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Research Article

Conventional and molecular differentiation between capsular types of *Pasteurella multocida* isolated from various animal hosts

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Abstract: In this study, ten strains of *P. multocida* isolated from chicken, cattle, buffalo and sheep had a clinical manifestation of pneumonia were identified by species-specific PCR (PM-PCR) and 460bp products were obtained. Capsular typing of *P. multocida* is useful for epidemiological evidence and has been assessed by conventional and genotyping assays. According to the results, only one out of the ten strains (10%) which isolated from chicken was detected as capsular type D based on acriflavine test and did not detect the capsular type A for all the ten strains (0%) by using hyaluronidase test. PCR was applied to identify the capsular types using specific primers for each type of *P. multocida*. The findings of this study showed that a uniform amplicon size was corresponding to 657bp, 851bp and 510bp indicating that it belongs to capsular type D, type F and type E, respectively. So, the ten strains were identified to a one strain isolated from chicken as a type D (10%), one strain of which isolated from cattle as a type F (10%) and eight strains of which isolated from sheep, cattle, buffalo and chicken as a type E (80%). Likewise, the data of multiplex PCR showed that capsular type D, one strain; capsular type F, one strain and capsular type E, eight strains. Thus, the multiplex PCR can be used as a simple, sensitive, rapid, reliable technique for capsular typing identification of *P. multocida*. We concluded that the *P. multocida* serogroup E is common in Egypt and has a wide host range.

Keywords: *Pasteurella multocida*; Hyaluronidase test; Acriflavine test; Capsular typing; PCR and Multiplex PCR.

1. Introduction

Pasteurella multocida, a non-motile bacteria, facultatively anaerobic, gram-negative coccobacilli, has been considered a highly heterogenic species due to different antigenic specificity [1]. Diseases caused by *P. multocida* include fowl cholera in chicken; hemorrhagic septicemia in cattle and buffalo; enzootic pneumonia in sheep [2].

Many *P. multocida* strains express polysaccharide capsules on their surface and can be classified into serogroups A, B, D, E and F based on capsule antigens [3,4]. For capsular serotyping, Carter [5,6] used indirect haemagglutination test. Michael *et al.*, [7] used the gel diffusion precipitation test. Capsular type D strains were identified using acriflavine assay [8]. While capsular type A strains can be identified by inhibition of growth in the presence of hyaluronidase [9].

Serotyping and biochemical characterization have indicated a high heterogeneity within the *P. multocida* species [10,11,12], which was later confirmed by a new-generation of typing system based on molecular techniques. Townsend *et al.*, [13] developed PCR for capsular typing of *P. multocida* strains. Townsend *et al.*, [14] used a multiplex PCR assay based on specific gene sequences for laboratory typing of *P. multocida* isolates.

Oligonucleotide primers designed during sequencing of the biosynthetic loci of the capsules of serogroups A and B Chung *et al.*, [15] and Boyce and Adler [16] were used to determine the nucleotide sequences of the region 2 genes from the remaining three capsular serogroups (D, E, and F). Serogroup-specific sequences were then identified for use as primers in a multiplex PCR assay. The genetic organization of the biosynthetic loci for all five capsule types has been determined [2,14].

Data on the nucleotide sequence of the second region in the locus for synthesis of *P. multocida* capsule allowed identifying unique genes for each capsular group which encodes proteins involved in the synthesis of group-specific capsular polysaccharides. The *hyaD* gene is responsible for synthesis of hyaluronic acid and is unique for group A strains, *fcbD* encodes chondroitin

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synthase in group F, *dcbF* is responsible for synthesis of heparan glycoside in D. Genes *bcbD* and *ecbJ* encode glycosyltransferase in strains of capsular groups B and E, respectively. Also, cell wall protein gene *KMT1* highly conserved and unique for the *P. multocida* is identified [14].

The elucidation of the genetic basis for capsule biosynthesis has facilitated the development of a much-improved method for laboratory typing of *P. multocida* isolates utilizing a multiplex PCR assay based on specific gene sequences [14]. Capsular genotyping was conducted based on amplification of 5 different capsular groups using multiplex PCR in the presence of each capsule specific primer along with *P. multocida* specific primers by Shayegh *et al.*, [17] and Ihab *et al.*, [18]**.** The PCR-based typing method has therefore supplanted the previously conventional serotyping [19,20,21] and is now used extensively worldwide as accurate standard.

