonia or septicaemia belonging to different geographical regions of Karnataka. All the 29 *P. multocida* strains were characterized by conventional methods as well as molecular methods which indicated homogeneity as they belonged to serogroup A and possessed highly conserved *ptfA* gene by phylogenetic analysis. The study highlighted the conservation of *ptfA* gene/fimbrial protein among *P. multocida* strains from identical/diverse clinical conditions and could be employed in rapid epidemiological studies in routine surveillance of circulating pathogenic bacterial strains as well as pasteurellosis outbreak investigations among animals and birds.

Keywords: Fimbrial protein, *Pasteurella multocida*, Pneumonia, *ptfA* gene, Sequence analysis, Sheep

Sheep with its multi-facet utility (for meat, wool, milk, hide and manure, etc.) form an important component of rural economy especially in the arid, semi-arid and mountainous regions of tropical countries including India. Sheep production system by nomadic or tribals as well as small and marginal farmers largely depend on conventional migratory system of rearing which is constantly threatened by chronic form of infectious diseases, one among them is 'pneumonic pastuerellosis' caused by *Pasteurella multocida*. Ubiquitous gram-negative bacterium, *P. multocida* with five capsular serogroups (A, B, D, E and F) and 16 somatic serotypes (1 to 16), known to affect multiple hosts (both animal/avians and humans) with wide range of disease manifestations (Shivachandra *et al.* 2011). Unlike large ruminants (Cattle and buffalo) which largely succumb to highly contagious, septicaemic and fatal disease referred as 'haemorrhagic septicaemia (HS)', small ruminants (sheep and goat) most commonly exhibit pneumonic pasteurellosis (Odugbo *et al.* 2003, Sarangi *et al.* 2014, Sahay *et al.* 2018, Bello *et al.* 2019), an economically significant form of chronic infectious disease than sporadically reported acute septicaemic form of infection. Although, a limited literature reported on circulation of different serotypes of *P. multocida* associated with ovine pnenumonic pasteurellosis (Kumar *et al.* 2014), owing to inherent lack of discriminatory power, labour intensivity and non-reproducibility of most commonly used conventional methodologies, several efforts in the past to differentiate the strains greatly hampered (Shivachandra *et al.* 2017).

Nucleic acid based typing approaches involving amplification of either targeted single/multiple virulence-associated genes (*ptfA*, *tbp*A, *pfh*A, *tox*A, *ompA*, *vacJ, ompH*, *oma87*, *sodA*, *sodC*, *hgb*A, *hgb*B, *exbBD-tonB*, *nanB*, *plbB*) or housekeeping genes (*adk, est*, *pmi, zwf*, *mdh*, *gdh, pgi*) in MLST, repetitive genes (REP-/ERIC-) and random sequence (RAPD) based PCR assays, and whole genome sequencing/REA/PFGE, etc. have greatly augmented the capability to differentiate phenotypically homogenous *P. multocida* strains especially those originating from similar clinical form of infections among livestock thereby assisting in rapid epidemiological studies (Ewers *et al*. 2006, Dziva *et al.* 2008, Shivachandra *et al*. 2008).

One among several virulence-associated genes (VAG) of *P. multocida* is *ptfA* (435 bp), which encodes for Type IV fimbrial protein (144 aa, 14.9 kDa), is an important 'epidemiological marker' for characterizing strains as it

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plays an important role in their colonization of host surface mucosa by adhesion (Craig *et al*. 2004, Shivachandra *et al.* 2012, Varma *et al*. 2013). Earlier, *ptfA* gene and proteins sequence analysis of *P. multocida* serogroups (A, B, D and F) isolated from different clinical conditions originating from diverse animal and avian hosts indicated the variability of *ptfA* gene among strains which could be used to determine the severity potential of infection (Sellyei *et al.* 2010, Shivachandra *et al.* 2013, Verma *et al.* 2013). In view of these, we considered *ptfA* gene sequencing to understand the variability among circulating *P. multocida* strains among common clinical condition, pneumonic pasteurellosis in sheep originated from different geographical regions of Karnataka, India.

