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ANTIGENIC VARIATION AMONG *PASTEURELLA MULTOCIDA* ISOLATES FROM DISEASED BUFFALOES BY PROTEIN PROFILING AND CLUSTER ANALYSIS

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ABSTRACT

Hemorrhagic septicemia is an acute bacterial disease of buffaloes and cattle caused by *Pasteurella multocida*. In the present study, *P. multocida* (n=11) were isolated from healthy carrier (04/200) and diseased buffaloes (07/200) from Sargodha division, Punjab. *Pasteurella multocida* were identified by culture characteristics and biochemical profile index (API) Kit. Isolates were confirmed by serological and molecular characterization by PCR. Antigenic variation among *P. multocida* (11 isolates) and one vaccine strain was revealed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Although SDS-PAGE revealed that five major proteins were present in all the isolates as well as in vaccine strain of *P. multocida* but isolates had variations in minor protein bands. Relatedness among the isolates was assessed by cluster analysis using fingerprint analysis of missing data (FAMD) and principal coordinate analysis (PCA) of 11 isolates and one vaccine strain. All isolates were clustered into 5 different groups namely I, II, III, IV and V on the basis of their electrophoretic profile. Group I, II, III, IV and V contained 1 (8.33%), 1(8.33%), 2 (16.67%), 6 (50%) and 2 (16.67%) isolates of *P. multocida*, respectively. Vaccine strain had high relatedness with group IV (S3, S6, S7, S8, and S9). It is concluded that distinct polypeptide variation is present among *P. multocida* isolates which insinuate for detailed proteomic and genomic analysis for the development of effective vaccines and control of hemorrhagic septicemia.

Keywords: Hemorrhagic septicemia, *Pasteurella multocida,* Buffaloes, PCR, Antigenic variation and protein profiling and cluster analysis.

INTRODUCTION

Buffalo (*Bubalusbubalis*) is the most important milch among livestock of Pakistan (Javaid *et al.*, 2009). Diseases like foot and mouth (FMD), hemorrhagic septicemia (HS) and brucellosis are major threats to dairy herd health (Afzal, 2009). HS is a contagious bacterial disease of cattle and buffaloes causing heavy economic losses (Afzal and Munir, 1988). Etiological agent of HS, *Pasteurella multocida* is a natural inhabitant of the nasopharynx and occasional resident of intestines of healthy domestic and wild ruminants (Jahferian, 2006). In Pakistan, *P. multocida* serotype B is involved in all out-breaks (Tasneem *et al.,* 2009). The incidence of the disease is higher in buffaloes as compared to the cattle (Hajikolaei *et al.*, 2008). The disease process is generally triggered in various stress conditions (Khan *et al.* 2011). The disease is seasonal in occurrence and its outbreaks are mostly experienced during the rainy seasons of the country (Farooq *et al.* 2011).

HS is diagnosed on the basis of clinical signs and laboratory tests i.e isolation and identification of *P. multocida* from the blood of diseased animals or from various organs like spleen, pharynx, larynx, lungs and bone marrow of dead/slaughtered animals (OIE, 2009). Brickel *et al.*, (1998) developed a molecular technique, polymerase chain reaction (PCR) for accurate and rapid diagnosis of HS in Asia. Five capsular serogroups (A, B, C, D and F) and 16 somatic serotypes (1-16) of *P. multocida* have been identified by PCR (Waheed *et al.*, 2009).

P. multocida strains, used for vaccine production, should be antigenically similar and immunologically homologous to prevalent field strains. Polypeptide mapping of different isolates reveal different patterns of protein bands of *P. multocida* ranging from 16 to 104.71kDa (Pal *et al.*, 2002; Numan *et al.*, 2008). Specific polypeptides (82 kDa, 44.37 kDa, 37 kDa, 32 kDa and 30 kDa), revealed by immunoblot assay, are considered as immunogenic (Tan *et al.*, 2010). Present study was planned to investigate the antigenic variation among *P. multocida* strain isolated from diseased and healthy buffaloes.

MATERIALS AND METHODS

Sample collection: Total of 400 **s**wab samples were collected from larynx/pharynx of diseased (n=200) and healthy (n=200) buffaloes as described by De-Alwis (1984). Sampling was carried out from animals in

Sargodha division. Primary isolation of bacteria was done on Tryptic soya broth and Tryptic soya agar (OIE, 2009). Preliminary, bacteria were identified biochemically in comparison with reference strain of *P. multocida* Robert's type I procured from Quality Operation Laboratory, University of Veterinary and Animal Sciences, Lahore.

