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ISOLATION AND MOLECULAR IDENTIFICATION OF *PASTEURELLA MULTOCIDA* FROM COMMERCIAL POULTRY FARMS SUFFERING FROM FOWL CHOLERA

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ABSTRACT

Fowl cholera (FC) a contagious infection, remains a major threat to the poultry industry, particularly in developing countries including Pakistan. This study was carried out on suspected field cases to isolate and identify local strains of *P. multocida*. A sum of 25 samples was collected from suspected poultry flocks from different regions of Pakistani.e. Lahore, Faisalabad, Mansehra, Abbottabad, Karachi, Lahore, and Vehari. The samples were processed and *P. multocida* strains were isolated. The primary identification of collected isolates was checked by colony morphology, microscopy, and biochemical characterization. The strains were molecularly characterized by PCR using 16S rRNA and *P. multocida* specific primers targeting OmpH gene. Samples positive for *P. multocida* showed gram-negative, cocco-bacilli, bipolar and non-motile characteristics. They were able to reduce nitrates but failed to produce hydrogen sulfide, consumed ornithine as a carbon source. The isolates consistently produced an acid while fermenting glucose and sucrose whereas lactose and maltose were not fermented. PCR successfully amplified the product of 500 bp and 993 bp against 16S rRNA and OmpH gene primers respectively. Results of molecular characterization confirmed the strains as *P. multocida* which can be used in the preparation of an inactivated vaccine against local isolates of fowl cholera.

Keywords: Fowl cholera, OmpH gene, Poultry flocks, *Pasteurella multocida*

INTRODUCTION

Pakistan's meat industry is a vibrant and is known to be the world's largest poultry producer with the production of 1.02 billion broiler meat annually. The share of poultry meat in overall meat production is 30% [1]. However, due to the some important infectious diseases, which pose a serious threat to the survival of poultry birds, significant economic losses have been imposed on this state [2].

Fowl cholera (FC) is the contagious and most common infection of poultry birds leading to substantial economic losses to poultry sectors of developing countries including Pakistan. The infectious agent is *Pasteurella multocida* and has been recognized as the major pathogen commonly found in mammals and birds [3]. *P. multocida* has been divided into five capsular serotypes i.e. A, B, D, E and F. The two severe infections i.e. hemorrhagic septicemia (HS) in cattle and fowl cholera (FC) in poultry and are solely caused by serotype B and serotype A respectively [4, 5]. Several other infections have also been caused by *P. multocida* in sheep, goats, rabbits, swine, dogs, and cats [6]. This bacterium belongs to family *Pasteurellaceae* and is a small, non-motile, gram-negative, facultatively anaerobic, Cocco-bacillary [7, 8].

The site of infection of *P. multocida* is the respiratory tract in many domestic and wild birds, which leads to high morbidity and mortality. Infected poultry birds are responsible for spreading bacteria through secretions from eyes, nose, and mouth and contaminate feed and water leading to per-acute and acute infections to chronic infections [9]. Previous literature has reported the significant economic losses associated with *P. multocida* in commercial poultry flocks in different regions of Pakistan [10, 11, 12]. Vaccination is an effective way to combat the prevalence andwidespread infections [13]. Pakistan's poultry industry possesses a remarkable contribution to the national economy [14]. Although, several imported vaccines are being practiced in Pakistan to control the incidence of disease. However, insufficient literature is available in this country for the productionof fowl cholera vaccine by using local isolates which can be cost-effective for poultry breeders. In general, it is usuallyaccepted that a local strain should be selected for effective vaccine production to control a particular disease like fowl cholera [15]. This study aimed to isolate *P. multocida* circulating in commercial poultry flocks in different regions of Pakistan, their identification by morphology, fermentation characteristics, and their confirmation by molecular characterization.

MATERIALS AND METHODS

Samples collection

P. multocida was isolated from liver, blood and spleen of infected birds. Sums of 25 samples were collected from different regions of Pakistan i.e. Abbottabad, Faisalabad, Karachi, Lahore, Mansehra, and Vehari. Obtained samples were homogenized in glass homogenizer together with 2 to 3 ml of PBS (phosphate saline buffer). These were then centrifuged for two minutes at 500 rpm. Supernatants were collected and stored at 4°C for further use.

Isolation of *P. multocida*

The supernatant obtained from collected samples were streaked at MacConkey, CSY Blood, and CSY agar plates. These plates were incubated at 37 °C for 24 hours in an aerobic environment. After incubation, on the basis of morphology (color, translucency, shape, margins and surface elevation), cultural and biochemical characteristics, isolated bacteria were identified. Based on colony characteristics, the isolates of primary culture were further streaked on blood agar to obtain pure colonies. Inoculated plates were incubated for 24 hours at 37 °C.