MATERIALS AND METHODS

Bacterial strains: A total of 29 *P. multocida* strains belonging to ovine origins maintained in the 'Bacterial Epidemiology Laboratory-3' of ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI)', Bengaluru, Karnataka, India, were used for the current study. All the strains were isolated from predominantly pneumonic pasteurellosis clinical condition among sheep farms during 2017–2018 belonging to different geographical regions of Karnataka (Table 1). All the *P. multocida* strains were revived from glycerol stock on BHI broth and blood agar plate and incubated aerobically at 37°C for 48 h. The genomic DNA was isolated using DNeasy Blood and Tissue Kit (Qiagen, USA) as per the manufacturer's instructions and used as template for *ptfA* gene amplifications.

Sequencing of ptfA genes and phylogenetic analysis: PCR amplification of *ptfA* gene from respective strains were carried out using the primer set and reaction conditions as described previously (Siju *et al.* 2007). The amplified *ptfA* gene products from all the ovine *P. multocida* strains were purified from the agarose gel using a gel extraction kit (Qiagen, USA) and were subjected for Sanger sequencing (Eurofins Genomics, Bengaluru, Karnataka, India). The complete nucleotide sequences of *ptfA* gene of *P. multocida* strains were submitted to GenBank. For comparative phylogenetic analysis, a total of 15 *ptfA* gene sequences of a representative *P. multocida* strains originated from other animal hosts belonging to different countries including India and one outgroup sequence from genus *Actinobacillus* belonging to Pasteurellaceae family were retrieved from the NCBI database (Table 2). Comparative multiple sequence alignment of TFF protein sequences (n=44) was carried out using pairwise ClustalW method of MegaAlign (DNASTAR). All the sequences were used in phylogenetic

Table 1. Details of ovine *P. multocida* strains used in this study and their *ptfA* gene sequences

Designation of *P. multocida* strain	Capsular type	Clinical condition	Sheep farm number	Name of district	Village	GenBank Acc. No.
NIVEDI/Pm1	A	Pneumonic	1	Haveri	KVK-H/S	MT371926
NIVEDI/Pm2	A	Pneumonic	1	Haveri	KVK-H/S	MT371927
NIVEDI/Pm3	A	Pneumonic	2	Haveri	Savanur	MT371928
NIVEDI/Pm4	A	Pneumonic	2	Haveri	Savanur	MT371929
NIVEDI/Pm5	A	Pneumonic	3	Haveri	Billahalli	MT371930
NIVEDI/Pm6	A	Pneumonic	3	Haveri	Billahalli	MT371931
NIVEDI/Pm7	A	Pneumonic	3	Haveri	Billahalli	MT371932
NIVEDI/Pm8	A	Pneumonic	4	Chickmanagluru	Hirenallur	MT371933
NIVEDI/Pm9	A	Pneumonic	5	Chickmanagluru	Hirenallur	MT371934
NIVEDI/Pm10	A	Pneumonic	5	Chickmanagluru	Hirenallur	MT371935
NIVEDI/Pm11	A	Pneumonic	6	Chickmanagluru	Hirenallur	MT371936
NIVEDI/Pm12	A	Pneumonic	7	Koppal	H. Mundi	MT371937
NIVEDI/Pm13	A	Pneumonic	8	Koppal	H. Mundi	MT371938
NIVEDI/Pm14	A	Pneumonic	9	Tumkuru	Tiptur city	MT371939
NIVEDI/Pm15	A	Pneumonic	10	Tumkuru	Tiptur city	MT371940
NIVEDI/Pm16	A	Pneumonic	10	Tumkuru	Tiptur city	MT371941
NIVEDI/Pm19	A	Septicaemic	11	Mysuru	Chikkanerale	MT371944
NIVEDI/Pm20	A	Pneumonic	12	Haveri	Ranebennur	MT371945
NIVEDI/Pm21	A	Pneumonic	13	Chamarajnagar	Bachehalli	MT371946
NIVEDI/Pm22	A	Pneumonic	14	Chamarajnagar	Bachehalli	MT371947
NIVEDI/Pm23	A	Pneumonic	14	Chamarajnagar	Bachehalli	MT371948
NIVEDI/Pm24	A	Pneumonic	15	Chamarajnagar	Bachehalli	MT371949
NIVEDI/Pm25	A	Pneumonic	15	Chamarajnagar	Bachehalli	MT371950
NIVEDI/Pm26	A	Pneumonic	15	Chamarajnagar	Kundekere	MT371951
NIVEDI/Pm27	A	Pneumonic	15	Chamarajnagar	Kundekere	MT371952
NIVEDI/Pm28	A	Pneumonic	16	Chamarajnagar	Kundekere	MT371953
NIVEDI/Pm29	A	Pneumonic	16	Chamarajnagar	Kundekere	MT371954
NIVEDI/Pm30	A	Pneumonic	17	Chamarajnagar	Kundekere	MT371955
NIVEDI/Pm31	A	Pneumonic	18	Bellary	Hampasagara	MT371956

