Article 126

Isolation and Characterization of *Pasteurella multocida* from Infected Animals

Rabia Durrani*[1], Fawad Ali Khan[2] and Qurban Ali[3]

Haemorrhagic septicemia is an acute and fatal disease among cattle and buffaloes caused by Pasteurella multocida. The prevalence of this disease is 49% in animals of Pakistan. The present study was undertaken to evaluate the prevalence, multidrug resistance and the molecular heterogeneity within clinical strains of P. multocida collected from different cities of Pakistan. Tissue samples of lung and spleen of dead animals were used for diagnostics. Rapid plate screening test for Hyaluronidase of clinical Isolates showing clear and white zones proved that given isolate is pathogenic. Amongst one hundred and ninety nine isolates, only one hundred and one isolates were found to be virulent as clinical isolate of P. multocida. Isolates were confirmed through amplification (PCR) of specie–specific and Hemorrhagic septicemia type B causing test. The primer pair of specie –specific test showed a band of 300bp in all subspecies of P. multocida whereas the primer pair of HS type B causing test showed a band of 460bp in all HS-causing serotype of P. multocida. The high prevalence was found only in Karachi. Whole cell and envelope protein profile of the isolates on SDS-PAGE showed presence of common proteins of 55, 43 and 26kDa indicating no molecular heterogeneity among strains. Representative samples of clinical Isolates from nine different cities were tested for antibiotics sensitivity test. Isolates from Karachi, Badin, Taxila and Abbotabad showed resistant against, Ciprofloxacin, Neomycin, Ofloxacin and Norfloxacin. This study concluded that organism was genetically conserved and there is no diversity within the number of samples tested.

KEYWORDS
Haemorrhagic septicemia, SDS-PAGE, hyaluronidase, BHI (brain heart infusion).

[1]*Microbiology Research Lab, Department of Microbiology, Faculty of Biological Sciences, Quaid-I-Azam University, Islamabad, Pakistan.
[2]Department of Biotechnology, University of Malakand Chakdara Dir Lower Khyber Pakhtunkhwa, Pakistan.
[3]National Veterinary Labs. Disease Diagnosis and Surveillance Section, Ministry of Livestock and Dairy Development Chak Shehzad Islamabad.

INTRODUCTION

In Pakistan Livestock, production is the second major economic activity that has engaged more than 92 million people in the rural areas. The number of large ruminants in the country ranges 45 million including 22.4 and 23.3 million cattle and buffaloes. This sector contributes almost 11% of national GDP and 14 % of the world's population of ruminants (Munir, R et al., 2007). Due to poor rearing conditions, these animals are prone to large number of infectious diseases like foot and mouth disease, Bovine mastitis, enterotoxaemia etc. Amongst these diseases, Haemorrhagic Septicemia (HS) of cattle and buffaloes has emerged as a disease inflicting huge economic losses. This disease has occurred as calamitous epizootics in different Asian and African countries, resulting in high mortality and morbidity in the animals. Prevalence of Haemorrhagic Septicemia (HS) disease is about 49% in the animals of Pakistan (Waheed Ullah et al., 2009). The causative agent of Haemorrhagic Septicemia is Pasteurella multocida (*P. multocida*) a heterogeneous gram-negative bacterium, commensal of the upper respiratory tract of a wide range of animal species including cattles, buffaloes etc. The pathogenesis associated with this organism

emerges in terms of complex interaction of host specific factors (age, diet, immune status and the environment) and bacterial virulence factors like Lipopolysaccharides (LPS, Capsule, adhesions, outer membrane). Most strains of Pasteurella form a capsule or envelope that constitutes polysaccharides, Lipopolysaccharides and variety of protein that significantly contributes in pathogenesis in the host. The overall bacterial envelop has been considered as a basic tool in classification that contribute versatility in host range and associated pathologies.