Molecular Characterization of *P. multocida:* All of the biochemically positive *P. multocida* isolates were confirmed by PCR whereas antigenic variations among isolates were determined by sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE).

Polymerase Chain Reaction (PCR): Pure cultures of all *P. multocida* isolates were processed for DNA extraction following Townsend *et al.*, (1998). DNA from bacterial samples was detected by 0.9% agarose gel electrophoresis, stained with ethidium bromide (0.05µg/ml) and visualized under UV light. PCR was performed in Master cycler Gradient machine (Eppendrof, Germany). Reaction mixture (50ul) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 µM of each dNTPs, 20 pmols of each of primer pair and 100 ng DNA, and 2.5 U of Taq DNA Polymerase. *P. multocida* specific primers were KMT1T7 5'-ATC-CGC-TAT-TTA-CCC-AGT-GG-3' KMT1SP6 5'-GCT-GTA-AAC-GAA-CTC-GCC-AC-3'.
Amplification procedure was programmed as 95°C for 5 min for initial denaturation followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 52 °C for 30 sec and extension step at 72°C for 30 sec followed by final extension at 72°C for 5 min. Amplicons were analyzed by 1.5 % agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light as described by Townsend *et al.* (1998).

Antigenic variation among *P. multocida* isolates: Protein profiling of *P. multocida* was done using SDS-PAGE as described by Laemmli *et al.* (1970). Discontinuous buffer system under denatured conditions was used for segregation of *P. multocida* proteins (Nauman *et al.*, 2008). Vertical gel electrophoresis system was used for the separation of polypeptides of *P. multocida*. Whole cell bacterial proteins were revealed using 12.5% separating polyacrylamide gel. Stacking polyacrylamide gel (4.5%) was layered over the separating gel to prepare the wells for loading of samples. Electrophoresis was carried out at 100 volts for 8 hours. Electrophoresed gel was stained by coomassie brilliant blue, placed on top of a light box and photographed with digital camera.

Data analysis: The banding pattern was used to calculate relationship indices among *P. multocida* isolates and vaccine strain. Relatedness among the isolates was assessed by cluster analysis using fingerprint analysis of missing data (FAMD) and principal coordinate analysis (PCA). Dendrograms were constructed using arithmetic averages (UPGMA) cluster analysis as described by Sneath and Sokal (1973).

RESULTS

P. multocida were isolated from 15 samples including diseased (09/200) and healthy (06/200) buffaloes, respectively. On the basis of biochemical similarities among positive isolates, reference numbers were traced from API and designated by symbols with reference to the districts. Fifteen bacterial isolates preliminarily identified as *P. multocida* on the basis of API kit were further confirmed by conventionally performed cultivation and biochemical tests. Out of fifteen isolates identified by API kit, thirteen were in agreement with the conventional identification scheme as followed by Burgey's Manual of Determinative Bacteriology (7th edition, 2007). Out of thirteen isolates of *P. multocida*, only eleven (84.62%), including 7 from diseased and 4 from carriers, were confirmed by AGPT.

Molecular characterization: Out of thirteen biochemically identified isolates eleven isolates and a vaccine strain were confirmed as *P. multocida* by PCR (~460 bp). (Figure 1)

Protein profiling: Protein profiling of *P. multocida* isolates was carried out using SDS-PAGE along with known protein marker for determination of polypeptides of bacteria revealed in separating gel (figure 2). Standard curve was plotted between log molecular weights of protein markers and their respective relative flow values. Molecular weights of *P. multocida* polypeptides of vaccine strain and field isolates were determined from standard curve of protein markers and are summarized in table (2). Variation in pattern of polypeptides was observed in *P. multocida* isolated from diseased and healthy buffaloes as well. Vaccine strain contained total ten (10) polypeptides upon electrophoresis with molecular weights ranging from 21.87 to 141.3 KDa. Two field isolates, KBC-02 and MNO-01 were closely related to vaccine strain of *P. multocida*. Both revealed ten polypeptides on segregation in resolving gel having molecular weights ranging from 16.21 to 213.79 KDa (KBC-02) and 21.87 to 213.79 KDa (MNO-01). Difference observed was in molecular weights of two large proteins and remaining polypeptides exhibited similar protein patterns. Two other isolates one from healthy buffaloes of Mianwali and one from diseased buffalo of Bhakkar showed similar pattern of polypeptides having molecular weights 16.21-213.79 and 10.96-112.20 KDa, respectively. These two isolates were again closely related to vaccine strain of *P. multocida*. One each field isolate from outbreak in Bhakkar and Sargodha were similar in their protein profiles having twelve polypeptides with molecular weights ranges of