Table 2. Details of *ptfA* gene sequences retrieved from GenBank for phylogenetic tree analysis

Bacterial strain/isolate	Host	Country/location	GenBank Ac No.
P. multocida VP161	Avian	Vietnam	AF154834
P. multocida Strain IndPm176	Avian	India	JX139091
P. multocida Strain PM14	Avian	Iran	KX781185
P. multocida P52	Bovine	India	AY644678
P. multocida IndPm94	Bovine	India	JX139092
P. multocida subsp. *septica* strain CIRMBP-0873	Rabbit	France	CP020347
P. multocida strain 12601	Cattle	Denmark	CP026859
P. multocida strain PMX	Cattle	Malaysia	FJ526996
P. multocida strain 161215033201-1	Human	Netherlands	CP026744
P. multocida strain PMb2	Ovine	Iran	KX679397
P. multocida strain 9N	Rodents	China	CP028927
P. multocida strain D2	Swine	China	KX161904
P. multocida strain NCTC10323	Bovine	UK	LR134532
P. multocida strain ATCC 43137	Pig	USA	CP008918
Actinobacillus pleuropneumoniae strain HK361	Pig	UK	AF302997

UK, United Kingdom; USA, United States of America.

tree construction by the neighbour-joining program of MEGA version X (bootstrap 1000).

RESULTS AND DISCUSSION

Primary differentiation of pathogenic bacterial strains such as *P. multocida* from diverse hosts largely depends on conventional bacteriological approaches involving phenotypic, biochemical, pathogenic and serological characteristics (Carter 1952, Heddleston *et al*. 1972, Prajapati *et al.* 2020b). However, inability to differentiate closely related (phenotypically as well as serologically) strains pose a greater challenge for rapid epidemiological studies in acute disease outbreaks and lingering chronic clinical conditions (Hotchkiss *et al.* 2011). *P. multocida* strains are being a causative agent for wide spectrum of diseases, clinical forms (both acute and chronic), and severity, varying with involvement of different multiple capsular and somatic types continue to challenge the strategies for prevention and control in endemic countries (Shivachandra *et al*. 2011, Wilson and Ho 2013). Fortunately, in recent time, multiphasic molecular approaches have been practiced by several researchers in order to better understand the diverse pathogenic bacterial strains (Dziva *et al.* 2008, Shivachandra *et al.* 2017).

In our study, all the 29 ovine *P. multocida* strains revived from glycerol stock were identical phenotypically as well as biochemically exhibiting no change in their morphological and cultural characteristics. Further, all the strains belonged to capsular serogroup A as identified previously by multiplex capsular PCR typing system. Further, all the ovine circulating *P. multocida* strains (n=28) from pneumonic pasturellosis and one strain from septicaemic form belonged to capsular serogroup A. Clinically, reports on circulating pathogenic *P. multocida* strains with varying serogroups from various clinical conditions (pneumonic and/or septicaemic) of small ruminants (sheep and goat) reported worldwide including India are scarce. However, serogroups such as A:1, A:3, A:3,4,12, A:12, B:2, D:3, F:1, F:4,12 have been reported in the earlier studies (Dwivedi *et al.* 1990, Das and Bhagwan 1997, Kumar *et al.* 2004, Sarangi *et al*. 2014). Most of *P. multocida* strains are commonly known to colonize upper respiratory tract of susceptible hosts and persist in the crypts of tonsils for several months even after use of antibiotics (De Alwis *et al.* 1990) which might have led to chronic pneumonia cases in small ruminants. Hence, there was high chance of homogeneity among strains isolated from chronic cases. Whereas, strains causing acute septicaemic form in host reckoned to be triggered by various influencing environment factors leading to high mortality, tend to show a very little genetic variations despite being phylogenetically similar (Orynbayev *et al.* 2019). Moreover, the severity of pneumonic form despite clonality of strains could be due to presence of dermonecrotoxin (*toxA* gene) as noted in the recent time (Cid *et al.* 2019, Prajapati *et al.* 2020a). Therefore, in chronic clinical cases, as observed in our study as well as others in the past, it was difficult to differentiate all the phenotypically and serologically similar *P. multocida* strains. Hence, we employed molecular approaches to delineate the differences among them, if any.

