RESEARCH ARTICLE

Multilocus Sequence Typing of *Pasturella multocida* Isolates from Different Animal Species

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Abstract

The present study was carried out to characterize six *Pasteurella multocida* isolates obtained from sheep, duck, pig, cow, poultry, and *P. multocida* P_{52} vaccine strain by multilocus sequence typing (MLST). All the isolates were first confirmed positive for *P. multocida* by culturally first and then by a biochemical method. The Deoxyribonucleic acid (DNAs) were extracted from the isolates and PM-PCR was carried out, which confirmed all the isolates as *P. multocida,* and further studied for capsular typing. Among them three isolates of *P. multocida* were typed as capsular type A (sheep, duck and poultry), and the other two were typed as capsular type B (cow and pig). Specific primers and protocols described in the Rural Industries Research and Development Corporation (RIRDC) MLST scheme for *P. multocida* were used to amplify the seven house-keeping genes, *viz., adk, est, pmi, zwf, mdh, gdh,* and *pgi.* The purified PCR products were subjected to sequencing by Sanger's method. The total length sequences of genes were trimmed to the length of 466 bp, 535 bp, 602 bp, 500 bp, 521 bp, 530 bp, and 560 bp for *adk, est, pmi, zwf, mdh, gdh* and *pgi* genes, respectively, as described in the RIRDC MLST scheme. Isolates of sheep, duck, pig, cattle, poultry, PAS-506/06, PAD-914/11, PAP-721/09, PAC-93/15, PAP-87/13 and P_{52} vaccine strain were grouped to sequence type 122, 307, 308, 309, 129 and 122, respectively.

Keywords: Multilocus sequence typing (MLST), *Pasteurella multocida*, PM-PCR.

Ind J Vet Sci and Biotech (2021): 10.21887/ijvsbt.17.1.2

Introduction

Pasteurella multocida infects a wide spectrum of domestic and wild animals such as poultry and wild birds, pigs, cattle, buffaloes, rabbits, small ruminants, dogs, cats (including house cats and large wild cats) and other mammals (Wilkie *et al*., 2012; Wilson and Ho, 2013). It is documented that *P. multocida* isolates' infection displays host predilection (Wilkie *et al*., 2012; Wilson and Ho, 2013). Isolates from non-avian hosts generally do not cause fowl cholera symptoms in birds (Peng *et al.,* 2017). However, little is known about the genetic characteristics of *P. multocida* strains circulating in different hosts.

The two key surface components, capsule, and LPS form the main typing basis of *P. multocida*. Serologically, the bacterium is classified into five capsular serogroups (A, B, D, E, F) and/or 16 somatic serotypes according to its capsule and/or LPS antigens (Heddleston *et al.,* 1972). These traditional serological typing methods are too complicated to be used, because the preparation of the high-sensitive antiserum required for these methods is very difficult (Peng *et al.,* 2016). Therefore, molecular typing methods have been developed to help assign *P. multocida* into five capsular genotypes (Townsend *et al*., 2001) and eight LPS genotypes (L1-L8) (Harper *et al*., 2014). Besides, the MLST method has also been designed for *P. multocida* (Subaaharan *et al.,* 2010), and it has been widely used in epidemiology and surveillance (Hotchkiss *et al.,* 2011; and Peng *et al.,* 2018)

Multilocus Sequence Typing (MLST) takes advantage of the speed and simplicity of automated DNA sequencing and has many important advantages over the other methods that are used for global epidemiology (Enright and Spratt, 1999). Identifying alleles directly from the nucleotide sequences of internal fragments of seven housekeeping genes, *viz., adk01, est, gdh, mdh, pgi, pmi* and *zwf* forms the basis for MLST (Bisgaard *et al*., 2013). Gene regions of approximately 450-500bp are sequenced, and those found unique within a species are assigned an allele number. The alleles then characterize each isolate at each of the seven loci, which constitute its allelic profile or sequence type (ST) (Perez-Losada *et al*., 2013). MLST databases offers mainly three queries: allele sequence identification and comparison, allele profile identification and comparison and matching of isolates. This study aimed to understand the sequence type

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How to cite this article: Mistry, SJ., Bhanderi, BB., Roy, A., Mathakiya, R.A., & Jhala, M.K. (2021). Multilocus Sequence Typing of *Pasturella multocida* Isolates from Different Animal Species. Ind J Vet Sci and Biotech, 17(1): 6-10.