13.18-134.89 and 19.49-114.81kDa, respectively. Three isolates belonged to same group with 13 polypeptide bands which were from Sargodha (02) and MNO-02. One isolate from Bhakkar and one from Khushab were entirely different other isolates in polypeptide numbers and molecular weights. However three proteins with molecular weights of 91.20-95.49, 30.19-38.90 and 20.41-22.90kDa were common in all field isolates and vaccine strain of *P. multocida*.

Proteomic relatedness among the isolates was assessed by cluster analysis using Fingerprint Analysis of Missing Data (FAMD) of the 12 isolates. The twelve (12) isolates were clustered into 5 groups namely I, II, III, IV and V (figure 3-a). Each group consisted of different number of isolates e.g. Group I=1, Group II=1, Group III=2, Group IV=6 and Group V=2. Group I and II consisted of only one isolate in each (8.33%), group III had two isolates (16.67%), group IV had five isolates and only two isolates were typed in group V (16.67%). PCA was done to define the overall grouping pattern of twelve isolates. According to this investigation total isolates were typed into 5 clusters (figure 3-b).

Table 1: *P. multocida* (n=11) isolated from buffaloes of different districts of Sargodha

Isolate	Designation	Origin	
		Carrier/Outbreak	Place
S1	SGC-01	Carrier	Sargodha
S2	KBC-02	Carrier	Khushab
S3	BkC-01	Carrier	Bhakhar
S4	MNO-01	Outbreak	Mianwali
S5	MNC-03	Carrier	Mianwali
S6	SGO-02	Outbreak	Sargodha
S7	SGO-01	Outbreak	Sargodha
S8	MNO-02	Outbreak	Mianwali
S9	KBO-01	Outbreak	Khushab
S10	BKO-01	Outbreak	Bhakhar
S11	BKO-02	Outbreak	Bhakhar

