MULTILOCUS SEQUENCE TYPING OF *P. MULTOCIDA* ISOLATES OF BUFFALO ORIGIN FROM GUJARAT STATE OF INDIA

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ABSTRACT

Haemorrhagic septicaemia in Indian buffaloes is mainly caused by *Pasteurella multocida* serotype B: 2. The present study was conducted to characterize the *P. multocida* serotype B: 2 isolates of buffalo origin by multilocus sequence typing (MLST) to identify the sequence types prevailing around Gujarat state in India. A total of seven field isolates collected from different regions across the state and a reference strain (P_{52}) were used for this study. Seven housekeeping genes were specifically amplified by PCR; the gene sequences obtained were trimmed to specific length and allelic profiles were assigned to these seven loci. The assigned allelic profiles were 23, 37, 21, 17, 4, 2 and 17 for *adk, est, pmi, zwf, mdh, gdh* and *pgi* genes, respectively and all the isolates were grouped in one sequence type, sequence type 122 (ST122). eBURST analysis classified ST122 into group number 23 when group definition was 5 or more matches. But when group definition was 6 or more matches it was classified into group number 22 along with ST63. Phylogenetic and Splits Tree analysis showed that ST122 is closely related to ST63, ST127, ST162 and ST147 and all five of them were grouped in a cluster. From the study it was concluded that ST122 may be the predominant sequence type in buffaloes of Gujarat state.

Keywords: buffaloes, *Bubalus bubalis*, *Pasteurella multocida*, multilocus sequence typing, Gujarat, sequence type, allelic profile

INTRODUCTION

In epidemiological studies involving *Pasteurella* species, the differentiation of strains of the same species has been done by serotyping, bacteriophage typing, determination of antibiogram, biotyping, plasmid DNA analysis, and whole-cell protein analysis. But many of these conventional typing relies on phenotypic traits which are inconsistently expressed. Also, the sensitivity level is inadequate to differentiate strains accurately (Snipes *et al*., 1989).

The limitations of traditional phenotypic procedures can be overcome by genotypic methods of bacterial identification. Highly sensitive and reproducible techniques that have proved its value for epidemiologic studies are restriction endonuclease analysis (REA) of whole-cell DNA, ribotyping, Polymerase chain reaction (PCR) based typing techniques like REP-PCR, ERIC-PCR, Amplified fragment length polymorphism (AFLP), Multilocus enzyme electrophoresis (MLEE), Multilocus sequence typing (MLST) etc. (Snipes *et al*., 1989; Savelkoul *et al.*, 1999; Townsend *et*

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al., 2001; Blackall and Miflin, 2000).

Multilocus sequence typing (MLST), a molecular typing method for characterizing the microbial isolates, is considered as a gold standard test for global epidemiology (Maiden *et al*., 1998; Enright and Spratt, 1999). 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).

Apart from playing a major role in diagnosing pathogens, MLST has proven to be a high resolution genetic approach that provides data amenable to sophisticated phylogenetic and population genetic analysis (Perez-Losada *et al*., 2013). The method has high discriminatory power and can be applied to all isolates of any species for which there is sufficient knowledge of relevant gene loci to allow DNA sequencing (Spratt, 1999). It is possible to compare typing results from laboratory to laboratory and use a centralized Web-based databank (Feil, 2004; Enright and Spratt, 1998). With the aid of MLST databases one can identify and compare allele sequence, identify the allele profile and also match and compare the isolates (Perez-Losada *et al.,* 2013).

MATERIALS AND METHODS

Bacterial strains

Seven *P. multocida* strains isolated from buffalo affected with haemorrhagic septicaemia and a vaccine strain P_{52} was used for the present study. The field isolates had been isolated and capsular typing was performed as per the method of Townsend *et al.* (2001) at the Department of Microbiology, College of Veterinary Science and Animal Husbandry, Anand. The details of the isolates used are furnished in Table 1. The reference vaccine strain P_{52} was procured from Animal Vaccine Institute, Gandhinagar.

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 37ºC for 18 h 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 resuspended in 100 µl of triple distilled water. Quality and quantity of DNA was calculated by using NanoDrop 1000 spectrophotometer at 260 and 280 nm with distilled water as reference. The pellet suspended in 100 µl triple distilled water was boiled for 10 minutes and immediately chilled on ice for 30 minute. The sample was then thawed and centrifuged at 3000 x g for 5 minutes. The supernatant was stored at -20ºC for further use as template DNA.

