DETERMINATION OF THE RELATIONSHIPS BETWEEN *Pasteurella multocida* ISOLATED FROM DIFFERENT FARM ANIMALS AND THEIR HOST RANGE CONTACTS IN EGYPT USING BIOCHEMICAL AND MOLECULAR TECHNIQUES

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Pasteurella multocida is a commensally in the upper respiratory tract, lungs, spleen, blood and liver of many infected animals. Members of the species *Pasteurella* are well recognized pathogens of many species of animals and cause a wide range of important diseases of cattle, sheep, buffaloes and goats (Jabbari and Esmaelizadeh, 2005). The animal pathogen *P. multocida* is associated with a wide range of diseases, including fowl cholera of poultry and wild fowl, atrophic rhinitis of swine, hemorrhagic septicemia of cattle and buffaloes. The fowl cholera disease causes serious significant economic losses to animal production due to deaths, condemnation losses, and costs of the vaccination (Hird *et al*., 1991; Rhoades and Rimler, 1991).

The complexity associated with conventional biochemical identification makes alternative approaches attractive. One such alternative is the use of the polymerase chain reaction (PCR) to detect the sequence of DNA unique to *P. multocida* (Miflin and Blackall, 2001). Molecular genetic methods of bacterial identification have proved beneficial in overcoming some limitations of traditional morphological methods. Nucleic acid-based assays allow the detection of organisms directly from clinical samples or from small amounts of cultured bacterial cells, thus dramatically improving sensitivity and decreasing the time required for bacterial identification (Townsend and Papadimetrious, 1997).

The biochemical activities of *P. multocida* have shown that the bacterium is positive for some specific tests like indole, catalase, oxidase, ornithine decarboxylase and it reduces nitrate to nitrite. On the other hand the organism is negative for using urease and citrate (CLSI, 2009). Hawari *et al*. (2008) found that, all the isolates of *P. multocida* and *P. manheimia* produced catalase, indole, presence of ornithine decarboxylase, fermented mannitol and produce acid by fermentation of glucose by using API-20 E Kit.

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 *et al*., 2007). PCR fingerprint-

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ing is feasible for any laboratory with PCR capability, with several methods previously used for *P. multocida* differentiation. RAPD analysis and arbitrarily primed PCR, respectively, have been shown to be useful for epidemiological studies of *P. multocida* isolated from rabbits (Chaslus-Dancla *et al.*, 1996). Repetitive sequence PCR analysis of *P. multocida has* provided useful tool for discrimination of avian and pig bacterial isolates. The development of DNA-based techniques has provided alternative methods of characterization that overcome the limitation of Phenotyping, while identifying precisely individual strains of closely related bacteria (Owen, 1989). Genomic characterization techniques have replaced the traditional typing methods for the discrimination of isolates from a wide range of bacterial pathogens. DNA fingerprinting has been shown to be useful for the differentiation between phenotypically similar *P. multocida* isolates (Gunarwardana *et al*., 2000).

Jared *et al*. (2010) recorded that PCR fingerprinting has been employed by numerous researchers for swine, poultry, and rabbit isolates of *P. multocida*. The result showed that all primers successfully amplified variable DNA fragments from the isolates and the bands obtained have been ranged from 12 to18 bands. This study aims to isolate *Pasteurella* spp. from apparently clinically sick different farm animals (sheep, buffalo and cattle) from Fayoum region. Aiming to identify *Pasteurella* spp. isolates using morphological, conventional biochemical methods and rapid API-20 E Kit., assess the sensitivity profiles of *P. multocida* isolates to panel of conventional antimicrobial agents and confirm isolated of *P. multocida* using molecular characterization, RAPD-PCR.

MATERIALS AND METHODS

Samples collection and isolates cultivation

Twelve isolates were isolated from privet farms in Fayoum Governorate, Egypt and used in this study were presented in Table (1). Samples were obtained from animals had a clinical manifestation of pneumonia according to the nasopharyngeal swabs method and transferred to the bacteriological laboratory, plated onto 10% sheep blood agar and incubated at 37°C overnight for culture and microbiological examination.

Isolation, purification and morphological characterization of Pasteurella spp.