To differentiate pathogenic strains, the most common approach has been targeting the virulence associated genes. In the past, different virulence factors of *P. multocida* belonging to categories of outer membrane proteins (OMPs)/ enzymes/ lipoproteins such as OmpW (Yogisharadhya *et al.* 2019), VacJ (Shivachandra *et al.* 2014), TbpA (Shivachandra *et al.* 2005), Omp16 (Kumar *et al*. 2015), Type IV fimbriae (Shivachandra *et al.* 2013), Skp (Sunadarraj *et al.* 2020) had been sequenced for characterization and found their maximum homogeneity (Hatfaludi *et al.* 2010, Wilson and Ho 2013, Peng *et al.* 2019). Of these virulence factors, Adhesins [fimA (fimbriae), *hsf-1,2* (autotransporter adhesins), *pfhA* (filamentous hemagglutinin), *tad* (non-specific tight adherence protein), and *ptfA* (subunit of type 4 fimbriae)] had been recognized to play a crucial role in mediating

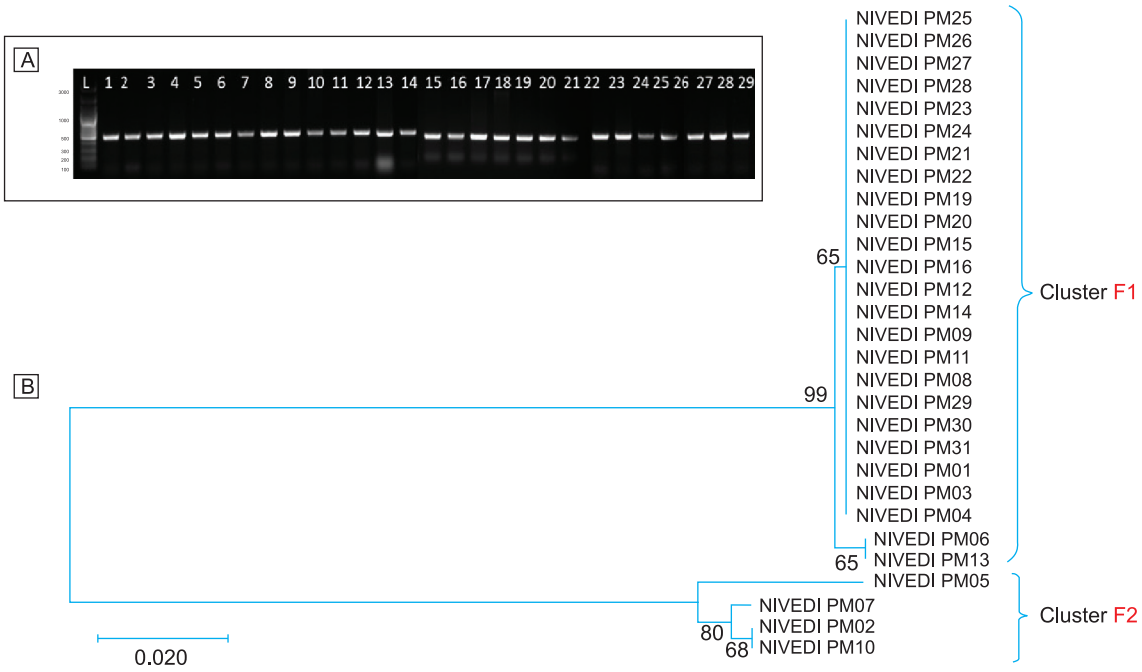

Fig. 1. Amplification of *ptfA* gene and phylogenetic analysis of ovine *P. multocida* isolates. Panel A. PCR amplification of *ptfA* gene (~435 bp) from *P. multocida* strains (n=29). The serial number on top of agarose gel electrograph denotes *P. multocida* strains sequentially as mentioned in Table 1. Lane L, denotes standard DNA ladder. Panel B. Phylogenetic tree of deduced amino acid of *ptfA* gene of ovine *P. multocida* isolates using neighbour-joining method in MEGA X. The number in the branch of phylogram indicates bootstrap value (%) by 1000-replication multiple, and scale indicates one per 1000 substitutions of amino acid sequence.

colonization and invasion of the host (Harper *et al.* 2006). Various studies had shown that *ptfA* genes encoding subunit of type 4 fimbriae is an important epidemiological markers of pathogenic *P. multocida* isolates from clinical cases (Verma *et al.* 2013, Orynbayev *et al.* 2019).