Confirmation of *P. multocida*

The isolates were reconfirmed as *P. multocida* by PM-PCR (Townsend *et al.*, 1998).

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 sited at University of Oxford. Genomic DNA from seven buffalo isolates and one vaccine strain of *P. multocida* was used as the template for

PCR assay. Primer sequences used for detection of seven housekeeping genes are given in Table 2.

Forward and reverse sequences of each representative sample of each gene locus were assembled against most closely related reference sequence of 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 for determination of the allele and sequence type of the isolates.

The sequence type was analysed by eBURST v3 software analysis. Concatenated sequences of ST122 and other related sequence types were downloaded from RIRDC MLST database. Phylogenetic tree analysis and split decomposition analysis were carried out for comparison of ST122 with these sequence types. Details of the sequence types used for phylogenetic analysis are given in Table 3. The phylogenetic organization of aligned concatenated sequences of various STs were analysed by neighbour-joining method available in MEGA programme. For split decomposition analysis using Splitstree V4 programme, allelic profile data were required.

RESULTS AND DISCUSSION

On PM-PCR, All the isolates showed an amplified product size of ~460bp (Figure 1). 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 analysed by agarose gel electrophoresis (Figure 2 to Figure 8).

The sequences of seven housekeeping genes obtained from all the seven isolates were 100% 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 23, 37, 21, 17, 4, 2 and 17 for *adk, est, pmi, zwf, mdh, gdh and pgi* genes, respectively and all the isolates and the vaccine strain were grouped in sequence type 122 (Table 4).

All the sequence types of *P. multocida* available in the RIRDC MLST database were subjected to eBURST analysis. In the database, as on 5th May 2015, 749 isolates belonging to 286 sequence types were available. These 286 STs belonged to 23 clonal complexes with 152 being unassigned. eBURST v3 divided them into groups according to their allelic profiles. When sequences were grouped based on sharing of five or more alleles, all the 286 sequence types could be clustered into 40 groups and 57 singletons. ST122 was placed in group 23 along with ST63, ST127, ST 147 and ST 162 which are referred to as H.S. clonal complex (Table 5). When group was defined for sharing of six or more alleles, the ST122 was placed in group 22 along with ST 63 (Table 6).

The phylogenetic analysis by neighbour-joining method revealed that ST122 formed a cluster along with ST63, ST127, ST162 and ST147 (Figure 8) which are also HS related sequence types and all five of them collectively referred to as HS clonal complex (Petersen *et al.*, 2014). Split

decomposition analysis revealed a network-like tree structure (Figure 9). Parallelograms suggest the evidence of recombination. In both split tree and phylogenetic tree, HS clonal complex formed a separate cluster. The percentage similarities of ST122 with other sequence types are given in Table 7.

Subaaharan *et al.* (2010) had developed an MLST scheme for avian strains of *P. multocida.* They recognized 39 sequence types (STs) among 63 Australian poultry isolates and three reference strains of *P. multocida.* Further MLST based investigations included *P. multocida* isolated from cattle, sheep, goats and pigs (Hotchkiss *et al.*, 2011; Pors *et al.*, 2011; Mir *et al.*, 2011; Cardoso-Toset *et al.*, 2013; Moustafa *et al.*, 2013 and Sarangi *et al.*, 2014). Hotchkiss *et al.* (2011) assigned 62 STs to the 195 *P. multocida* isolates used in their study. They found that majority of the HS isolates belong to the ST122 and it included isolates from cattle, buffalo, elephant and bison. Moustafa *et al.* (2013) noticed that ST122 is the predominant HS-associated strain from countries like India, East Timor, Indonesia, Thailand, Myanmar and Pakistan, based on which they suggested that there is potential for region-wide strategies to be adopted aimed at regional control of HS. Petersen *et al.* (2014) carried out MLST of 64 isolates of *P.*

Table 1. Details of the *P.multocida* isolates used for multilocus sequence typing.

Sr. No.	Isolate ID	Host	Year	Sample used for isolation	Region	Capsular type
1	PAB-12-3/08	Buffalo	2008	Bone marrow	Khambat	B:2
2	PAB-33-5/09	Buffalo	2009	Tissue	Kapadvanj	B:2
3	PAB-36-8/09	Buffalo	2009	Tissue	Mehsana	B:2
4	PAB-78-1/13	Buffalo	2013	Tissue	Patan	B:2
5	PAB-83-6/13	Buffalo	2013	Bone marrow	Amreli	B:2
6	PAB-86-9/13	Buffalo	2013	Tissue	Ahmedabad	B:2
7	PAB-89-1/14	Buffalo	2014	Tissue	Gandhinagar	B:2

Table 2. Details of primers used for the amplification of the seven housekeeping genes (http://pubmlst.org/pmultocida_rirdc/info/primers.shtml).