Swabs from sheep, buffalo and cattle were inoculated onto brain heart infusion agar and blood agar selective medium and incubated aerobically at 37°C for 24 h. Cultural and morphological examinations were conducted as described by Cowan and Steel (2004).

Identification by API Kit

All *Pasteurella* spp. isolates identified by biochemical tests were further subjected to analytical profile test using commercially available Kit (API-20 E),

according to the manufacturer's instruction.

Antibiotic sensitivity test

Five isolates (*P. multocida*) which identified out of 12 *pasteurella* spp. according to their morphological criteria and biochemical tests were examined for their sensitivity against ten most commonly used antibiotics. The antibiogram of all isolates were supplemented with nutrient agar according to the disc diffusion method as reported by Bauer *et al*. (1966).

Genomic DNA extraction of P. multocida strains

Genomic DNA isolation was carried out according to Ozbey *et al*. (2004), with some modification as following: single colony was inoculated in brain-heart infusion broth and grown overnight at 37°C in shaking incubator. Cells were harvested from 1ml culture in eppendorf tube by centrifuging then; 300µl of distilled water was added. The tubes were vortexed and incubated at 56°C for 10 min; The suspension was then added to 300 µl of K-buffer and 200 µl of proteinase-K (200 µg/ml) following 10 min boiling; Equal volume of phenol was added to the suspension which vigorously shaking by hand for 5 min then, centrifuged at 11600 rpm for 10 min. The upper phase was transferred to a new eppendorf tube. Genomic DNA was precipitated with absolute ethanol and 0.3 M sodium acetate at -20°C for one h. The mixture was centrifuged at 11600 rpm for 5 min and the upper phase discarded. The pellet was washed by ethanol 70% and centrifuged for 5 min then, the pellet was dried and suspended in 50 µl of sterile distilled water and used as a template DNA in PCR.

Polymerase chain reaction (PCR)

In the present study nine random primers OPA-01 → OPA-09 (BioTeke Corporation) were used in order to identify the five strains of *P. multocida*. The nucleotide sequences of nine random primers are shown in Table (2). The reaction mixture (25 µl) contained 10 mM Tris-HCI (pH 8.4), 50 mM KCI, 0.2 mM of each deoxy nucleoside triphosphate (dNTPs), 20 pmol of each primer, and 1.0 U of *Taq* DNA polymerase and 3µl of template DNA preparation. RAPD fingerprinting patterns were carried out with computer assisted analysis using RAPD software package, version 1.4 (Armstrong *et al*., 1994). Similarity of the band profiles was based on Excoffier matrix (Excoffier *et al*., 1992). The correlation coefficient was used to compare the number of DNA patterns obtained. The clustering of the strains was determined by the unweighted pair group method using arithmetic average (UPGMA).

RESULTS AND DISCUSION

Isolation and morphological identification of Pasteurella spp.

A total of 12 isolates of *Pasteurella* spp. were isolated from farm animals (sheep, buffalo and cattle) had clinical manifestation of pneumonia as shown in Table (1). The *Pasteurella* isolates FUP-2,

FUP-5, FUP-8, FUP-9 and FUP-12 were growing on blood agar, non-hemolysis of blood, failed to grow on MacConkey medium and proved to be negative to gram test, these results indicated that this isolates are *P. multocida*. On the other hand the isolates FUP-1, FUP-3, FUP-4, FUP-6, FUP-7, FUP-10 and FUP-11 were grown on blood agar media with non- hemolysis grew on MacConkey agar and is gram negative thus these isolates probability is *P. haemolytica* (Table 1). The findings regarding general characteristics of *Pasteurella* spp. are in agreement with Dewani (2000) who described the cultural characteristics of *P. multocida* as non hemolytic, but some isolates produced brownish discoloration on blood agar medium. Balakrishnan and Parimal (2012) isolated eight negative coccobacillary isolates of *P. multocida* typical characteristics of dew drop, mucoid on MacConke agar.

Biochemical identification of Pasteurella spp.