Upon completion of *ptfA* gene targeted PCR assay, an amplified product of ~435 bp was noticed in all the *P. multocida* strains from sheep (Fig. 1A). The details of all *ptfA* gene sequences with their GenBank accession numbers are listed in Table 1. Multiple sequence alignments revealed that deduced protein sequences were almost conserved in most of strains at N-terminal (1-203aa). Further, the percentage of identity among the *P. multocida* strains of Indian sheep was 76.4 to 100%. Among 29 strains of this study, 25 strains were absolutely conserved despite origin from different sheep farm locations. Whereas, with other *P. multocida* strains from different host origin, the identity ranged from 79.2 to 100%. C-terminal (204–430aa) was showing variability in different strains and it was more frequent in ovine strains of Haveri and Chickmangaluru districts. All strains have variability at 360nt position and most have thymine in place of cytosine. Deduced amino acids sequence analysis revealed absolute conservation of N-terminal sequences between 1–67 positions. Upon multiple sequence alignment, C-terminal of 4 strains of Haveri and Chickmanagluru districts had variation at 32 different positions in comparison to Indian HS vaccine strain (P52) (Fig. 1A). Only one strain of Koppal district had variability at 122aa position, wherein Serine was present in place of Valine/Alanine.

Phylogenetic analysis based on the deduced amino acid sequences of *ptfA* genes, grouped 29 *P. multocida* strains into 2 clusters (F1 and F2) with each having two sub-branches (Fig. 1B). Of two clusters, a larger cluster F1 included 25 strains, whereas smaller sized cluster included 4 strains only. Phylogenetic analysis with representative NIVEDI_Pm sequences (10) with sequences (15) representative of *P. multocida* strains from different regions of world and host species also formed two clusters (I and II) (Fig. 2).

Comparative sequence analysis of *ptfA* gene of 29 *P. multocida* strains showed that all sequences had conserved ORF (435 nucleotide) coding for 144 amino acids, which were similar to the previous reports (Hatfaludi *et al.* 2010, Shivachandra *et al.* 2013). Notably, a few of the strains were showing C-terminal variability (between 204–430) and variability was found more in strains of Haveri and Chickmangaluru districts in comparison to HS vaccine strain (P52). N-terminal deduced amino acid sequence was found highly conserved among all the strains until 67 amino acid which was in concordance with previous finding of conserved N-terminus α-1 helix region and heterogeneous C-terminus (68–137 aa) that comprised of β-strand regions in the *ptfA* sequences of *P. multocida* (Shivachandra *et al.* 2013). It was quite interesting to note that in spite of variation at nucleotide level, C-terminal of protein sequences of all the strains except 6 strains, were well conserved. Previously, sequence variability of *ptfA* gene form different alleles group of strains were directly correlated with infectivity of strains (Sellyei *et al.* 2010).