Sr. No.	Gene	Primer Sequence (5' to 3') Forward	Primer Sequence (5' to 3') Reverse	Size (bp)
1	*adk*	TTTTTCGTCCCGTCTAAGC	GGGGAAAGGGACACAAGC	570
2	*est*	TCTGGCAAAAGATGTTGTCG	CCAAATTCTTGGTTGGTTGG	641
3	*pmi*	TGCCTTGAGACAGGGTAAGC	GCCTTAACAAGTCCCATTCG	739
4	*zwf-1*	AATCGGTCGTTTGACTGAGC	TGCTTCACCTTCAACTGTGC	808
5	*mdh*	ATTTCGGGATCAGGGTTAGC	GGAAAACCGGTAATGGAAGG	620
6	*gdh*	ATCGACTTCTTCCGCAGACC	GCGGGTGATATTGGTGTAGG	702
7	*pgi*	ACCACGCTATTTTTGGTTGC	ATGGCACAACCTCTTTCACC	784

Table. 3. Details of the sequence types used for phylogenetic analysis and Split decomposition analysis as per the data in RIRDC MLST database for *P. multocida* and other available literatures.

ST	Host	Lesion	Country/ Continent	Capsular serotype	Reference
63	Bovine	Haemorrhagic septicaemia	no information	B	Petersen *et al.* (2014)
162	Bovine	Haemorrhagic septicaemia	Africa	E	Petersen *et al.* (2014)
147	Bovine	Haemorrhagic septicaemia	Vietnam, South Africa	E	Petersen *et al.* (2014)
127	Bovine	Haemorrhagic septicaemia	Africa	E	Hotchkiss *et al.* (2011)
51	Bovine	Haemorrhagic septicaemia	No information	A	Hotchkiss *et al.* (2011)
50	Swine, Bovine and others	Pneumonia, atropic rhinitis, Bronchopneumonia	Czeck Republic, Denmark, Germany, UK	A, B, D	Hotchkiss *et al.* (2011) and Bisgaard *et al.* (2013)
163	Antelope	no information	USA	B	RIRDC MLST database for *P. multocida*
164	Elk	no information	USA	B	RIRDC MLST database for *P. multocida*
129	Bovine	Haemorrhagic septicaemia	Sri Lanka	B	Hotchkiss *et al.* (2011)
277	Cattle	Pneumonia (HS like symptoms)	India	A	Sarangi *et al.* (2014)
9	Cattle, Goat	Pneumonia	India	F	Sarangi *et al.* (2014)
229	Sheep, Cattle	no information	India	F	Sarangi *et al.* (2014)

Table 4. Allelic profiles of *P. multocida* serotype B: 2 isolates of Gujarat.

Isolate details								Allelic profiles							
Isolate	**Database ID**	**Host**	**Year**	**Country**	**Disease**	**Heddleston serovar**	**Carter serotype**	***adk***	***est***	***pmi***	***zwf***	***mdh***	***gdh***	***pgi***	**ST**
PAB-12-3/08	743	Buffalo	2008	India	HS	2	B	23	37	21	17	4	2	17	122
PAB-33-5/09	744	Buffalo	2009	India	HS	2	B	23	37	21	17	4	2	17	122
PAB-36-8/09	745	Buffalo	2009	India	HS	2	B	23	37	21	17	4	2	17	122
PAB-78-1/13	746	Buffalo	2009	India	HS	2	B	23	37	21	17	4	2	17	122
PAB-83-6/13	747	Buffalo	2013	India	HS	2	B	23	37	21	17	4	2	17	122
PAB-86-9/13	748	Buffalo	2013	India	HS	2	B	23	37	21	17	4	2	17	122
PAB-89-1/14	749	Buffalo	2014	India	HS	2	B	23	37	21	17	4	2	17	122
P_{52}*	253	Buffalo	-	India	HS	2	B	23	37	21	17	4	2	17	122

***** Submitted by Mir *et al.* (2011).

Table 5. eBURST analysis of the isolates (Group definition: 5 or more matches).

Group: 23				
ST	**Frequency**	**SLV**	**DLV**	**SAT**
63	1	1	1	2
122	1	1	1	2
127	1	1	1	2
147	1	-	2	2
162	1	1	3	-

Table 6. eBURST analysis of the isolates (Group definition: 6 or more matches).