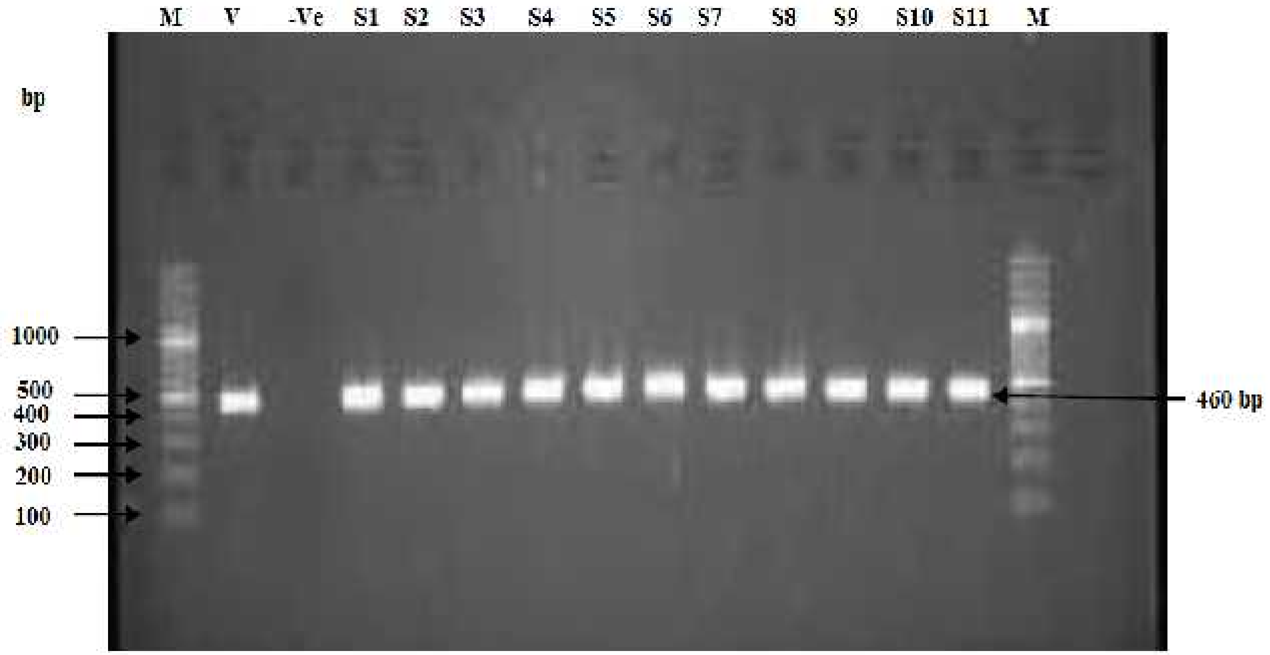


Figure 1: Amplification of *P. multocida* specific DNA by polymerase chain reaction

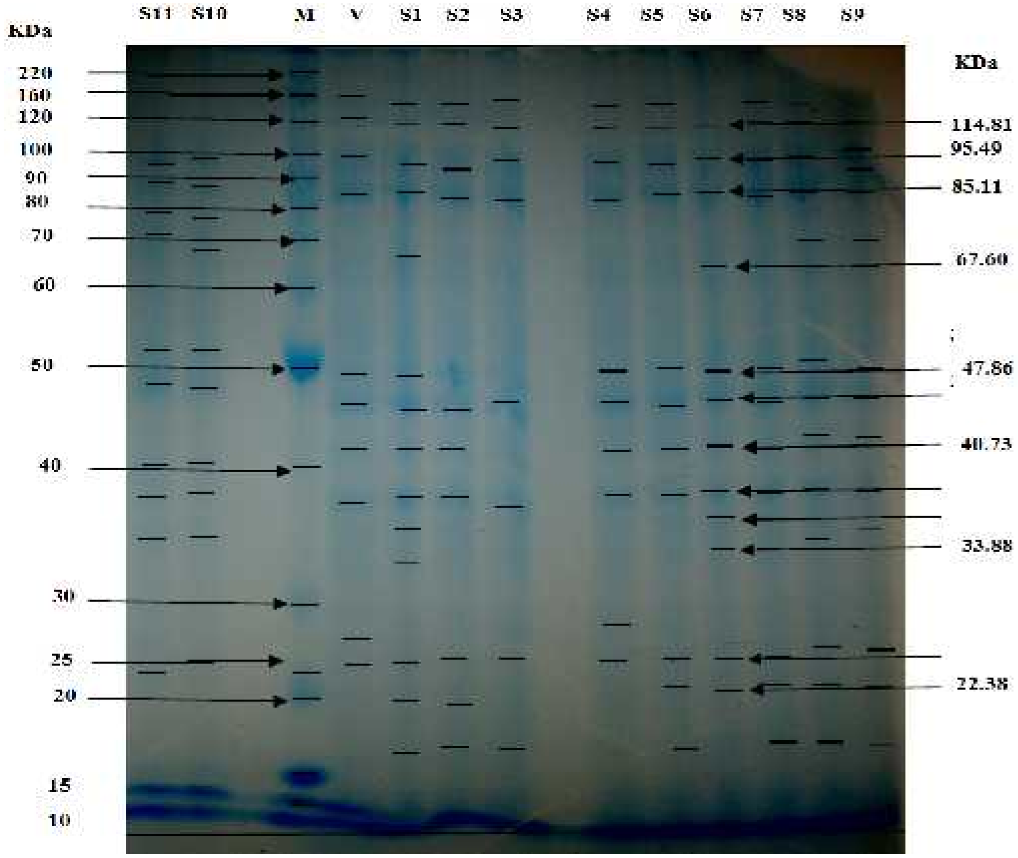
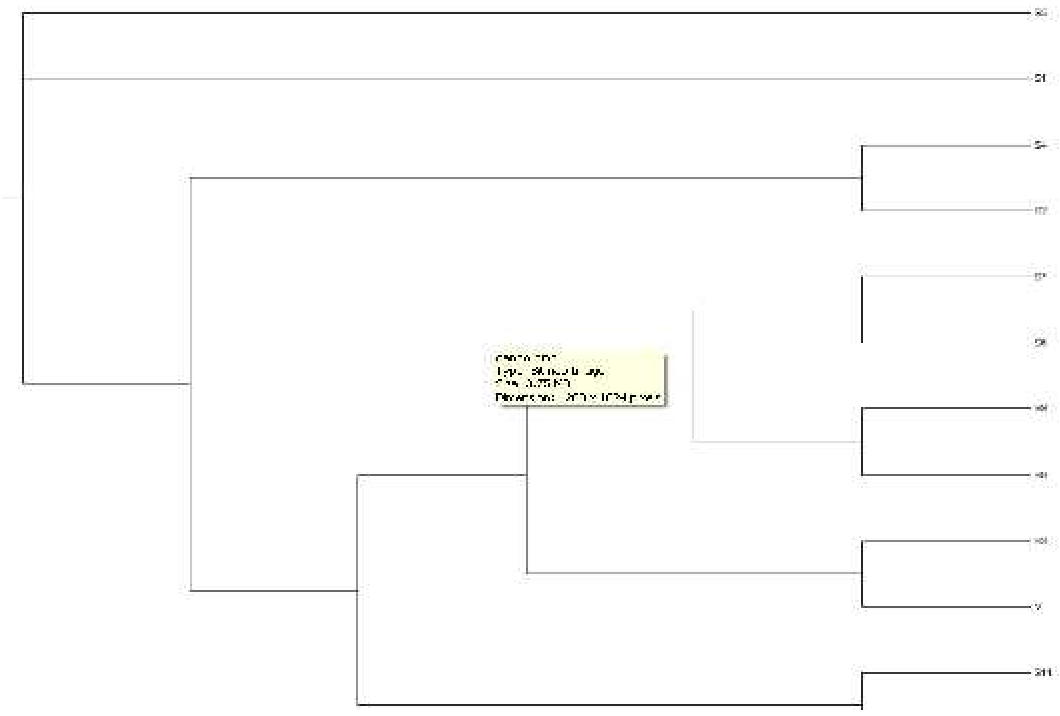