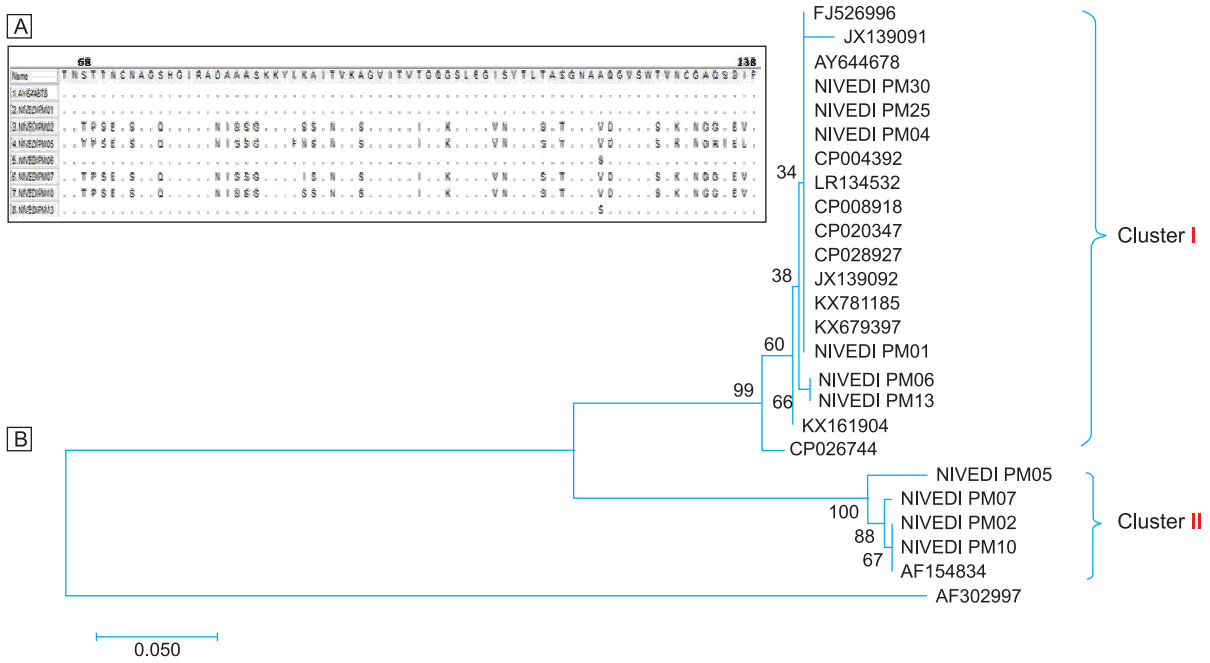

Fig. 2. Comparative Multiple sequence alignment and phylogenetic tree analysis. Panel A. Multiple sequences alignment using deduced amino acid of *ptfA* genes of selected six ovine *P. multocida* isolates showing variability at C-terminal variation between the amino acids 68–138 region in comparison with HS vaccine strain (P52). Panel B. Phylogenetic tree constructed using deduced amino acid sequences of *ptfA* gene from representative ovine *P. multocida* strains (n=10) along with representative *ptfA* sequences (n=15) retrieved from GenBank using neighbour-joining method in MEGAX. The number in the branch of phylogram indicates bootstrap value (%) by 1000-replication multiple, and scale indicates one per 1000 substitutions of amino acid sequence. *Actinobacillus pleuropneumoniae* used as an outgroup taxon.

All strains analyzed in our study other than 6 of two districts mentioned had absolute homogeneity at amino acid level. Notably, C-terminal sequences of four strains; Haveri (04) Chickmangaluru (01) and Koppal (01) districts of Karnataka, were found to be different from other strains reflecting the circulation of diverse strains in the population. Despite the fact that distance between districts were far away from each other, presence of same strains in the districts indicated that migratory sheep movements and potential mixing in that area are natural part of sheep production system practiced by the local farmers. All the *ptfA* gene sequences had two pairs of highly conserved Cysteine residues (residues 62 and 72, and residues 131 and 143) as described earlier (Shivachandra *et al.* 2013).

Phylogenetically, all the 29 strains formed two clusters (F1 and F2) with majority (n=25) clustered in F1, while 4 strains formed cluster-F2. This indicated two different genotype of *P. multocida* circulating in that geographic area. Global phylogenetic analysis also grouped the strains into only two different clusters. Most strains formed one group (Cluster-I) along with strains of USA, Asia, and Europe and Middle East countries belonged to different host except 4 strains of Haveri and Chickmanagluru districts (NIVEDI_PM02, NIVEDI_PM05, NIVEDI_PM07, NIVEDI_PM10) that formed separate group (Cluster-II) with avian strain (AF154834) of Vietnam. Similarly, we found strong significant correlation between cluster F2 and *P. multocida* strains of Haveri in model based correlation analysis. Within the *P. multocida* cluster, preferential geographical wise no grouping was observed which indicated that protein is highly conserved among strains of various geographical regions in spite of having allelic variation at genetic level. Overall, out of 29 *P. multocida* strains, 25 strains were found to be absolutely homogenous by *ptfA* gene based sequence analysis, reflecting the need for another typing tool to discern the genetic variability, if any, among them. Conclusively, all the pathogenic *P. multocida* strains of ovine origin were possessing *pftA* gene encoded type IV fimbrial protein invariably. Further, majority (86%) of the circulating ovine *P. multocida* serogroup A strains belonged to pneumonic pasteurellosis exhibited absolute homogeneity by *ptfA* gene based sequencing.

ACKNOWLEDGEMENTS

Authors are thankful to the Indian Council of Agricultural Research (ICAR), New Delhi, and ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Benglauru, Karnataka, India, for providing the necessary facilities and financial assistance to carry out the research on *Pasteurella multocida,* under the institute project (Code: IXX12176) 'Epidemiology of Haemorrhagic septicaemia (HS) in India'.

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