Envelope profile has assorted this organism into four serotypes (A, B, D, E or F), (Munir, R et al., 2007). There are two specific serotypes of *P. multocida*, B: 2 and E: 2 which are Asian and African in origin (Waheed Ullah et al., 2009). Besides, there is non- hemorrhagic septicemia type B, 3:4 strain of Australian origin(18).These serotypes were also classified as 6: B and 6: E (Waheed Ullah et al., 2009) . The serogroup A capsule contains hyaluronic acid which contributes to the pathogenicity of the organism and imparts a watery to mucoid character to the colonies(3) .Vaccination has been recognized as the most practical and reliable method to stimulate host immunity(Lebrun .A et al., 1992; Bain et al ., 1982; Carter 1967). Despite using different antibiotics in controlling the disease caused by *P. multocida*, the problem still persists due topresence of molecular heterogeneity within the strains of this organism that makes it resistance and difficult to be treated. This may be due to the vaccination failure that is administered to the animal before it suffers from disease. Pasteurella are generally susceptible to antibiotics like, penicillin and tetracycline. However, the organism has been found resistant to a variety of antibiotics including; Ciprofloxacin, Chlortetracycline, Cotrimoxazole, Furazolidone, Lincomycin, Ampicillin, Augmentin, Kanamycin, Apramycin and Cefatoxime. Penicillin and Tetracycline are considered as antibiotics of choice and cephalosporin are acceptable alternative for combating the infection (Munir, R et al., 2007). Molecular characterization of *P. multocida* would be quite effective in the phylogenetic categorization of the organism at subspecies level within the family Pasteurellaceae. This could help in understanding the clinical manifestation dealing with the transmission and control of disease (Munir, R et al., 2007). Direct identification of toxigenic strains of *P. multocida* using PCR proved to be a more rapid and sensitive method compared to conventional biochemical procedures. In addition, genome hybridization has been proved quite effective in the identification of unique DNA sequences involved in pathogenesis of the organism. Considering the prevalence, multidrug resistance pattern and the associated molecular heterogeneity within the different strains of *P. multocida* the detailed understanding of the concerned organism becomes vital. Therefore, the present study is focusing on characterizations of clinical isolates *P. multocida* from different cities of Pakistan. This would ultimately contribute towards basic understanding of the indigenous strains. It would ultimately contribute in establishing futuristic approach for controlling of this pathogenic organism.

MATERIALS & METHODS

Sample collection and Biochemical tests

The morbid material (Tissue samples of lung and spleen of dead animals) was brought to the lab for diagnosis Haemorrhagic Septicemia .Organism was confirmed through staining, Biochemical tests (Catalase test, Oxidase, Urease production test, H2S production test, Nitrate reduction, and Motility test) (Brough 1984) and API-20NE (Analytical profile Index for Non-Enterococcus Biomeurix 2000).

The tests applied for the general biochemical characterization of *P. multocida* isolates in this study were performed, following the (Karaivanov 1984).

Antibiotic sensitivity test

Antibiotics sensitivity test was performed to check the resistance of clinical isolates. Isolates that were susceptible to at least three different antibiotic classes were classified as multidrug resistant.

Hyaluronidase Enzyme Production Test

Rapid plate screening test for Hyaluronidase production by *P. multocida* was performed to check the pathogenicity of clinical isolates.

DNA Extraction of P. multocida

The DNA extraction method for *P. multocida* was done according to (1) protocol. PCR assay (Thermocycler, BioRads USA, PTA-200 with alpha engine) for analysis of both species – specific and HS-causing Type B-specific *P. multocida* conformation was performed according to (Waheed Ullah et al., 2009).

a) *P. multocida* specific PCR assay: The primer pair KMT1SP6 and KMT1T7 was used which specifically amplified a product of approximately 460base pair in all subspecies of *P. multocida*.

The primer sequences were:

KMT1SP6 5'- GCTGTAAACGAACTCGTCGTCGCCAC3-3'
KMT1T7 5'- ATCCGCTATTTACCCAGTGG-3'

b) PCR assay for HS associated type B serotype of *P. multocida*: Primer pair KTSP61 and KTT72 were used which specifically amplify a product of approximately 560 base pair (bp) in all HS causing serotype of *P. multocida*. Primer sequences were:

KTT72 5'- AGGCTCGTTTGGATTATGAAG-3'
KTSP61 5' – ATCCGCTAACACACTCTC-3'

The thermal cycling parameters included the initial denaturation at 94°C for 5minutes; 30 cycles of 94°C for 1 minute, 53°C for 1 minute and 72°C for 1 minute and final extension at 72°C for 9 minutes.

