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Full Length Research Paper

Characterization of *Pasteurella multocida* strains isolated from cattle and buffaloes in Karachi, Pakistan

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Pasteurella multocida **is a Gram-negative, non-motile coccobacillus. The organism is of special significance in the tropical countries including Pakistan where only in Punjab Province, it caused heavy economics losses as Rs.2.17 billion per year. We Isolated and identified the disease causing serotypes in Pakistan by using cultural, morphological and biochemical tests. Pathogenicity was confirmed by inoculation of the isolates in rabbits. Serotyping of the isolates was done and our isolates were found to be *P. multocida*, B: 2 serotype. Polymerase chain reaction (PCR) of 16s ribosomal deoxyribonucleic acid (rDNA) was done which amplified 1500 bp deoxyribonucleic acid (DNA) fragment. DNA sequencing and phylogenetic analysis of the gene revealed that our isolates are different from each other and they belong to two different groups. Whereas these isolates are closely related with *P. multocida,* sub species multocida, *P. multocida and* sub species septica.**

Key words: *Pasteurella multocida,* phylogenetic analysis, PCR, 16S ribosomal DNA, serotyping.

INTRODUCTION

Pasteurella multocida is a pathogenic organism and is one of the most common economically significant animal diseases occurring in both developed and developing countries while human infections associated with this bacterium are not frequent (Dziva et al., 2008). The pathogenic organism of haemorrhagic septicaemia (HS), a lethal disease of cattle and buffaloes, is *P. multocida* serotype B:2 (Ataei et al., 2009). This microorganism is pathogenic for a wide variety of mammals and birds (Marina et al., 2004). It is associated with atrophic rhinitis in Swine, fowl cholera in poultry and HS in cattle and buffaloes (Pedersen and Bradford, 1981). The most common hosts of HS infection and disease are cattle and buffalo but some other animals such as goats, pigs, deer, sheep and camel are also susceptible to it (Blackall et al., 2000). The disease is particularly endemic in Asia and Africa but outbreaks have also occurred in Europe and North America (Rimler, 2000).

In South East Asia it is the most important economic disease of livestock that has caused huge economic losses in Africa and India (Verma et al., 1998). Pakistan has 31.8 million cattle population and a population of 29.0 million buffalo heads (Anonymous, 2007, 2008). HS is the most common and foremost infectious disease of dairy animals (cattle and buffalo) in Pakistan, having mortality rate of about 70% and is causing several hundred million dollars annual losses to animal production (May et al., 2001). The highest prevalence of HS was recorded in Khanewal district (49%) and highest disease importance (75.6%) was recorded in Faisalabad district during 2000 to 2005 (Farooq et al., 2007). HS is prevalent in south and south East Asia (serotypes B: 2 and B: 2, 5) and (serotypes E: 2 and E: 2, 5) in tropical Africa (Farooq et al., 2007). PCR technology can be applied for rapid, sensitive and specific detection of *P. multocida*. There-fore the present study was designed for the isolation and

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Abbreviations: PCR, Polymerase chain reaction; **rDNA,** ribosomal deoxyribonucleic acid ; **DNA,** deoxyribonucleic acid; **HS,** haemorrhagic septicaemia; **BLAST,** basic local alignment search tool; **NCBI,** national center for biotechnology information.

characterization of Pasteurella strains in Karachi, Pakistan.

MATERIALS AND METHODS

Five milliliter blood was collected aseptically from the jugular vein of each diseased cattle and buffalo showing typical signs of HS (clinically positive cases) and also from animals died due to HS. On arrival at the laboratory, the isolation of organism was done by culturing the samples on 5% blood agar and Tryptose yeast extract agar (Oxoid). We carried out sugar fermentation test, Indole production test, Methyl red test, Nitrate reduction test, Hydrogen sulfide production test, Catalase test and Gelatin liquification test. To test the pathogenicity of strains, six male rabbits of four month age were selected and 0.5 ml growth suspension having 1×10^{9} cfu/ml of each sample was injected intraperitoneally. PCR amplify-cation of 16S ribosomal DNA of isolates was carried out using the following primers: AGAGTTTGATCCTGGCTCAG (forward) and ACGG (ACT) TACCTTGTTACGACTT (reverse). Then amplified PCR product was run on 1.5% agarose gel at 80 Volts for 30 min. Amplified 16S rDNA gene having 1,500 bp size was sequenced commercially (Genelink^{TM}, Hawthorne, New York). The phylogenetic analysis of sequences was carried out by comparing them with GenBank databases using the National Center for Biotechnology Information (NCBI) and Basic Local Alignment Search Tool. (BLAST).