Identification of selected *Pasteurella* isolates to species level was carried out on the basis of their carbohydrate fermentation patterns obtained by biochemical tests. Data in Table (3) showed that isolates were subjected to biochemical tests and proved to be positive for oxidase, nitrate reduction, H_2S production and fermented sucrose. However all isolates were positive for catalase production except for FUP-7 and FUP-10, fermented glucose except for FUP-6, FUP-7, FUP-10 and FUP-11, fermented fructose except for FUP-7, FUP-10 and FUP-11and fermented lactose except for FUP-2, FUP-5, FUP-9 and FUP-12. All isolates were negative for urease production (Table 3). From the previous review it can be seen that there is no absolute biochemical characteristic that can be used to identify all cultures of *P. multocida* but their usual characteristics are fermentation of glucose, sucrose, mannitol, sorbitol and fructose without production of gas. They do not usually ferment lactose, maltose, inositol, inulin, rhaminose and salicin. A great majority of *P. multocida* isolates produce indole and are positive for oxidase and catalase (Tefera, 2002; Oie manual, 2004).

Antibiotic sensitivity test

The results of antibiotic sensitivity test (Table 4) revealed that most of *Pasteurella* isolates showed highly sensitive (100%) to a wide range of antibiotics namely tetracycline, chloramphenicol, clindamycin, erythromycin and gentamicin. However, *P. multocida* isolates showed minimal sensitivity against penicillin and ceftazidime at the rate of 25%. The results of *in vitro* sensitivity of *P. multocida* against various antibiotics were summarized in Table (4) and Fig. (1). However all isolates showed resistance (100%) to ampicillin, trimethoprim/sulfamethoxazole and flucloxacillin and resistance of 75% to penicillin and ceftazidime. In clinical aspects antibiotic sensitive assay serves as a guide to choose the correct antibiotic to be used in the animal farms (Coates and

Hoopes, 1980). 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).

Molecular identification

In the present study nine random primers were used to identify the selective five isolates of *P. multocida*. These primers generated reproducible and easily securable RAPD profiles (Figs. 2a and b) with a number of amplified DNA fragments ranging from 1 to 8 amplicons per primer as shown in Table (5). The applicability of this method for determining genome similarities among *P. multocida* strains was investigated by performing cluster analysis on the RAPD data. The total number of amplified polymorphic and monomorphic bands by the nine primers with five strains of *P. multocida* was 53 bands. The number of amplified DNA fragments was scored for each primer as shown in Table (5). Primer OPA-01 and OPA-04 amplified the highest number of amplicons (seven polymorphic and one monomorphic), (eight polymorphic and zero monomorphic), respectively. While the lowest number was one with the primer OPA-08.The polymorphism percentage per primer ranged from zero% by primer OPA-08 to 100% by primers OPA-04 and OPA-06. Moreover, the present data revealed that the size of amplified fragments varied with different primers, ranging from 100 to 1500 bp.

RAPD analysis is considered an important molecular biology technique, which is used for the identification of indigenous *P. multocida* strains. In comparison to other molecular typing methods, RAPD is faster, less labor-intensive only a small amount of template DNA is required for amplification reaction (Sikora *et al*., 1997).

Computer analysis of RAPD dendrogram

Cluster analysis of RAPD showed distinct genetic heterogeneity among the selective five isolates of *P. multocida* that isolated from sheep, buffalo and cattle. The dendrogram was constructed considering all bands generated by nine primers and suggested two primary genetic clusters as shown in Fig. (3). The first cluster consisted of two sub-clusters that include *P. multocida* isolated from buffalo and cattle FUP-5, FUP-8 and FUP-9, respectively and the other sub-cluster include the isolate from sheep (FUP-2). The second cluster includes one strain (FUP-12) from cattle. The two strains isolated from buffalo (FUP-5 and FUP-8) were closely related in one linage; while the two strains isolated from cattle (FUP-9 and FUP-12) were highly distantly. The results of Ozbey *et al*. (2004) indicated that little genetic heterogeneity among *P. multocida* isolates from cattle and sheep but not efficient in differentiating strains of *Manheimia haemolytica*. However, Jared *et al*. (2010) reported that RAPD-PCR is perfect to characterize isolates of *p. multocida* and precludes quantitative assessment of the relationship between iso-

lates, potentially concealing relationships between strains. Also, Sabiel *et al.* (2012) indicated that RAPD-PCR methods were used to confirm the identities of six strains of *Manheimia haemolytica* and *P. multocida.* The amplification of genomic DNA obtained was corresponding to the anticipated sizes of 200 bp and 237 bp for *M. Haemolytica* and *P. multocida*, respectively. RAPD-PCR of all the 15 *P. multocida* isolates provided a range of amplicons between 400-1400 bp with a high degree of discrimination between capsular type A and D, and all of nine isolates of capsular type A showed similar banding pattern, and similarly, all six isolates of capsular typed exhibited the same banding pattern recorded by Zomuankima *et al.* (2014).