Source of support: Nil

Conflict of interest: None.

Submitted: 15/11/2020 **Accepted:** 20/01/2021 **Published:** 25/01/2021

and allelic profile of different *P. multocida* strains infecting different host species from India's different locations by MLST.

Materials and Methods

Bacterial Strains

Two isolates from Cow PAC 93/15 (Gujarat) and Poultry (Gujarat), other three obtained from Sheep (Chennai), Duck (Thrissur), Pig (IVRI, Izatnagar), and a vaccine strain *P. multocida* P52 were used for this study. The isolates were identified by morphological, cultural, and biochemical methods and further confirmed by specific *P. multocida* PM-PCR. Further study was carried out by capsular typing and then MLST analysis. The reference strain of *P. multocida* (P52 strain) was procured from Animal Vaccine Institute, Gandhinagar, Gujarat. The details of the isolates are given in Table 1. The isolates from sheep, duck and pig were obtained under the AINP-HS Network Programme.

DNA Extraction

The genomic DNA of *P. multocida* isolates was extracted according to Antony *et al.* (2006) with minor modifications. A pure colony of *P. multocida* was inoculated into 5 mL of BHI broth and incubated at 37°C for 18 hours. 1.5 mL of this broth culture was transferred into an Eppendorf tube and it was centrifuged at 3000 x g for 10 minutes. The pellet was washed twice in phosphate-buffered saline and the final pellet was re-suspended in 100 µL of triple distilled water. Quality and quantity of DNA were calculated using NanoDrop 1000 spectro-photometer at 260 and 280 nm with distilled water as reference. The pellet suspended in 100 µL triple distilled water was boiled for 10 min and immediately chilled on ice for 30 min. The sample was then thawed and centrifuged at 3000 x g for 5 min. The supernatant was stored at -20°C for further use as template DNA and this DNA was used as template for further confirmation by PM-PCR capsular typing and MLST analysis.

Confirmation of *P. multocida* by PM-PCR and Capsular Typing of Isolates by Specific Primer

The isolates were further confirmed as *P. multocida* by PM-PCR using specific PCR primers and PCR conditions developed by Townsend *et al.* (1998). The capsular typing of the isolates was also carried out using specific primers and PCR conditions developed by Townsend *et al.* (2001).

Multilocus Sequence Typing

All the isolates were subjected to MLST scheme developed by Subaaharan *et al.* (2010) for *P. multocida*. PCR amplification and sequencing was carried out for seven housekeeping genes using primers and protocols available at RIRDC MLST Database (http://pubmlst.org/ pmultocida_rirdc/) of PUBMLST developed by Jolley *et al.* (2004) and cited at the University of Oxford. Genomic DNA from two isolates from Cow (Gujarat) and Poultry (Gujarat), other three obtained from Sheep (Chennai), Duck (Thrissur), Pig (IVRI, Izatnagar) and a vaccine strain P52 of *P. multocida* was used as the template for PCR assay. Details of primers used to amplify the seven housekeeping genes targeted were as given in the website http://pubmlst.org/pmultocida_rirdc/info/primers.shtml.

Forward and reverse sequences of each gene locus's representative sample were assembled against most closely related reference sequence of a respective gene by using SeqScape (V2.5) software, and total length sequence was obtained. The obtained sequences of genes were trimmed to the length of 466, 535, 602, 500, 521, 530, and 560 bp for *adk, est, pmi, zwf, mdh, gdh* and *pgi* genes, respectively, as described in the Rural Industries Research and Development Corporation (RIRDC) MLST scheme for *P. multocida*. The sequence of each locus was checked in RIRDC MLST Database website to determine the allele and sequence type of the isolates.