Group: 22				
ST	**Frequency**	**SLV**	**DLV**	**SAT**
63	1	1	-	-
122	1	1	-	-

SLV-Single locus variant DLV- Double locus variant SAT- satellites

Table 7. Percentage similarity of ST122 of *Pasteurella multocida* with other sequence types.

Sequence type	Percentage similarity
ST63	99.97%
ST162	99.89%
ST127	99.86%
ST147	99.65%
ST229	99.49%
ST9	99.16%
ST51	98.92%
ST71	98.73%
ST50	98.62%
ST277	98.73%
ST163	98.65%
ST164	98.78%
ST129	98.84%

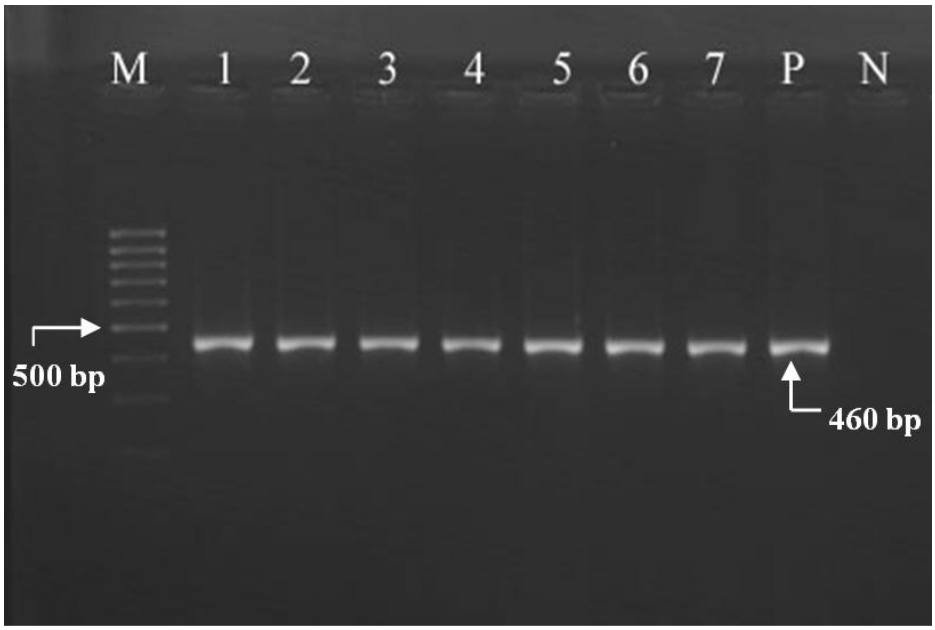

Figure 1. PM-PCR amplification (460 bp) of field isolates on 2% agarose gel. M- Molecular marker, P- Positive control, N- Negative control, Lane 1-7: field isolates.

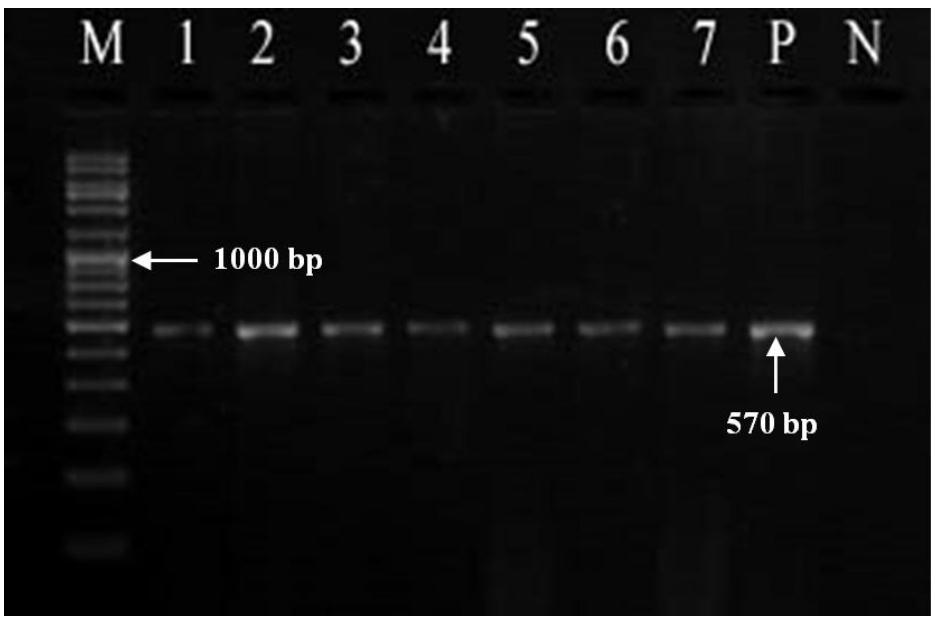

Figure 2. PCR amplification of *adk* gene (570 bp) on 2% agarose gel. M- Molecular marker, P-Positive control, N-Negative control, Lane 1-7: field isolates.