Venkatesan *et al.* (2006) indicated that the relationship between the source, place of isolate and RAPD type reveals different *P. multocida* RAPD types in the same place at any one time during the year.

SUMMARY

The aim of the present study was to identify and compare the variations among clinical of *P. multocida* isolates originating from different hosts, sheep (n = 4), buffalo (n = 4) and cattle (n = 4). These isolates were obtained from various locations in the Fayoum governorate, Egypt and they were analyzed by morphological, API kit and RAPD PCR at molecular level to check the distribution of Pneumonia and hemorrhagic septicemia in different farm animals. The chosen isolates exhibited luxuriant growth on blood agar with translucent grayish or yellowish green colonies. However, they showed no growth on MacConkey and no hemolysis of blood; also isolates were positive for oxidase, catalase and H_2S production, negative of urease and can ferment glucose, fructose and glucose but can't ferment lactose. Antibiotic sensitivity showed that isolates were sensitive to tetracycline, clindamycin and erythromycin but resistant to ampicillin, trimethoprim/sulfamethoxazole and flucloxacillin. To determine the genetic diversity nine random primers OPA-01 → OPA09 were used to which generate different profiles. The distinct RAPD profiles of *p. multocida* strains showed specificity with regard to the host and the disease induced.

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Table (1): Isolates of *Pasteurella* spp. used in the present study and their origin.

Isolates code	Source of isolates	Isolate date
FUP1	sheep	April 2010
FUP2	sheep	April 2010
FUP3	sheep	April 2010
FUP4	sheep	April 2010
FUP5	buffalo	April 2010
FUP6	buffalo	April 2010
FUP7	buffalo	April 2009
FUP8	buffalo	April 2010
FUP9	cattle	April 2010
FUP10	cattle	April 2010
FUP11	cattle	April 2010
FUP12	cattle	April 2010

Table (2): The nucleotide sequence of nine random primers used for RAPD analysis of *P. multocida*.

Primer code	Primers sequence5'-3'
OPA-01	5′ CAGGCCCTTC 3′
OPA-02	5′ TGCCGAGCTG 3′
OPA-03	5′ AGTCAGCCAC 3′
OPA-04	5′ AATCGGGCTG 3′
OPA-05	5′ AGGGGTCTTG3′
OPA-06	5′ GCTCCCTGAC 3′
OPA-07	5′ GAAACGGGTG 3′
OPA-09	5′ GGGTAACGCC 3′
OPA-010	5′ GTGATCGCAG 3′

Table (3): Biochemical reactions results of *Pasteurella* spp.

Isolate No.	H_2S production	Urease production	Oxidase production	Nitrate reduction	Catalase production	Fermentation of			
						glucose	fructose	sucrose	lactose
FUP-1	+	-	+	+	+	+	+	+	+
FUP-2	+	-	+	+	+	+	+	+	-
FUP-3	+	-	+	+	+	+	+	+	+
FUP-4	+	-	+	+	+	+	+	+	+
FUP-5	-	-	+	+	+	+	+	+	-
FUP-6	-	-	+	+	+	-	+	+	+
FUP-7	-	-	+	+	-	-	-	+	+
FUP-8	-	-	+	+	+	+	+	+	+
FUP-9	+	-	+	+	+	+	+	+	-
FUP-10	-	-	+	+	-	-	-	+	+
FUP-11	+	-	+	+	+	-	-	+	+
FUP-12	-	-	+	+	+	+	+	+	-

Table (4): Sensitivity pattern of *P. multocida* isolates to various antibiotics.