Results and Discussion

All the isolates yielded an amplified 460 bp product in PM-PCR. The 460 bp amplicon band's presence indicated that all the isolates were of *P. multocida* (Fig. 1). Further studies were carried out for capsular typing. Among them, three isolates PAS-506/06, PAP-87/13 and PAD-914/11 typed capsular typing A with amplification of 1044 bp (Fig. 2) and other two isolates PAP-721/09, PAC-93/15 and a vaccine strain P52 were typed as capsular type B by amplification of 760 bp (Fig. 3).

Table 1: Details of the *P. multocida* isolates and allelic profile of *P. multocida* isolates from sheep, duck, pig, cow, poultry and P52 vaccine strain (as submitted to the RIRDC MLST isolate database)

Isolate details						*Allelic profiles*							
Isolate	*Database ID*	*Host*	*Year*	*Place*	*Capsular type*	*adk*	*est*	*pmi*	*zwf*	*mdh*	*gdh*	*pgi*	*ST*
PAS-506/06	962	Sheep	2006	Chennai	A	23	37	21	17	4	2	17	122
PAD-914/11	963	Duck	2011	Thrissur	A	23	82	18	8	17	46	11	307
PAP-721/09	964	Pig	2009	IVRI	B	45	10	20	19	1	2	8	308
PAC-93/15	965	Cow	2015	Gujarat	B	7	37	21	10	4	7	8	309
PAP-87/13	966	Poultry	2013	Gujarat	A	21	33	26	2	17	20	20	129
P52 Vaccine strain	253	Buffalo	-	-	B	23	37	21	17	4	2	17	122

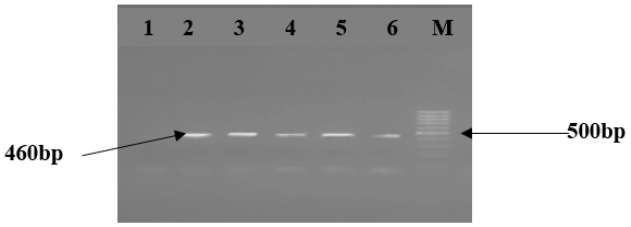

Fig. 1: PM-PCR showing 460bp amplified product specific for *P. multocida*
Lane 1: Blank control, Lane 2-6 : *P. multocida* from different host
Lane M: Marker, 100bp DNA ladder

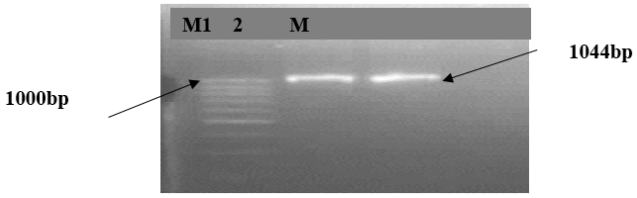

Fig 2: PCR for detection of capsular type of *P. multocida* isolates showing amplified product of 1044 bp (Capsular type A)
Lane M: Marker, 100bp DNA ladder
Lane 1: *P. multocida* isolate from sheep and poultry

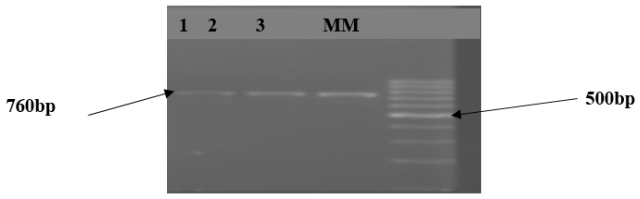

Fig. 3: PCR for detection of capsular type of *P. multocida* isolates showing amplified product of 760 bp (Capsular type B)
Lane 1-3: *P. multocida* isolates from different host Pig, Cow and a P_{52}
Lane M: Marker 100bp DNA ladder