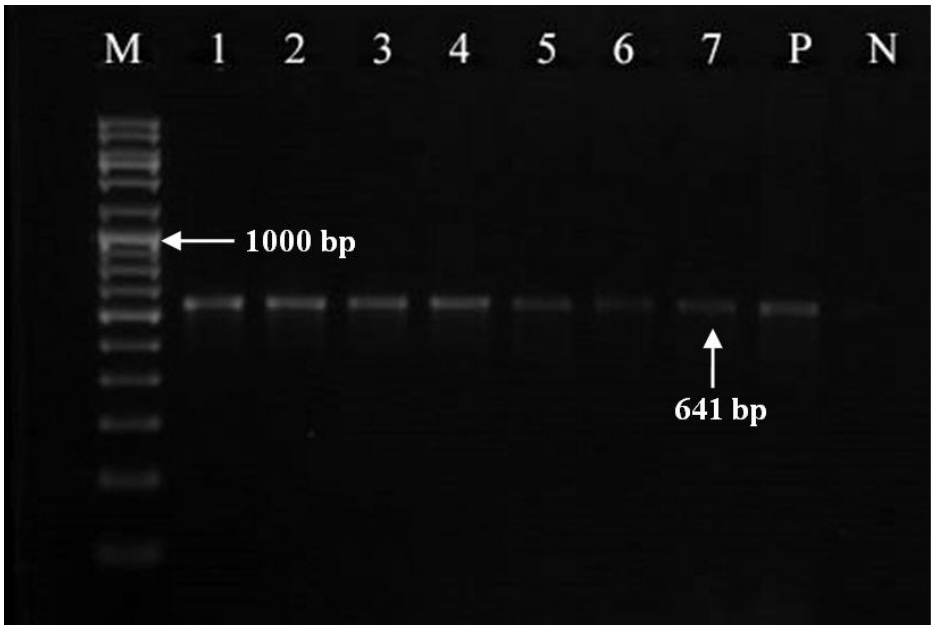

Figure 3. PCR amplification of *est* gene (641 bp) on 2% agarose gel. M- Molecular marker, + Positive control, - Negative control, Lane 1-7 field isolates.

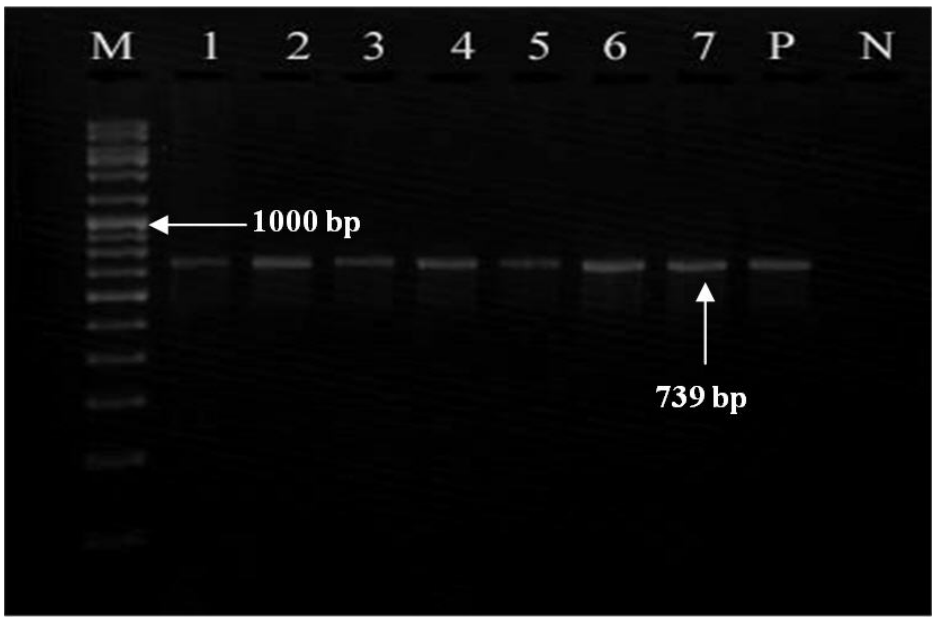

Figure 4. PCR amplification of *pmi* gene (739 bp) on 2% agarose gel. M- Molecular marker, + Positive control, - Negative control, Lane 1-7 field isolates.