Antibiotics	Conc. (µg)	Resistance%	Sensitivity%
Penicillin	10	80	20
Tetracycline	30	0	100
Chloramphenicol	30	0	100
Ampicillin	10	100	0
Ceftazidime	30	80	20
Clindamycin	2	0	100
Erythromycin	15	0	100
Trimethoprim/Sulfamethoxazole	25	100	0
Gentamicin	10	0	100
Flucloxacillin	5	100	0

Table (5): Random primers showing polymorphism among five *P. multocida* strains.

Primer	Molecular weight (bp)	Total band obtained	Polymor-phic bands	Monomor-phic bands	%Polymor-phism
OPA-01	500- >1500	8	7	1	87.5
OPA-02	250- >1500	7	5	2	71.4
OPA-03	600->1500	7	6	1	85.7
OPA-04	500- >1500	8	8	0	100.0
OPA-05	200>1000	4	3	1	75.0
OPA-06	750>1000	5	5	0	100.0
OPA-07	200->1500	7	6	1	85.7
OPA-09	800	1	0	1	0.0
OPA-10	700->1500	6	4	2	66.6
Total		53	44	9	671.9
Average		5.9	4.9	1	74.65

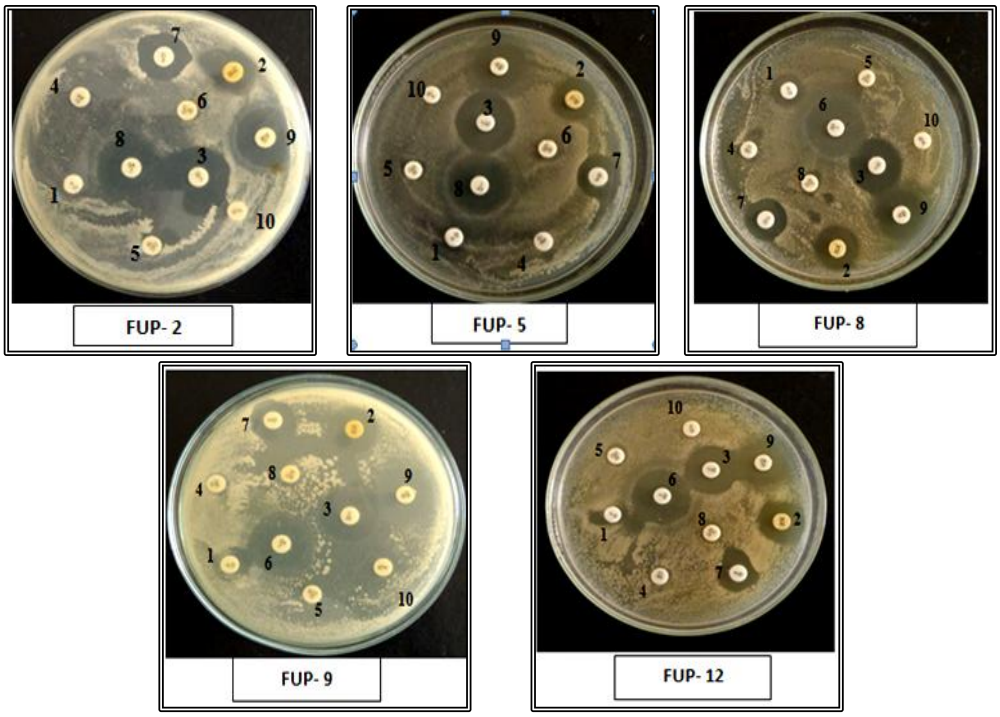

Fig (1): Sensitivity of five isolates from *P. multocida* to ten different antibiotics.
1: penicillin; 2: Tetracycline; 3: Chloramphenicol; 4: Ampicillin; 5: Ceftazidime; 6: Clindamycin; 7: Erythromycin; 8:Trimethoprim/Sulfamethoxazole; 9: Gentamicin and 10: Flucloxacillin.