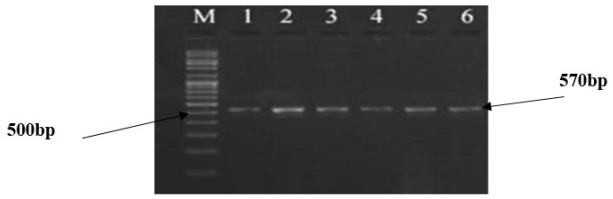

Fig.4: *PCR* amplification of *adk*gene (570 bp) on 2% agarose gel
M: Molecular marker,
1-6*: P. multocida* from different host

PCR amplification of seven housekeeping genes revealed amplified products of ~570, 620, 641, 702, 739, 784 and 808 bp for the genes *adk, mdh, est, gdh, pmi, pgi* and *zwf,* respectively, as analyzed by agarose gel electrophoresis (Fig. 4 to Fig. 10).

The sequences of seven housekeeping genes obtained from all the *P. multocida* isolates from different host were not similar. The gene sequences were then trimmed to the length of 466, 535, 602, 500, 521, 530 and 560 bp for *adk, est, pmi, zwf, mdh, gdh* and *pgi* genes, respectively, as described in the Rural Industries Research and Development Corporation (RIRDC) MLST scheme for *P. multocida*. The allelic profiles assigned to all the isolates at the seven loci were different and sequence type was also different, as shown in Table 1.

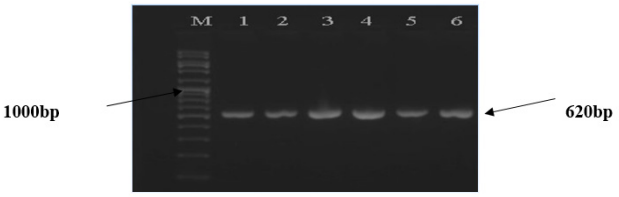

Fig. 5: PCR amplification of *mdh*gene (620 bp) on 2% agarose gel
M: Molecular marker,
1-6*: P. multocida* from different host

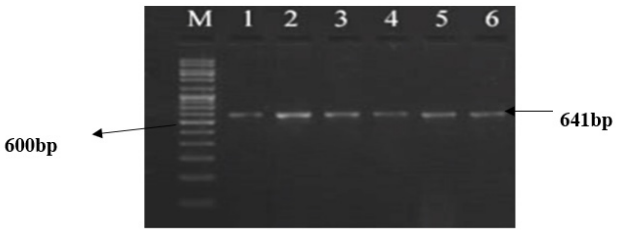

Fig. 6: PCR amplification of *est* gene (641 bp) on 2% agarose gel
M: Molecular marker,
1-6*: P. multocida* from different host

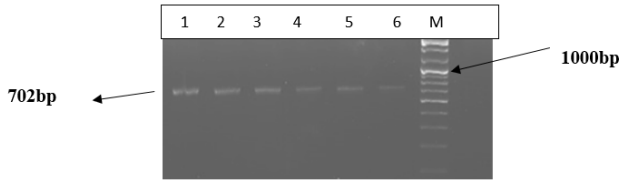

Fig. 7: PCR amplification of *gdh* gene (702 bp) on 2% agarose gel
M: Molecular marker,
1-6*: P. multocida* from different host

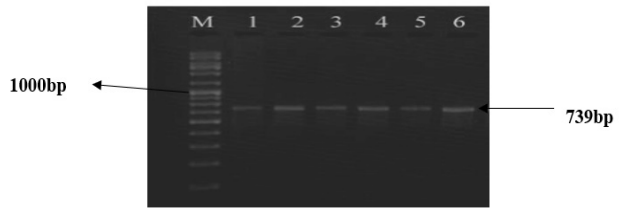

Fig. 8: PCR amplification of *pmi*gene (739 bp) on 2% agarose gel
M: Molecular marker,
1-6*: P. multocida* from different host