Figure 2: SDS-PAGE pattern of *Pasteurella multocida* isolates and a vaccine strain
M: Marker, V: Vaccine strain



b:

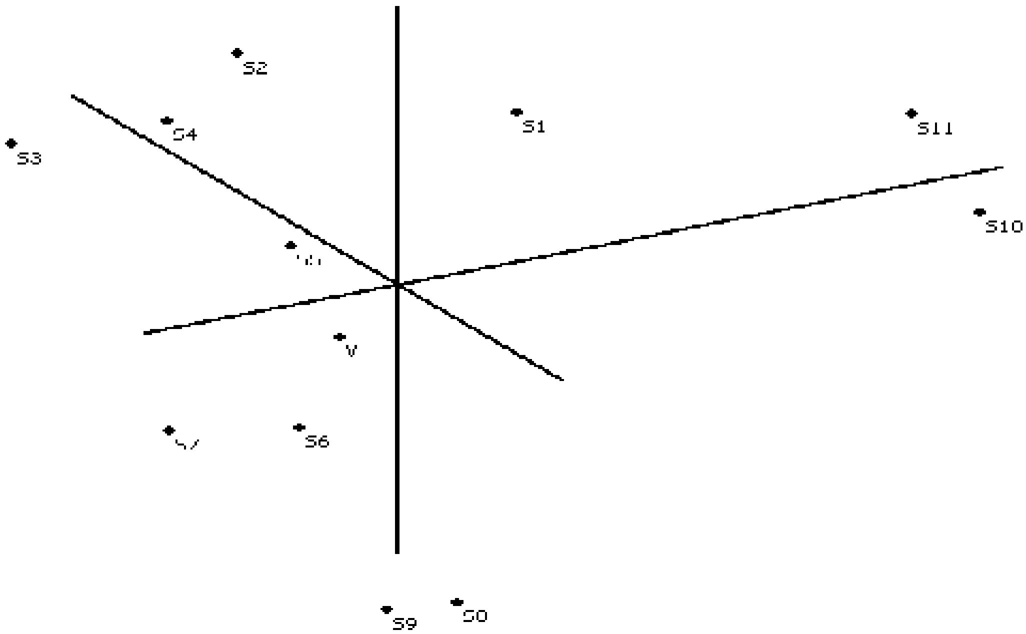


Figure 3: Polypeptides relatedness among *P. multocida* isolates (11) and vaccine strain (01) assessed by cluster analysis using Fingerprint Analysis of Missing Data (FAMD) and Principle coordinate analysis (PCA).
a: Fingerprint Analysis of Missing Data b: Principle coordinate analysis