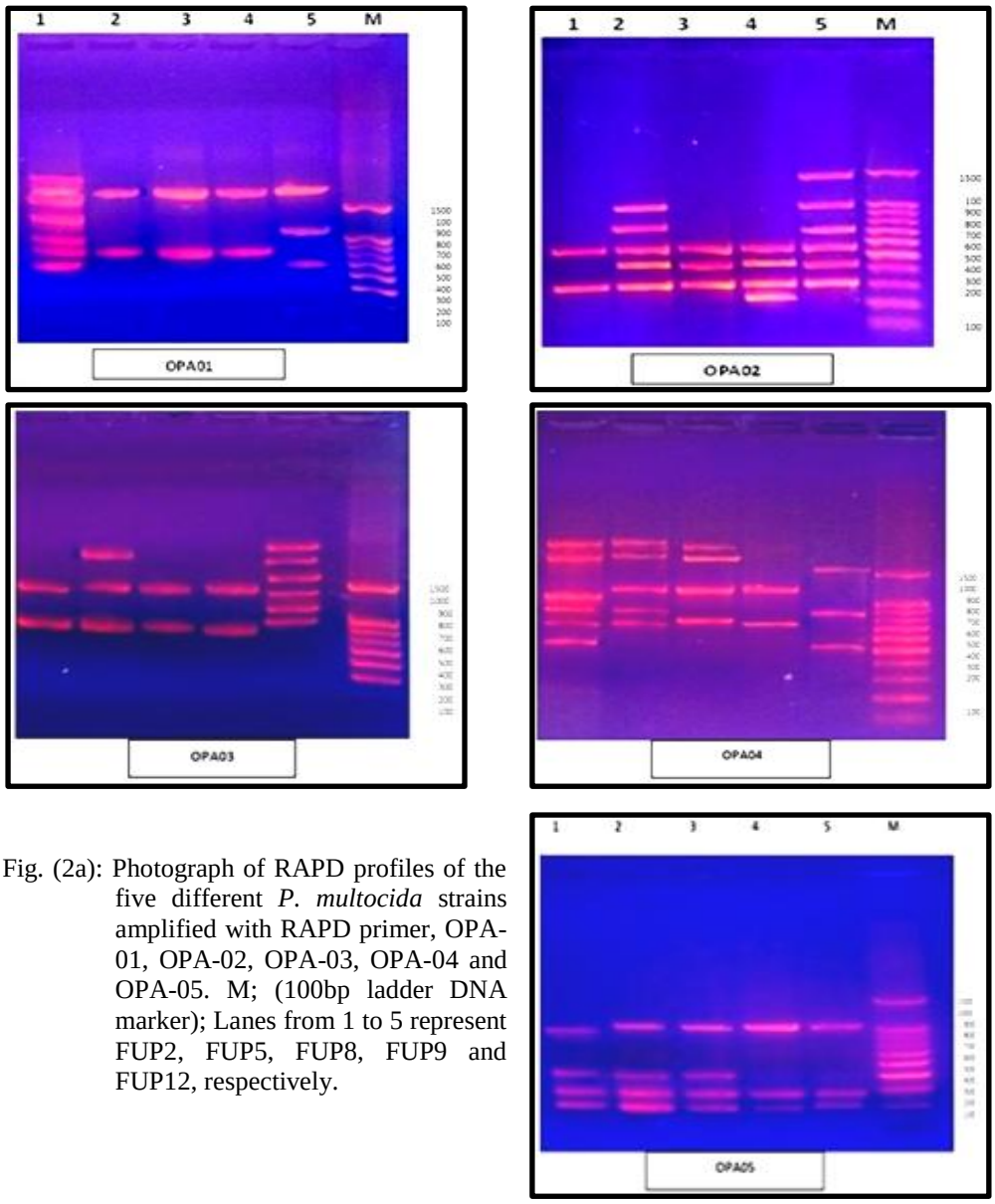

Fig. (2a): Photograph of RAPD profiles of the five different *P. multocida* strains amplified with RAPD primer, OPA-01, OPA-02, OPA-03, OPA-04 and OPA-05. M; (100bp ladder DNA marker); Lanes from 1 to 5 represent FUP2, FUP5, FUP8, FUP9 and FUP12, respectively.