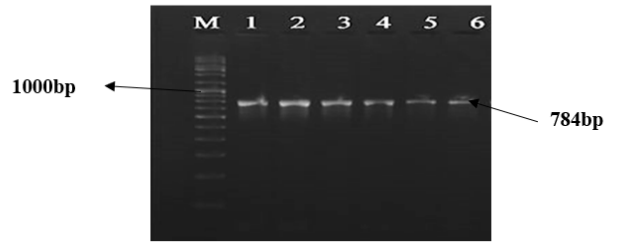

Fig. 9: PCR amplification of *pgi* gene (784bp) on 2% agarose gel
M: Molecular marker,
1-6*: P. multocida* from different host

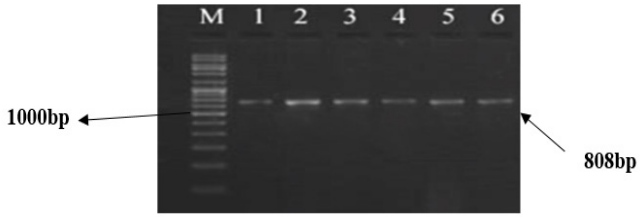

Fig. 10: PCR amplification of *zwf-1* gene (808 bp) on 2% agarose gel
M: Molecular marker,
1-6*: P. multocida* from different host

The sequence of each locus was checked on the RIRDC MLST Database website for determination of the allele and sequence type of the isolates. The allelic profiles assigned to all five isolates were Sheep PAS-506/06-(23, 37, 21, 17, 4, 2, 17), PAD-914/11 (23, 82, 18, 8, 17, 46, 11), PAP-721/09 (45, 10, 20, 19, 1, 2, 8), PAC-93/15 (7, 37, 21, 10, 4, 7, 8), PAP-87/13 (21, 33, 26, 2, 17, 20, 20) and P_{52} vaccine strain (23, 37, 21, 17, 4, 2, 17) for *adk, est, pmi, zwf, mdh, gdh* and *pgi* genes, respectively, and isolates PAS-506/06, PAD-914/11, PAP-721/09, PAC-93/15, PAP-87/13 and the vaccine strain P_{52} were grouped in sequence type 122, 307, 308, 309, 129 and 122, respectively (Table 1). The five *P. multocida* isolates profile of sheep, duck, pig, cow and poultry isolates were submitted to the RIRDC MLST database (ID 962-966). Peng *et al*. (2018) and Wang *et al*. (2013) studied genetic and phylogenetic characteristics of *P. multocida* isolates from different host species and they genotyped *P. multocida* strains from different host species using the predominate MLST from avian species and found them as ST129. MLST genotypes such as ST8, ST9, ST27, ST53, and ST60 are also common in avian *P. multocida* (Subaaharan *et al.,* 2010).

In the present study, a total of two field isolates Cow (Gujarat), and Poultry (Gujarat), three isolates from different locations sheep (Chennai), Duck (Thrissur), Pig (IVRI, Izatnagar), and P_{52}vaccine strain were grouped in ST309, ST129, ST122, ST307, ST308 and ST122. Hotchkiss *et al*. (2011*)* and Aiswarya *et al*. (2017) also carried out MLST of *P. multocida* from cattle buffalo and other host species and found that majority had their own unique sequence types (STs). In present study different ST types of *P. multocida* were obtained by MLST typing which is also important as within *P. multocida* there is a difference in ST circulating in different species which is an important epidemiological survey.

Conclusion

P. multocida isolates from sheep, duck, and pig contained different sequence types: ST122, ST307 and ST308 from different locations and two isolates from cow and poultry from Gujarat contained another sequence type which was ST309 and ST129.

Acknowledgement

The work was carried out under ICAR's All India Network Programme on Haemorrhagic Septicaemia (AINP-HS) and the authors duly extend thanks for the fund provided by ICAR, New Delhi, India. The authors thankfully acknowledge the laboratory facilities extended by the Department of Agriculture Biotechnology, AAU, Anand.

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