Table 2: Molecular weights of *P. multocida* polypeptides of vaccine strain and field isolates from diseased and healthy buffaloes with designated symbols from four districts of Punjab

Band No.	Vaccine Strain	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
1	141.3	213.79	213.79	141.25	213.79	213.79	114.81	141.25	141.25	141.25	134.89	112.20
2	134.89	117.48	117.48	114.81	114.81	114.81	95.49	114.81	117.48	117.48	93.32	95.49
3	95.49	95.49	91.20	95.49	95.49	95.49	85.11	95.49	95.49	112.20	87.09	93.32
4	85.11	85.11	85.11	83.17	83.17	85.11	67.60	85.11	85.11	91.20	79.43	74.13
5	56.23	70.79	53.70	52.48	57.54	57.54	56.23	69.18	72.44	85.11	72.44	57.54
6	52.48	56.23	40.73	36.30	52.48	53.70	52.48	58.88	67.60	74.13	57.54	53.70
7	46.77	53.70	37.15	22.90	46.77	46.77	47.86	51.28	58.88	67.60	53.70	44.66
8	38.01	44.66	22.38	16.21	37.15	37.15	40.73	47.86	51.28	57.54	44.66	37.15
9	22.90	38.01	18.62		22.38	22.38	33.88	40.73	48.97	51.28	40.73	28.84
10	21.87	31.62	16.21		21.87	19.49	30.19	30.19	40.73	48.97	30.19	21.87
11		27.54				16.21	22.38	21.37	30.19	40.73	20.41	10.96
12		21.87					19.49	19.95	21.37	38.90	13.18	
13		18.62						17.37	19.05	21.37		
14		16.59							15.84	19.95		
15										16.59		

DISCUSSION

In the present study, *P. multocida* isolates from carrier and healthy buffaloes, identified biochemically and serologically were characterized using PCR. Isolates were compared on the basis of their protein profiles. Percentage of *P. multocida* determined biochemical reactions in buffalo samples lower than the percent prevalence (44.4) of *P. multocida* reported in different areas of Punjab by Mustafa *et al.* (1978). Highest prevalence (49.00%) was observed at Khanewal and lowest (5.37%) in Attock (Patel, 2004). Jindal *et al.* (2002) carried out isolation from trachea, lungs and heart blood of affected cattle and buffaloes at Hariana, India. Kapoor *et al.* (2004) and Javia, (2004) identified 11 and 13 isolates of *P. multocida* from cattle and buffaloes on the basis of culture characteristics. In Pakistan, the percentage of *P. multocida* identified using API 20 NE kit was 93 which was reported as good identification (Nawaz *et al.* 2006).

Molecular characterization: Genomic DNA analysis of 11 field isolates of *P. multocida* and vaccine strain yielded band of 460 base pairs in accord with the findings of Townsend *et al.* (1998). The method reported was efficient for the identification of *P. multocida* isolates. Dutta *et al.* (2001) identified bacteria using PCR for 18 isolates in India compared to 11 isolates in this present study. Nauman *et al.* (1998) compared molecular characterization using PCR with routine microbiological techniques for *P. multocida* isolates and declared PCR as a reliable technique for detection. In agreement with the present work Lee *et al.* (2000) used PCR with minor modifications of method reported by Townsend *et al.* (1998) for molecular characterization of *P. multocida.* Results observed were in agreement with the findings of present study with a similar amplicon of approximately 460 base pairs. Successful amplification of the pathogen has been reported by Townsend *et al.* (2001) who observed a product of 465 base pairs using KMT1SP6 and KMT1T7 primers for *P. multocida,* Shivshankara *et al.* (2001) using the same set of primers and reported an amplified product of 590 base pairs. On the other hand, Anupama *et al.* (2003) used IPFWD and IPREV primers for detection of pathogenic *P. multocida* and declared this technique a reliable tool for rapid identification. Similar to the present study, Javia (2004) obtained amplified product of 465 base pairs while working on 13 field isolates and one vaccine strain of *P. multocida* by PCR as well as Patel (2004) who reported a 465 base pair band. High specificity of PCR has made this technique an efficient tool for early diagnosis of bacterial problems (Blackall and Miflin, 2000).

Protein analysis: Protein profiling of *P. multocida* isolates was carried out using SDS-PAGE along with known protein marker to be used for determination of polypeptides of bacteria revealed in separating gel.