Protein Profiling of Pasteurella multocida

Representative samples from two cities were taken clinical isolates i.e.; HS causing strains of *P. multocida* and non-clinical isolates were then subjected to SDS-PAGE for their whole cell and envelope protein profile comparison to determine the common and some non-common proteins in them. Two groups were selected from each, and then whole cell and envelope proteins were extracted by the method of (9).

RESULTS

The present study was conducted to characterize intra-strain variation among clinical and non-clinical (vaccinal) isolates of *P. multocida* at molecular level to check the distribution of Hamemorrhagic septicemia in some cities of Pakistan. The isolates exhibited luxuriant growth on blood agar with translucent grayish or yellowish green colonies; however, they showed no growth and haemolysis on MacConkey and blood agar respectively. Clinical isolates were confirmed through different biochemical tests and by Analytical profile Index (API -20NE) kits as shown in Table 1. Clinical isolates of Pasteurella multocida collected from nine cities were screened for antibiotic sensitivity test. The isolates from Karachi, Badin, Taxila, and Abbotabad showed multidrug resistant pattern i.e. they were resistant to three antibiotics i.e., Ciprofloxacin, Neomycin, Ofloxacin and Norfloxacin. Fifty eight isolates were resistant Ciprofloxacin (CIP), Chloramphenicol, Neomycin, Ofloxacin, Norfloxacin, and Ceftiofur. Twenty eight isolates from Peshawar, Islamabad and Toba Tek Singh were sensitive to Augmentin, Ciprofloxacin and Gentamycin as shown in Table 3.Rapid plate screening test for Hyaluronidase production test was performed to check the virulency of clinical isolates of Pasteurella multocida. The appearance of white precipitate on noble agar plate showed the pathogenicity of clinical isolates. Amongst one hundred and ninety nine, only one hundred and one isolates were found to be virulent as clinical isolate of *P. multocida* as shown in Table 2.The DNA extracted from clinical isolates were run on agarose gel. All positive samples showed DNA band on gel. The first set of primer pair KMT1T7 and KMT1SP6, which were species-specific amplified all the clinical strains of *P. multocida* showed a band of 300bp as shown in Fig.2.The second set of primer pair KTT72 and KTSP61 confirmed Hemorrhagic septicemia type B strain of Pasteurella multocida, amplified a product of 460bp as shown in Fig.1 in all HS-causing serotype of *P. multocida*. From 180 Isolates, only 104 were found to be positive as shown in Table 4 for the given two types of PCR tests. The concordance of PCR results indicates 100% specificity and sensitivity. Comparative protein profiling of clinical and non-clinical isolates were performed on SDS PAGE, selecting two cities i.e., Karachi and Kahutta as shown in Table 5 and 6. At least six protein bands of 55, 43, 34, 26, 17 & 10 were observed in complex whole cell profile as shown in Table 5, 6 and Fig. 3. During envelop protein profiling, band of 130, 43 and 26kDa were appeared on SDS-PAGE as shown in Fig. 4. Common proteins of 43 and 26kDa were observed in clinical and non-clinical isolates of

P. multocida as shown in Table 5 & 6. The results concluded that no significant variations were observed due to complex protein pattern at whole cell and envelop profile.

DISCUSSION

This study was carried out to observe the inter-strain variation between clinical isolates and non-clinical (vaccinal isolates) at molecular level to check the distribution of Haemorrhagic septicemia in different cities of Pakistan. The results of gram reaction, biochemical test including; Catalase, Oxidase, indole, H2S production, nitrate reduction, haemolysis on 5% sheep blood agar were consistent with other studies (Knights 1990). The isolates from Karachi, Badin, Taxila, and Abbotabad showed multi drug resistant pattern i.e. they were resistant to three antibiotics i.e., ciprofloxacin, Neomycin, Ofloxacin and Norfloxacin. And sensitive to Ciprofloxacin, Gentamycin, Ofloxacin and Augmentin were. Antibiotic resistance in bacterial strain may be attributed to the movement of animals across the border and distribution of virulent genes in these animals (Tang et al., 2009).

Amongst one hundred and ninety nine total isolates, only one hundred and one isolates were found to be virulent as clinical isolate of *P. multocida*. These findings were in agreement with earlier reports of (Carter 1980).