RESULTS

Culture characteristics

All the samples were examined for their cultural characteristics and observations were recorded after 24 h aerobic incubation at 37ºC, as favorable temperature for *P. multocida* growth was recorded at 35±5°C (1.15 mg mL-1), whereas poor activities (<1.15 mg ml^{-1}) were observed at below 25±5°C and the organism did not g row at 50° (Atta et al., 2008). The isolates that exhib ited luxuriant growth having off white mucoid and sticky colonies of a large size being 2 mm in diameter and also not producing haemolysis on blood agar were subjected to staining and biochemical reactions for isolation of typical *P. multocida*. Such bacterial growth was observed in 27 out of 40 samples. While in case of TYE agar the colonies showing round, sticky mucoid consistency slightly elevated in the center about 2 to 3 mm in diameter were selected for staining and biochemical reactions for the isolation and confirmation of *P. multocida*. The organism with Gram's staining method appeared Gram negative under microscope, as coccobacillary thin rods with rounded ends. The size of the organism was variable with repeated sub culturing but the shape remained almost consistent with a tendency to bipolar staining. The capsule of the organism was observed after staining with India ink and dumple shaped bipolar organisms were observed.

The suspected 3 samples out of 4 samples showing characteristics of *P. multocida* were selected and cultured on CSY agar to purify the culture. Results of morphological and culture characteristics are shown in Table 1. The sugar fermentation was uniform for all the suspected isolates as they fermented glucose, sucrose, mannose and fructose with the production of acid only and no gas. The sugar fermentation results of all the isolates are given in Table 1. Similarly all the suspected isolates showed uniform biochemical reaction, for which they were tested, showing a positive reaction for catalase test, indole production test, nitrate reduction test and Hydrogen sulphide production test and showed negative reaction for methyl red test, gelatin liquefaction test and not showed urease activity on urea agar plates of showing typical signs of *P. multocida.* Results of all the samples are shown in Table 1. Pathogenicity of *P. multocida* was confirmed in rabbits. It proved highly pathogenic to rabbits and they died within 24 to 48 h. No death was recorded in control group. The isolation and identification of the organism was done from the heart blood of each dead rabbit. The postmortem lesions observed were generalized septicaemia, congestion of internal organs, excessive haemorrhages particularly on laryngeotracheal region, accumulation of inflammatory fluid in the thoracic and peritoneal cavities. Results of morphological and culture characteristics, sugar fermentation and biochemical tests are shown in Table 1. The phylogenetic tree showed that our isolate had 100% homology with *P. multocida* PM 64 strain while other isolate had 87, 99 and 72% homology with *Pasteurella multocida* PM 64, *Pasteurella multocida* subsp. Septica and *P. multocida* isolate 06 respectively, as shown in Figures 1 and 2.

DISCUSSION

HS is one of the most common bacterial diseases of buffaloes, cattles, sheep and goat. HS is caused by the bacterium *P. multocida (P. septica)*. Buffaloes are more susceptible to it than cattle. It causes heavy mortalities. The common name of HS is *galghotu,* in most parts of Nepal and India (Tabatabaei et al., 2002). This disease is caused by *P. multocida* serotype B: 2 and E:2 in Asia and Africa, respectively (Nawaz et al., 2006). *P. multocida* toxin can be used to create immunity against HS, because it is clear that LPS of *P. multocida* stimulates humoral immunity and it is considered to be protective antigen (Harper et al., 2004). Morbidity and mortality due to this disease is >45 and 92%, respectively. The incidence is seasonal, occurs in July and August in Pakistan due to stress and humidity (Marz et al., 1979). Blood samples of cattle and buffaloes showing typical signs of HS were collected with sterilized syringe and examined for the presence of *P. multocida.* The isolation of *P. multocida* from blood samples of diseased animal was quite possible if aseptically drawn out. The isolation of *P. multocida* from blood samples of animals suffering from HS is a common practice. (Mohan et al., 1994) also studied the phenotypic characteristics of 60 Zimbabwean isolates of *P. multocida* from disease syndromes in

Table 1. Results of morphology, cultural characteristics, sugar fermentation, biochemical, pathogenecity and serotyping tests of field isolates collected from clinically positive cases of HS (1, 2 and 4).

	Samples (1,2 and 4)	Samples (3)
Staining reaction	Gram –ve, Small coco bacilli, thin rods with rounded ends	Gram –ve, Small coco bacilli, thin rods with rounded ends
Capsule	Present	Absent
Bipolarity	Dumble shape	Absent
Blood agar	Luxuriant growth without haemolysis	Haemolysis showed
TYE agar	Smooth, slightly raised in the center, mucoid and sticky in nature	Small colonies
McConkey agar	No growth	Small whitish round colonies
CSY agar	Smooth, yellowish, mucoid and larger in size	Rounded sticky colonies
Sugar fermentation test		
i. Glucose	+	-
ii. lactose	-	+
iii.maltose	-	+
iv. Sucrose	+	-
v. Salicin	-	-
vi. Mannose	+	+
vii. Fructose	+	-
H2S production test	+	-
Methyl red test	-	-
Gelatin liquification test	-	+
Indole production test	+	-
Nitrate reduction test	+	+
Urease test	-	+
Catalase test	+	-
Mice inoculation test (Pathogenecity test)	+	-
Serotyping (IHA titer)	1:256-1:512	-
Inference	*P. multocida* (B)	Unknown