Two field isolates KbC-02 and MnO-01 were closely related to vaccine strain of *P. multocida* vaccine strain containing a total 10 polypeptides upon electrophoresis with molecular weights ranging from 21.87 to 141.3 kDa. Difference observed was in molecular weights of two large proteins and remaining polypeptides exhibited similar protein profiles. Two other isolates from healthy buffaloes of Mianwali and diseased buffalo of Bhakkar showed similar pattern of polypeptides with molecular weights 16.21-213.79 and 10.96-112.20 kDa, respectively. These two isolates were again closely related to vaccine strain of *P. multocida*. Field isolates from outbreaks in Bhakkar and Sargodha respectively were similar in protein profiles with 12 polypeptides with molecular weight ranges from 13.18-134.89 and 19.49-114.81kDa, respectively. Three isolates belonged to the same group with 13 polypeptide bands from SGO-02 and MNO-02. One isolate from Bhakkar and one from Khushab were entirely different from other isolates in polypeptide numbers and molecular weights. However, 3 proteins having molecular weights ranges of 91.20-95.49, 30.19-38.90 and 20.41-22.90kDa were common in all field isolates and vaccine strain of *P. multocida*.

Different researchers reported different number of polypeptide bands as well as different range of molecular weights of polypeptides. The results of Johnson (1991) and Singh *et al.* (2011) are similar to the findings of the present research where variable polypeptide patterns were reported (13, 12 and 15 bands). Noman *et al.* (2008) and Munir *et al.* (2007) reported fewer number of polypeptides (7, 6 and 8) ranging from 15 to 104.7 KDa whereas Nawaz *et al.* (2006) documented 31 polypeptide bands ranging from 11 to 125 kDa molecular weights. Pati *et al.* (1996) characterized *P. multocida*B: 2 and noted 10 major polypeptides with molecular weight range of 88 to 25kDa. In another study a total of six polypeptides with molecular weights from 15 to 91 kDa were revealed (Granoff *et al.* 1982). Pal *et al.* (2002) declared that major polypeptide with molecular weight of 32 kDa and two minor proteins of 39 and 28 kDa were characteristics of *P. multocida* in contrast to the findings of present study. Finally, comparative electrophoretic profiling of polypeptides of vaccine and isolates was performed and 12 protein polypeptides were exhibited in resolving gel by PAGE of boiled bacterial suspension with molecular weights range of 16-150kDa (Kennett *et al.* 1992).

In agreement results had been reported by Lee *et al.* (1991) by segregation of different *P. multocida* isolates. Morton *et al.* (1996) visualized a common polypeptide with molecular weight of 43 kDa out of eight proteins in different field isolates. Srivastava (1998) and Sridevi *et al.* (1999) also reported 12 proteins with molecular weights ranging from 26 to 120 kDa by SDS-PAGE in whole cell lysates of *P. multocida*. In contrast Lee *et al.* (1991) observed similar protein profile in 21 isolates of *P. multocida* belonging to 5 different serotypes. Twenty six polypeptides were reported with molecular weight range of 14 to 200 kDa and pattern of segregation was homologous with variation in 30-40 kDa proteins.

Differences in segregation of proteins of *P. multocida* had been reported by a number of other workers. Variations both in whole bacterial lysates and outer membrane proteins of *P. multocida* had been documented. Zhao *et al.* (1995) observed outer membranes with molecular weights of 74, 87 and 99 kDa; Snipes *et al.* (1988) with 80, 84 and 96 KDa proteins; Glisson *et al.* (1993) with 63, 77, 91 and 99 KDa polypeptides and Ruffolo *et al.* (1998) with 38, 40, 57, 90, 94 and 98 kda proteins in *P. multocida* isolates of different origins. Others like Syuto and Matsumoto (1982); Wang and Glisson (1994) had reported differences in polypeptides of *P. multocida* isolates.

Similarly differences of polypeptides were observed by Knights *et al.* (1990) by SDS-PAGE of *P. multocida* isolates of different serotypes.

Results of polypeptide segregation by SDS-PAGE vary a lot depending upon the location of isolates, technique used, conditions provided for the experimentation. Culture medium provided for the growth of bacteria and sample processing techniques play key role in differences of polypeptides visualized by SDS-PAGE (Irfanullah *et al.* 2008; Tabatabai and Zehr, 2004).

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