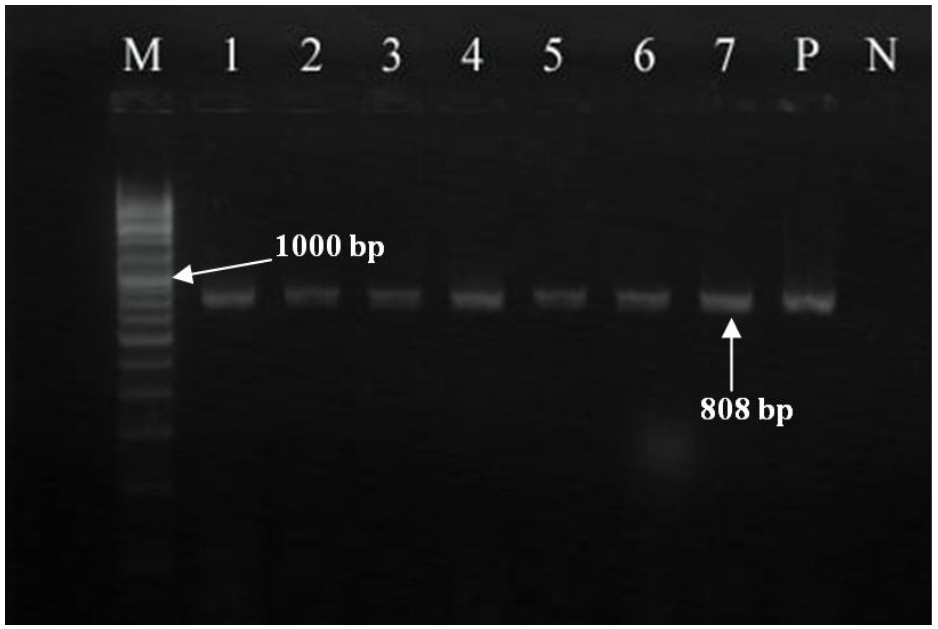

Figure 5. PCR amplification of *zwf* gene (808 bp) on 2% agarose gel. M- Molecular marker, + Positive control, - Negative control, Lane 1-7 field isolates.

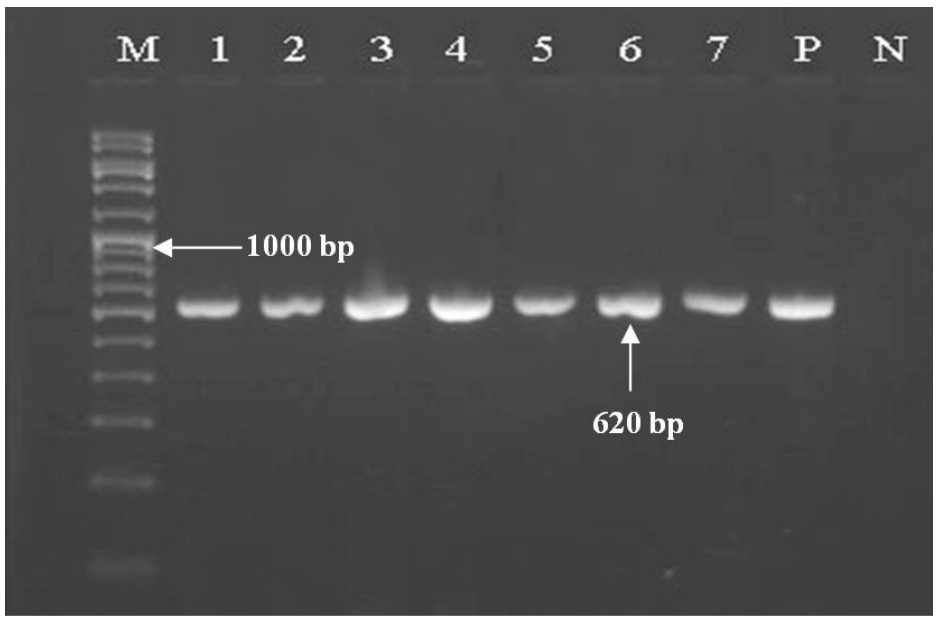

Figure 6. PCR amplification of *mdh* gene (620 bp) on 2% agarose gel. M- Molecular marker, + Positive control, - Negative control, Lane 1-7 field isolates.