Primary identification and biochemical characterization

P. multocida was primarily identified for its morphology by performing Gram staining as described by Islam *et al*., [16]. Giemsa staining was carried out to check bipolarity and LPS (Lipopolysaccharide) around bacterial isolates as described by OIE Manual [17]. Biochemical characterization of pure growth colonies was performed by using API (Analytical Profile Index) 20E (BioMerieux, France) system.

Total DNA extraction and quantification

The extraction of genomic DNA fromperformed from purified broth culture of each sample by following the procedure described by Sambrook *et al*., [18]. Extracted DNA obtained from all isolates was quantified by using NanoDrop Spectrophotometer (Thermo scientific Company, USA).

Polymerase Chain Reaction (PCR)

The isolates were subjected to PCR for molecular characterization of *P. multocida* organism by targeting the 16S ribosomal RNA gene and OmpH gene specific for *P. multocida* as described by Gamal *et al*., [19]. The primers were designed **(Table 1)** by using Primer3 Plus software (http://www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi).

Table 1: Primers used for characterization of *P. multocida*

Strain	Gene	Primer's Name	Primer Sequence	Product Size
P. multocida	16S	16S-F	GCTTACAAGCACATAGGAA	500 bp
		16S-R	ATTACAGCATTACAGCTTAT	
	Outer Membrane Protein (OmpH)	OmpH-F	GCGTTTCATTCAAAGCATCTC	993 bp
		OmpH-R	ATGACCGCGTAACGACTTTC	

Polymerase chain reaction was performed under optimized conditions using programmable Thermal cycler (Bio-Rad T100™ Thermo Cycler). To amplify ribosomal RNA gene, a reaction mixture of 50 µlwas prepared, containing *Taq* Buffer KCl, 10X (5 µl), $MgCl_2$ (25 mM)6 µl, dNTPs (2.5 mM) 3 µl, forward and reverse primer (10 pmol) 3 µl each, 1 µl of *Taq* polymerase (5U / µl), template (extracted DNA) 2 µl and sterilized water sufficient to 50 µl was added. Amplification conditions for16S rRNA gene were optimized at 94°C for 5 min, 94°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec, as initial denaturation, denaturation, annealing and extension temperatures respectively. The cycles were run for 36 times followed by 72°C as final elongation for 10 min.

To amplify OmpH gene, a reaction mixture of 25 ul was prepared, containing *Taq* Buffer KCl, 10X (2 µl), $MgCl_2$ (25 mM)2 µl, dNTPs (2.5 mM)2 µl, forward and reverse primer (10 pmol)1 µl each,0.5 µl of *Taq* polymerase (5U / µl), DNA template 2 µl and sterilized water sufficient to 25 µl was added. Amplification conditions for OmpH gene were optimized at 94°C for 5 min, 94°C for 15 sec, 55°C for 30 sec, 72°C for 1 min, as initial denaturation, denaturation, annealing and extension temperatures respectively. The cycles were run for 35 times followed by 72°C as final elongation for 10 min.

Agarose gel electrophoresis was performed to observe the amplification of both genes (16S rRNA and OmpH). Amplified PCR products were purified by using purification kit (Monarch DNA Gel Extraction Kit, BioLabs) as instructed by the manufacturer. Purified PCR products were sequenced by First BASE Laboratories SdnBhd, Selangor, Malaysia. The obtained nucleotide sequences were subjected to NCBI BLASTn network service from GenBank (http://www.ncbi.nlm.nih.gov/).

RESULTS AND DISCUSSION

FC a contagious infection leads to substantial economic losses to commercial poultry farms in Pakistan. This observation is in line with Tahir *et al*., [11], Zahoor and Siddique [20] and Mohamed and Mageed [21] who reported the occurrence of *P. multocida* isolates in commercial poultry farms as an endemic agent in various districts of the country. This strain can be isolated from visceral organs of infected poultry birds such as liver, blood, heart,

blood, spleen, and bone marrow [22, 17]. The positive samples (Sample ID; FC2, FC8, FC11, FC14, FC22) under study showed *P. multocida* colony characteristics like mucoid, viscous, Grey, translucent and non-hemolytic colonies, with the diameter of 1 millimeter approximately, were obtained on CSY based blood agar after incubation of 24 h at 37°C as described in OIE Terrestrial Manual [17].

Different growth media have been reported previously, which support the growth of *P. multocida* for instance; trypticase soy agar, Blood agar, CSY (Casein-sucrose-yeast), BHI (Brain heart infusion) [23, 24]. In the present study, the isolation of *P. multocida* was performed on CSY media and gram-negative, cocco-bacillary, and bipolar characteristics of bacteria were observed. Similar observations have been described formerly [25, 26].