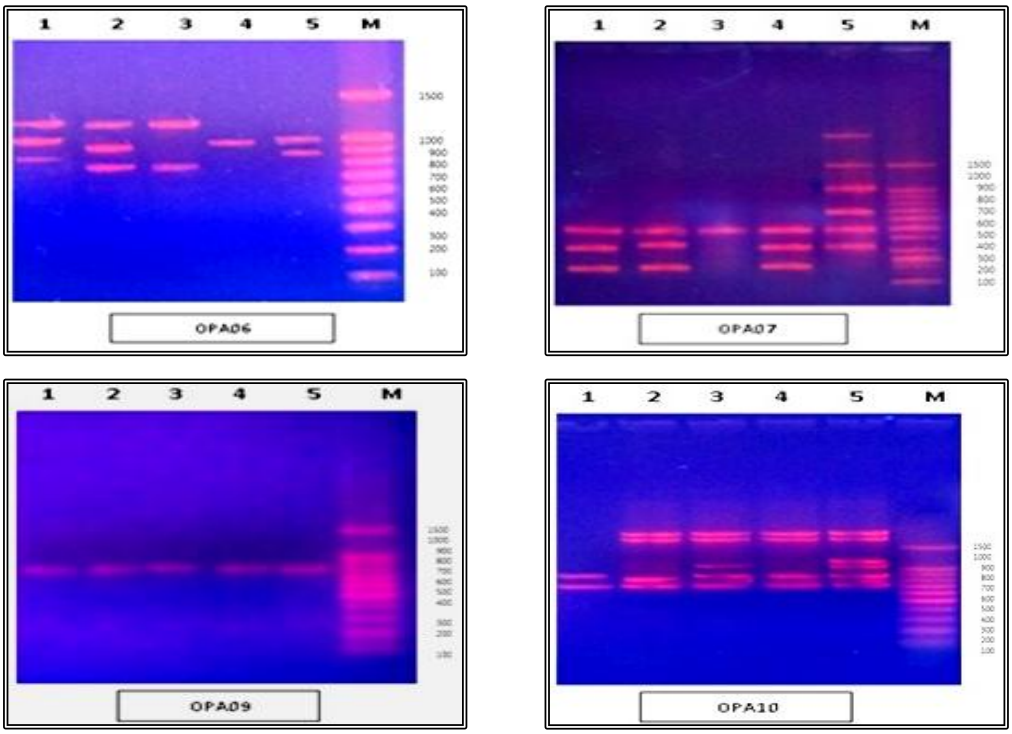

Fig. (2b): Photograph of RAPD profiles of the five different *P. multocida* strains amplified with RAPD primer, OPA-06, OPA-07, OPA-09, and OPA-10. M; (100bp ladder DNA marker); Lanes from 1 to 5 represent FUP2, FUP5, FUP8, FUP9 and FUP12, respectively.

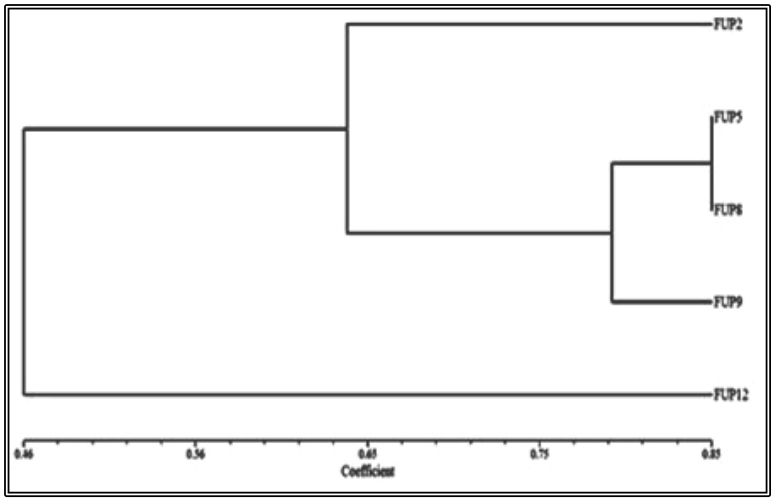

Fig. (3): Dendrogram showing genetic relatedness of *P. multocida* isolates constructed considering all bands of nine random primers (OPA-01 ⟶ OPA-10) using UPGMA and similarity matrices.