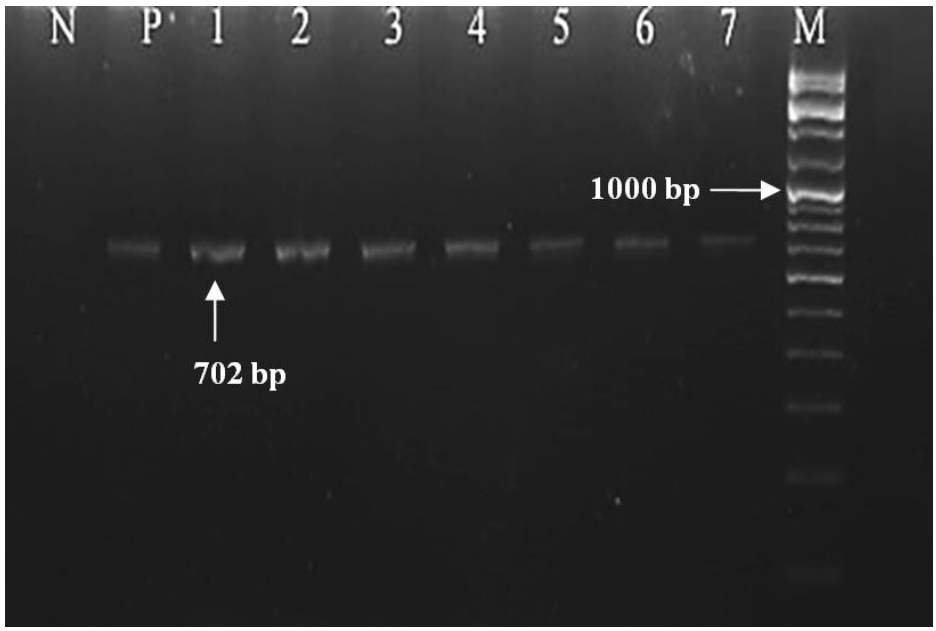

Figure 7. PCR amplification of *gdh* gene (702 bp) on 2% agarose gel. M- Molecular marker, + Positive control, - Negative control, Lane 1-7 field isolates.

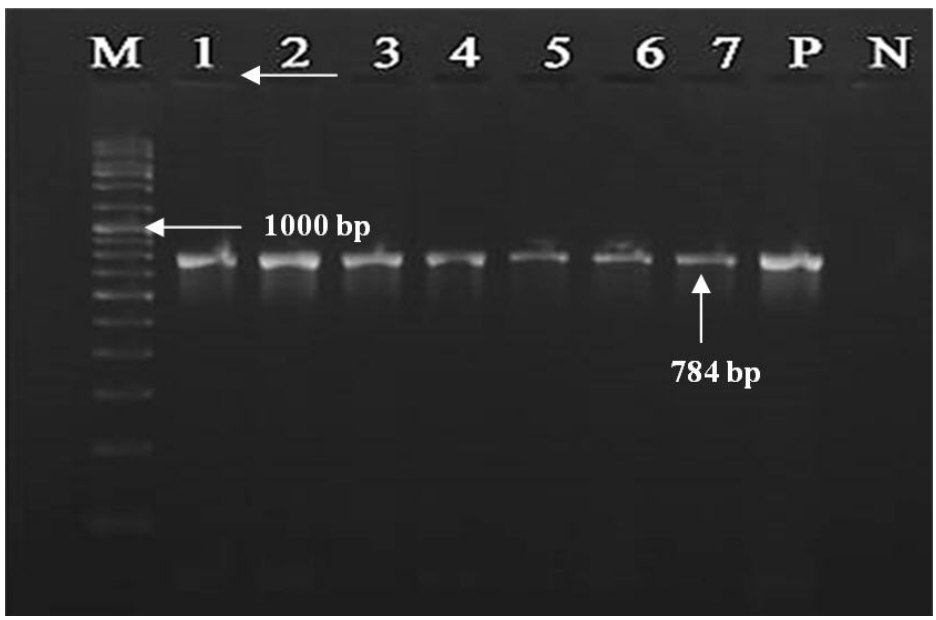

Figure 8. PCR amplification of *pgi* gene (784 bp) on 2% agarose gel. M- Molecular marker, + Positive control, - Negative control, Lane 1-7 field isolates.