The isolated bacteria grew well and showed mucoid, viscus, translucent and non-hemolytic colonies on CSY blood and CSY agar plates (Figure 1A ad 1B) while no growth was obtained on MacConkey's agar (Figure 1C). Previous data also described the same characteristics [27, 28]. The results of the microscopic analysis revealed that after gram staining, the *P. multocida* bacterium was gram-negative and *coccobacillus* (**Figure 1D**). The bipolarity of bacterial strains with the presence of LPS (Lipopolysaccharide) around the cell wall was observed with Giemsa staining. On the basis of these characteristics,the isolates were primarily identified as *P.multocida*. The results of biochemical characterization (**Table 2**) showed that the isolates were able to ferment glucose and sucrosewhile lactose and maltose were not fermented, with any production of hydrogen sulfide gas [8]. The isolates showed positive result for catalase and indole test [7, 8]. Isolates were able to reduce nitrates but showed no urease activity and no gelatin liquefaction was observed [3, 7]. Analytical profile index results of this study isolates are in accordance with Ievy *et al*., [22] and Christensen *et al.*, [29].

Biochemically characterized strains were confirmed as *P. multocida* by amplification of the 16S rRNA gene [30] as shown in **Figure 2A**. PCR was also carried out successfully with specific primers of OmpH gene. The findings of PCR showed that the under study isolates (Figure 2B) amplified the product of around 993bpwith OmpH specific gene primers of*P. multocida* as reported earlier [31,19]. Whereas, in case ofnegative control, there was no amplification with the same set of primer. OmpH (Outer membrane protein H) is a protein which is involved in the production of bacterial LPS [32]or in the exportation of macromolecules into and out of bacterial envelope as illustrated

previously [33]. Porin H or protein H of outer membrane proteinis a crucial component of the *P.multocida* envelope [34].

Various previous studies reported the molecular characterization of *P. multocida* isolates by amplifying this (Outer membrane protein H) gene [35, 36, 37]. ThePCR results revealed the organism as *P. multocida*, isolated from poultry birds. Sequenced samples compared with OmpH sequences of *P. multocida* present in NCBI showed 95 to 96% similarity with the previous isolates, confirmed the studied strains as *P. multocida*.