different host species. The blood drawn from clinical cases prior to death will yield positive cultures only at terminal septicaemic stage (Carter and De Alwis, 1989). Aslam et al. (1988) and Islam (1975) reported that *P. multocida* Robert's type-1 is exclusively involved in all HS outbreaks in Pakistan and is being used for vaccine production. According to Islam (1975) the virulent strains of *Pasteurella multocida* isolated from nasopharyngeal and tonsilar region of healthy buffaloes and cattle. All the isolates which were collected from field cases were grown on different media and cultural characteristics of the isolates observed on various media, concerning size and pigmentation of colonies, development of turbidity in liquid media and the extent of growth were in line with those observed by Bain (1957). Similar results were described by Wijewardana (1992). Blood samples were difficult to grow directly on solid media; the colonies were very small, transparent and were difficult to distinguish. Colonies cultured on CSY agar or on tryptose yeast extract agar were round, sticky and were of mucoid consistency about 2 to 3 mm in diameter and were slightly raised in the center.

These observations were also in agreement with Bain et al. (1982). The organism was identified on the basis of morphology, staining reactions and culture characteris-tics, on microscopic examination; the organism appeared as coccobacillus, capsulated, gram negative and stained bipolar. With repeated sub culturing organism tend to diminish in size and became somewhat rounded and sometimes even lost its bipolar character. These finding were in complete agreement to those reported by Topley and Wilson (1998) and Kumar et al. (2004). In wet India ink staining method, capsule appeared as white lined with India ink. The results are also in agreement with those of Bain et al. (1982). On blood agar after 24 h of inoculation at 37°C non-hemolytic luxuriant growth appeared. T hese results are in agreement with those observed by (Bain et al., 1982) and (Buxton and Fraser, 1977). On MacConkey

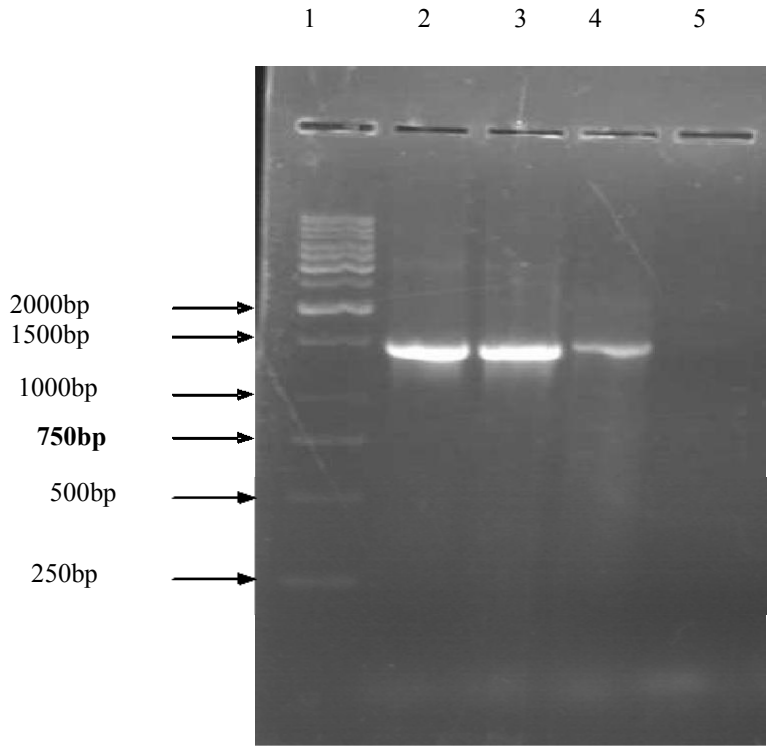

Figure 1. Analysis of PCR product on agarose gel, Lane 1:1 Kb DNA ladder, lane 2.sample 1; lane 3, sample 2; lane 4, sample 3; lane 5, -ve control.