On subsequent investigation, it was found that within type B strains, the property of Hyaluronidase production was restricted to serogroup B: 2, which is the classical Asian serotype. Differentiation of type B serotypes with the primer KMT1 requires additional hybridization. The first set of primer KMT1T7 and KMT1SP6 was species specific and amplified all clinical strains of *P. multocida*. In another reaction, PCR reaction was carried for HS-causing strain conformation by using primer pair, KTT72 and KTSP61 .In this reaction, only HS-causing strain was amplified while Non-HS causing was not amplified. So it suggested that the PCR assay was specific and sensitive for the given type strains of *P. multocida*. The concordance of PCR results with defined toxigenic status indicated 100% specificity and sensitivity. It is clear that this assay will not identify all HS –causing strains of *P. multocida*, as these primers do not amplify DNA from type E strains that cause HS in Africa.(Townsend et al., 2000) Obtained same results, that type B specific primers didn't amplify the type B, 3-4. The ability of PCR assay described in the present study provided rapid identification of *P. multocida* and confirmation of the H S–causing serotype has potential to reform HS diagnosis in Southeast Asia. The techniques in molecular biology have significantly increased understanding of the epidemiology of Pasteurella. In this reference SDS-PAGE has been extensively used to establish the unique properties of the bacterial proteins in *P. multocida* (Karaivanov 1984). SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) was used to observe the inter strain variation using whole cells (total protein) preparations of Pasteurella multocida. There appeared a number of bands in the gel although they could not differentiate Non-HS from the HS. Similar differential pattern was also observed by (Bardley 1970) in *P. multocida* serotypes based on polypeptide profiles. So these results concluded that during protein profiling no heterogeneity was found in the whole cell and envelope proteins of *P. multocida*. The clinical isolates of *P. multocida* confirmed no molecular and biochemical heterogeneity. However samples from animals having Hemorrhagic Septicemia from various ecological setups should be tested to find molecular and biochemical heterogeneity within strains of *P. multocida*. This study has been useful in highlighting the extent of similarities and dissimilarities among the strains of *P. multocida*. Therefore it is suggested that such study may be extended further with larger number of strains / isolates /serotypes of *P. multocida* to gain more insight into the disease and associated the morphological and physiological factors playing role in its prevalence. Detailed epidemiological studies can help control the diseases to safeguard animal wealth of the country.

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*Address for correspondence:
Rabia Durrani, Microbiology Research Lab.
Quaid-I-Azam University,
Islamabad, Pakistan.
e-mail: rabiadurrani_kust@yahoo.co.uk

TABLES

Table 1: Analytical profile Index tests results

Name of city	Number of Isolates	% Identification of Organism by API 20NE kit
Karachi	37	96%
Badin	4	92%
Tando Jam	7	96%
Taxila	7	96%
Islamabad	6	96%
Samundri	4	54%
Khurrianwala	7	67%
Toba Tek Singh	3	95%
Jaranwala	15	96%
Bakkar	11	67%
Sargodha	15	67%
Lahore	12	67%
Bahawalpur	10	55%
Peshawar	15	60%
Abbotabad	5	98%
Kahutta	6	39%
Faisalabad	7	96%

Table 2: Hyaluronidase production by *P. multocida.*

S.No	Name of City	Appearance of white precipitate Hyaluronidase +
1	Karachi	6
2	Kahutta	4
3	Badin	All positive
4	Peshawar	4
5	Taxila	2
6	Islamabad	4
7	Toba Tek Singh	2
8	Sargodha	3
9	Bahawalpur	4
10	Samundri	2
11	Faisalabad	8
12	Khurrianwala	All positive
13	Jaranwala	10
14	Bakkar	9
15	Lahore	6
16	Abbotabad	All positive
17	Khushab	All positive
18	Faisalabad	8
19	Tando Jam	All positive
Total Cities	19	Positive Isolates 101