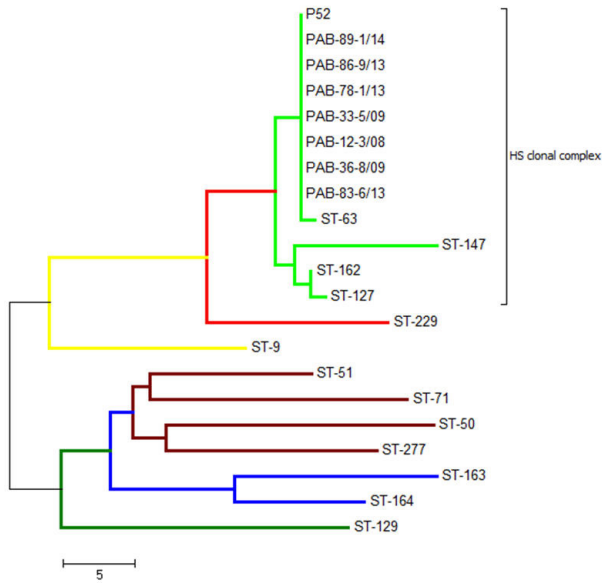

Figure 9. Unrooted Phylogenetic tree showing relationship between concatenated sequences of Gujarat isolates and other sequence types which can produce HS like symptoms reported from various parts of the world.

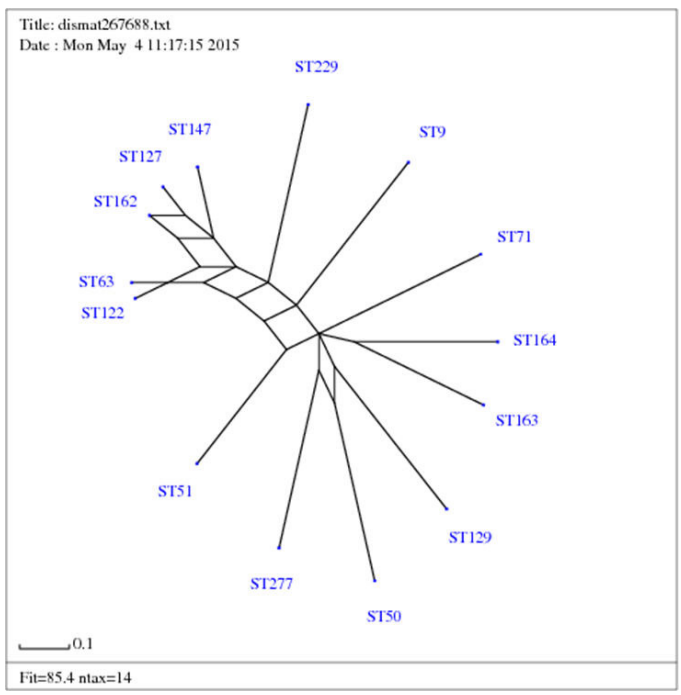

Figure 10. Splitstree analysis of ST122 and other sequence types of *P. multocida* obtained from RIRDC MLST database revealing a network-like tree. The parallelograms suggest the evidence of recombination.

multocida associated with HS in bovines, pigs and buffaloes. They found that the HS isolates were grouped into ST122, ST63, ST147 and ST162; and these four STs along with ST127 formed a HS clonal complex. Sarangi *et al.* (2014) studied the diversity of *P. multocida* isolates circulating in India and they identified ST122 from capsular type B isolates recovered from cattle, mithun, goat and pig which showed the widened host range of ST122. They suggested that the inclusion of pigs and small ruminants in the preventive vaccination policy may help to reduce the overall susceptible population.

In the present study, seven field isolates and vaccine strain P_{52} were grouped in ST122. Mir *et al.* (2011) also carried out MLST of *P. multocida* isolates (n=14) of buffalo origin in India. All the fourteen isolates (including P52 vaccine strain) used in their study were also grouped in ST122. Presently, the RIRDC MLST isolate database contain 114 isolates belonging to ST122 including 7 from the current study (P52 vaccine strain was already submitted to the database by Mir *et al.*, 2011). Remaining 107 isolates include submissions by Mir *et al.* (2011), Moustafa *et al.* (2013), Petersen *et al.* (2014) and Sarangi *et al.* (2014).

In conclusion, ST122 was found to be the dominant sequence type in *P. multocida* isolated from HS suspected buffaloes from Gujarat. Further studies incorporating different animal and bird species can give a clear picture of other sequence types circulating in the state.

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