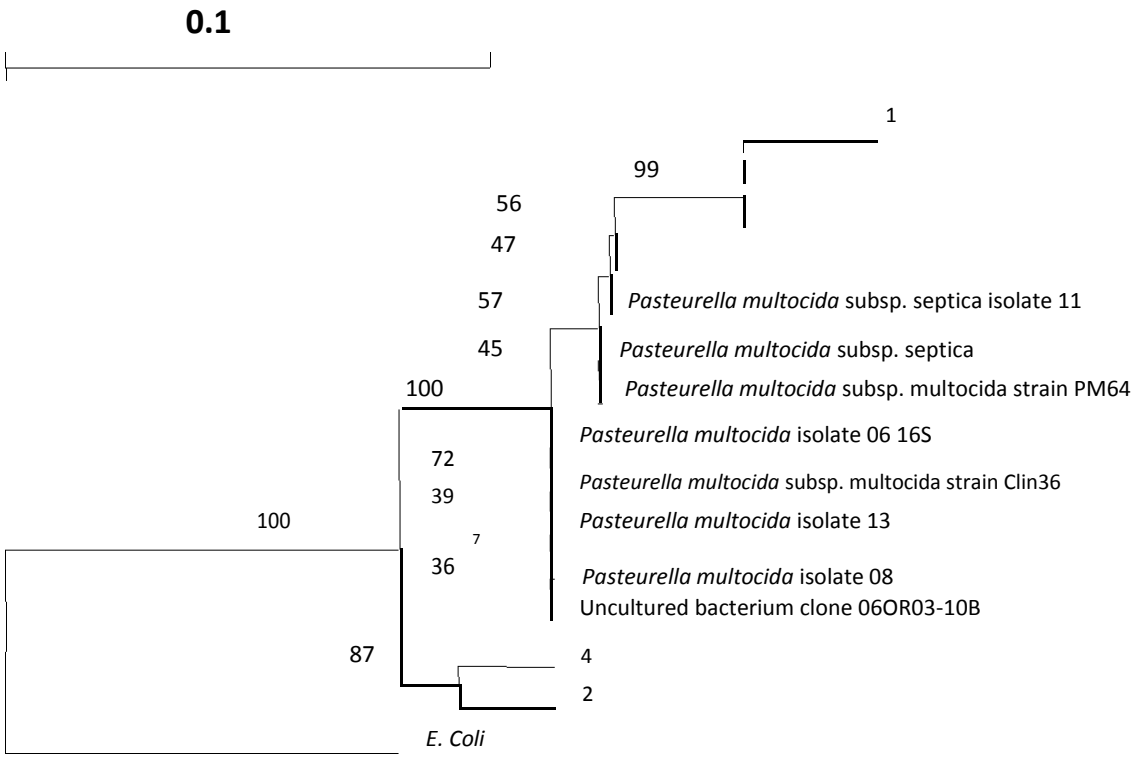

Figure 2. The inferred relationship, based on partial 16S rDNA sequence, of hemorrhagic septicemia causing local isolates of *Pasteuralla multocida* type B (1, 2 and 4) Phylogenetic analysis of isolates (1, 2 and 4).

agar and citrate agar no growth could be observed even after incubation of 24 h at 37°C. These results are also in line with Buxton and Fraser (1977). All the isolates fermented the glucose. This has also been reported by Aslam et al. (1988) and Mohan et al. (1994). Lactose gave variable results as reported by Kozarev and Mamadudian (1988) that variability is found in patterns of fermentation of levulose, arabinose. Xylose, maltose, and raphnose. In case of sucrose all the isolates were able to ferment it. These results are similar to Aslam et al. (1988) and Mohan et al. (1994) who reported that consistent results were obtained in the test for glucose, inositol. Salicin and sucrose. There was obvious relation-ship between serotype, host or disease and their pattern of utilization of certain substrates by an isolate. Mannose was also fermented by all the isolates. Same results were obtained by Bain et al. (1982). Salicin was not fermented by any of isolates. These results were in agreement with Bain et al. (1982). Mohan et al. (1994) also reported that salicin was not fermented by *P. multocida.* Fermentation of maltose was variable in our study. Same results were also discussed by Kozarev and Mamadudian (1988).

All the isolates were positive for indole production test and also for catalase test. Mohan et al. (1994) obtained similar results who reported that *P. multocida* gave consistent results for catalase and indole production test. All of the isolates were negative for methyl red test, urease activity and gelatin liquification test as observed by Chandrasekaran et al. (1981). Mohan et al. (1994) also obtained consistent results for urease test. Hydrogen sulphide was produced by all isolates. The similar results were observed by Aslam et al. (1988).PCR technology can be applied for rapid, sensitive and specific and/or detection of *P. multocida* (Natalia and Priadi, 2001). The rapidity and high specificity of two of the *P. multocida*-specific assays (Miflin and Blackall, 2001) provide optimal efficiency without the need for additional hybridisation. The phylogenetic tree showed that our isolate had 100% homology with *P. multocida* PM 64 strain while other isolate had 87, 99 and 72% homology with *P. multocida* PM 64, *P. multocida* subsp. Septica and *P. multocida* isolate 06, respectively. In summary, Phylogenetic analysis of the gene revealed that our isolates are different from each other and they belong from two different groups. While these isolates are closely related with *P. multocida,* sub species multocida and *P. multocida,* sub species septica.

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