Table 3: Antibiotic sensitivity test for clinical isolates of *P. multocida.*

S.No	Name of City	Number of isolates(clinical strains)	Name of Antibiotics	Measured zone of inhibition(cm)	Sensitivity Pattern
1	Karachi	8	Ciprofloxacin(CIP)	**1cm**	Resistance
2	Islamabad	7	Augmentin(AMC)	5cm	sensitive
3	Kahutta	8	Chloramphenicol(CMP)	1cm	Resistance
4	Peshawar	10	Ciprofloxacin (CIP)	2.1cm	Mild sensitive
5	Abbotabad	9	Neomycin(N)	0.9cm	Resistance
6	Taxila	11	Ofloxacin(OFX)	1.7cm	Resistance
7	Badin	10	Norfloxacin (NOR)	1.3cm	Resistance
8	Toba Tek Singh	11	Norfloxacin (NOR)	2.2cm	Mild sensitive
9	Sargodha	12	Ceftiofur (ACC)	1cm	Resistance
Number of cities	9	Total number of isolates :86	Total Antibiotics 9	Resistance 58	Sensitive 28

Table 4: Polymerase Chain Reaction Results for *P. multocida.*

S.No	Name of city	Number of isolates	Positive for PCR	Prevalence
01	**Karachi**	**37**	**19**	**8%**
02	**Badin**	**4**	**3**	**0%**
03	Tando jam	7	4	0.20%
04	Taxila	5	3	0.10%
05	Islamabad	12	9	1%
06	**Samundri**	**4**	**1**	**0.04%**
07	**Khurrianwala**	**7**	**6**	**0.40%**
08	**Toba Tek Singh**	**3**	**2**	**0.06%**
09	Jaranwala	15	5	0.70%
10	Bakkar	11	5	0.50%
11	Sargodha	15	10	1.50%
12	**Lahore**	**12**	**8**	**0.90%**
13	Bahawalpur	10	9	0.90%
14	Peshawar	15	9	1.35%
15	**Abbotabad**	**5**	**1**	**0.05%**
16	Khushab	6	3	0.18%
17	Faisalabad	12	7	0.80%
Total cities:17		Total 180	104	High prevalence Karachi

Table 5. Protein profile pattern of *P. multocida.*

S. No	Major and Minor proteins in kDa	Whole cell		Envelop		Comparison
		Isolate I (Karachi)	Isolate II (Kahutta)	Isolate I	Isolate II	
1.	130	2	2	3	3	Non-Common
2.	55	3	3	2	2	Non-Common
3.	43	3	3	3	3	Common
4.	34	3	3	2	2	Non-Common
5.	26	3	3	3	3	Common
6.	17	3	3	2	2	Non-Common
7.	10	3	3	2	2	Non-Common

Table 6: Comparison of clinical and non-clinical isolates of *P. multocida* in SDS-PAGE

S.No	Major and Minor protein bands kDa of non-clinical isolate B,3:4	Whole Cell profile	Envelope Profile	Comparison
01	130	2	3	Non-common
02	55	3	2	Non-common
03	43	3	3	Common
04	34	3	2	Non-common
05	26	3	3	Common
06	17	3	2	Non-common
07	10	3	2	Common

FIGURES

Fig 1. Haemorrhagic Septicemia Type B causing test. The experiment was run in triplicate with 1% low melting point Agarose gel. The thermal cycling parameters were ,initial denaturation at 94°C for 5minutes; 30cycles of 94°C for 1minute; 53°C for 1 minute and 72°C for 1minute and final extension at 72°C for 9 minutes.