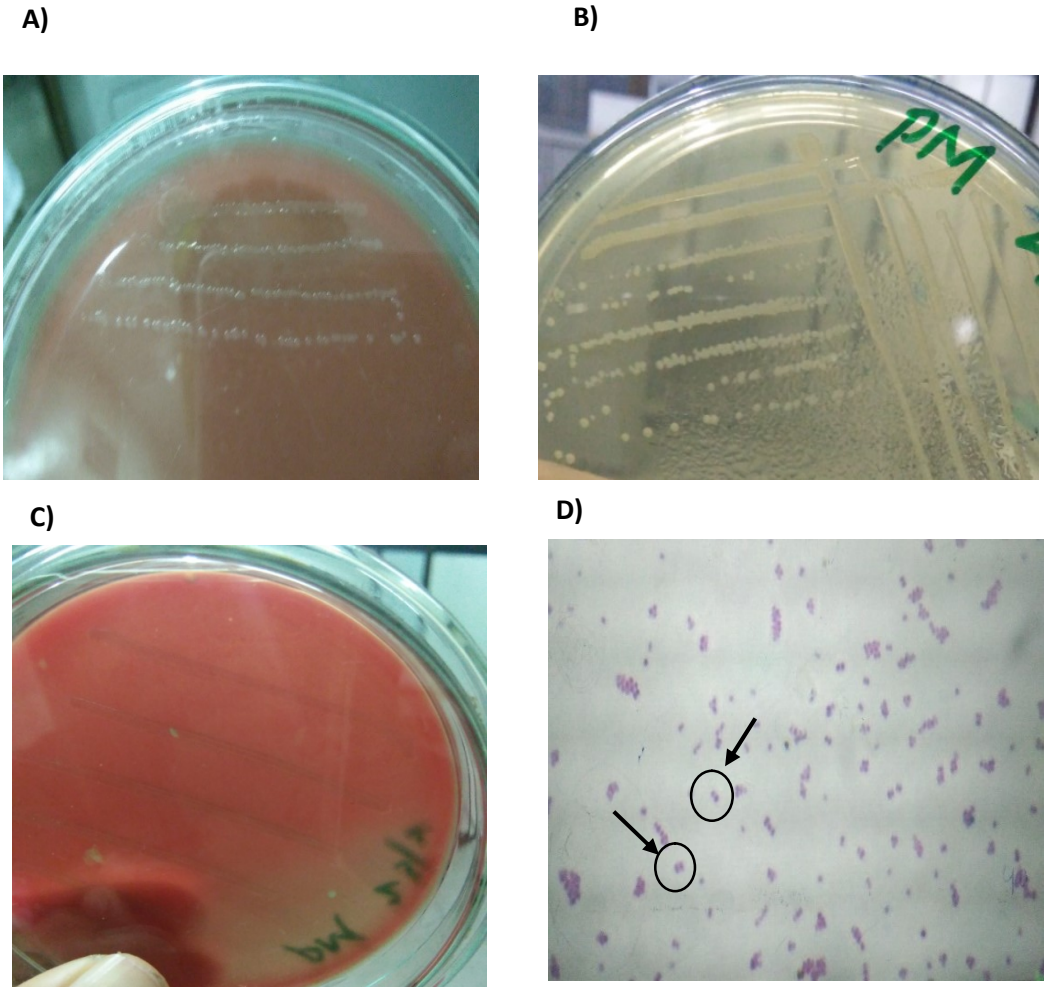

Fig 1: Culturing of *P. multocida* field samplesA) Culturing of*P. multocida* (FC2) isolate on CSY blood agar. B) Colonies of *P. multocida* (FC2) on CSY agar. C) Inoculation of*P. multocida*on MacConkey agar (No growth observed). D)Gram staining showing gram negative coccobacilli strains; *P. multocida* (FC2) visualized at magnification of 100X (Light microscope, MEIJI)

Table 2: Results of biochemical tests performed on samples positive for *P. multocida*

S. No.	Test	Results of Biochemical Tests					
		Control	FC2	FC8	FC11	FC14	FC22
1	Indole Production	+ve	+ve	+ve	+ve	+ve	+ve
2	Urease Production	-ve	-ve	-ve	-ve	-ve	-ve
3	Hydrogen Sulphide Production	-ve	-ve	-ve	-ve	-ve	-ve
4	GelatineLiquification	-ve	-ve	-ve	-ve	-ve	-ve
5	Citrate Utilization	-ve	-ve	-ve	-ve	-ve	-ve
6	Catalase Production	+ve	+ve	+ve	+ve	+ve	+ve
7	Glucose fermentation	+ve	+ve	+ve	+ve	+ve	+ve
8	Lactose Fermentation	-ve	-ve	-ve	-ve	-ve	-ve
9	Sucrose Fermentation	+ve	+ve	+ve	+ve	+ve	+ve
10	Sorbitol Fermentataion	+ve	+ve	+ve	+ve	+ve	+ve
11	Maltose Fermentation	-ve	-ve	-ve	-ve	-ve	-ve
12	Rhamnose	-ve	-ve	-ve	-ve	-ve	-ve
13	Ornithine decarboxylase	+ve	+ve	+ve	+ve	+ve	+ve

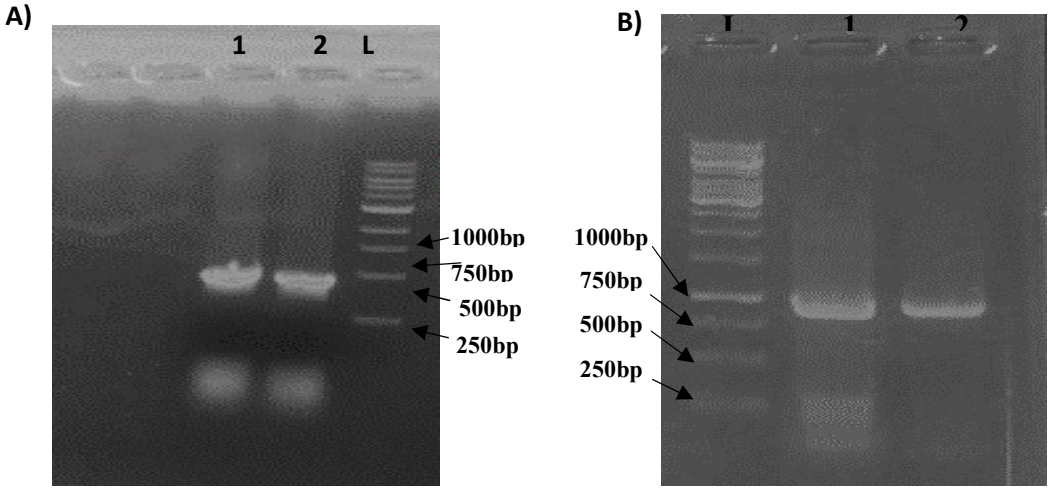

Fig 2: Amplification of 16S rRNA and OmpH gene of *P. multocida*A)*P. multocida*16S rRNA PCR; L: DNA Ladder (1kb), 1-2: PCR product of 16S rRNA (500bp, expected size)B)amplification of *P. multocida*OmpHspecific gene; L: DNA Ladder (1kb), 1-2: PCR product of OmpH gene (993bp, expected size)

CONCLUSION

P. multocida is an endemic agent for the poultry industry of developing countries including Pakistan which needs crucial and effective attention to control fowl cholera infection. In this study, isolation and identification and molecular characterization of *Pasteurella multocida* were successfully done from suspected poultry flocks which can be used for inactivated vaccine production against local isolates of fowl cholera.

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