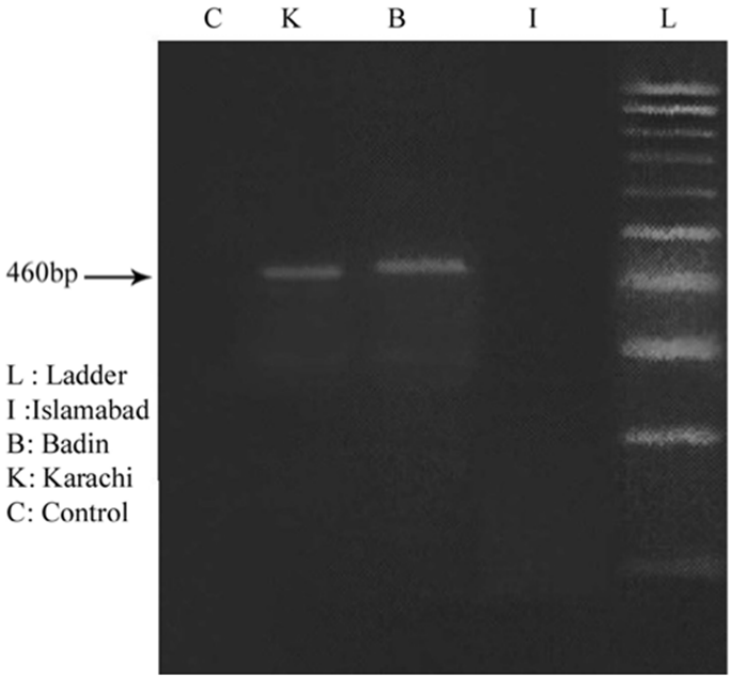

Fig 2. *P. multocida* species–specific test. The experiment was run in triplicate with 1% low melting Agarose gel. The thermal cycling parameters were initial denaturation at 94°C for 5minutes; 30cycles of 94°C for 1minute, primer annealing at 53°C for 1minute and extension at 72°C for 1 minute and final extension at 72°C for 9 minutes.

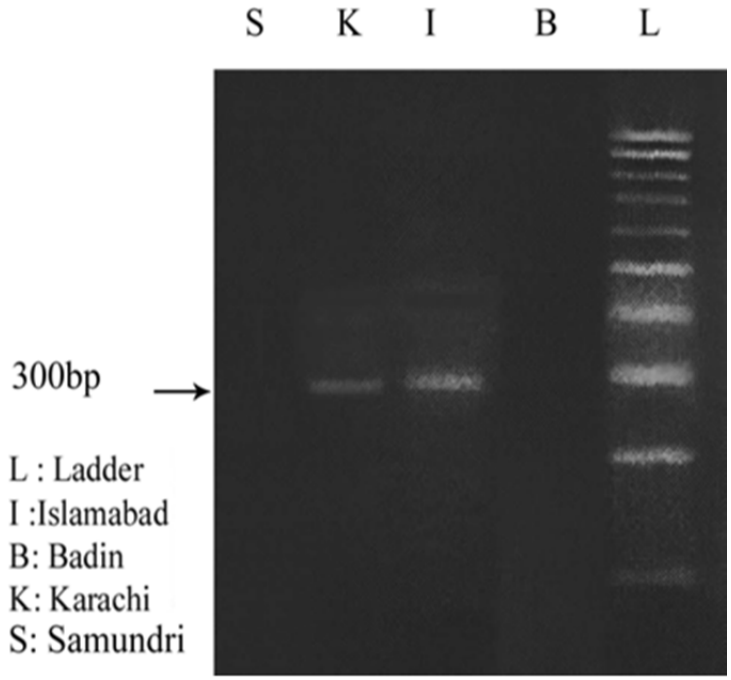

Fig 3. Whole Cell protein profile of *Pasteurella multocida*. The experiment was repeated five times with 12% separating gel w/v and 5 %(w/v) stacking gel , Coomassie brilliant blue (CBR) R-250 dissolved in 50% (w\v) methanol (Scharlau), 10%(v\v) acetic acid (Merck, Germany) in water i.e. the double distilled water.

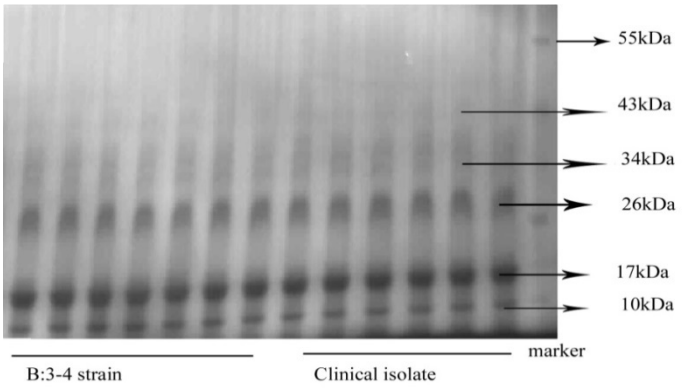

Fig 4. Envelop profile of *Pasteurella multocida*. The experiment was repeated five times with 12% separating gel w/v and 5 %(w/v) stacking gel , Coomassie brilliant blue (CBR) R-250 dissolved in 50% (w\v) methanol (Scharlau), 10%(v\v) acetic acid (Merck, Germany) in water i.e. the double distilled water.