Although the limitation of conventional serological or non-serological methods for capsular typing of *P. multocida* most laboratories still used this method. The objectives of this study were to apply the conventional methods and PCR protocols for the identification of the *hyaD-hyaC*, *bcbD*, *dcbF*, *ecbJ* and *fcbD* genes specific to capsular types A, B, D, E and F, respectively on *p. multocida* strains, and to determine the relationship between animal host and capsular type of *P. multocida* strains based on molecular capsular typing methods.

2. Material and Methods

2.1 Bacterial strains and growth conditions

Ten strains of *P. multocida* were used in this study. Some phenotypic and molecular characters of the five of *P. multocida* strains isolated from sheep, buffalo and cattle have been described previously [22] where another five strains isolated from chicken were kindly provided by Prof. Dr. Selim Salama Selim, Professor of Microbiology and Immunology, ARC, Egypt. *P. multocida* were grown in Brain Heart Infusion (BHI) Broth and Blood agar (BA) plates. The cultures were stored at 4°C and subcultured monthly.

2.2 Genomic DNA extraction from *P. multocida* strains

Genomic DNA of the ten *P. multocida* strains was extracted following the method described by Tillett and Neilan [23]. The purity and the concentration of DNA were estimated by spectrophotometry at 260 and 280nm.

2.3 Molecular confirmation of bacterial strains by species-specific PCR (PM-PCR)

The species-specific primers KMT1T7 and KMT1SP6 designed by Townsend *et al.*, [13], F-ATCCGCTATTTACCCAGTGG and R-GCTGTAAACGAACTCGCCAC were used to amplify the *KMT1* gene sequence in *P. multocida*. The PCR reaction was performed in the thermal cycler 2720 (Applied Biosystems, USA) in a total volume of 25µl containing 3µl of template DNA (50ng/µl), 1µl of each primer, 12.5µl of 1x PCR master mix (GeneDireX) and 7.5µL of DNase free water. The amplification program consisted of an initial denaturation step at 95°C for 5 min followed by 30 cycles of 30 sec denaturation at 94°C, 30 sec primer annealing at 50°C, 1 min extension at 72°C and a final extension of 10 min at 72°C.

2.4 Conventional capsular typing of *P. multocida*

Two aliquots of a BHI overnight culture were selected for the identification of serogroup D strains with the acriflavine test and for identification of serogroup A strains with the hyaluronidase test according to Carter and Subronto [8] and Carter and Rundell [9], respectively. Hyaluronic acid is the main chemical component of the capsular type A structure, If there was a diminution in the size of the *P. multocida* colonies in the region adjacent to the *Staphylococcus* streak, the isolate was assigned to serotype A. Serotype D was determined by the characteristic flocculation with acriflavine neutral (0.1%, Sigma, USA). If an isolate gave flocculation at the bottom of the test tube, then the isolate was assigned to serotype D.

2.5 Capsular typing via single and multiplex polymerase chain reaction

The separate singleplex PCR was used according to the method described by Townsend *et al.*, [14] using a specific pair of primers for each *capA*, *capB*, *capE*, *capD* or *capF* (Table 1). The PCR mix consisted of 12.5µL of 1X PCR master mix (GeneDireX), 3µL of template DNA (50ng/µl), 1µL of each primer and sterile deionized water for a final volume of 25µL. Amplification was performed in a thermal cycler 2720 (Applied Biosystems, USA) under the following reaction conditions: initial denaturation (95°C for 5 min) followed by 30 cycles of denaturation (95°C for 30 sec), annealing (50°C, 45°C, 42°C, 40°C and 45°C for 30 seconds) for *capA*, *capB*, *capE*, *capD* and *capF* respectively, and elongation (72°C for 60 sec) and a final elongation stage (72°C for 10 min).

The multiplex-PCR was used according to the technique described by Townsend *et al.*, [14] using 3 specific primer pairs for E, D and F capsular type (Table 1). PCR mix consisted of 12.5µl of 1X PCR master mix (GeneDireX), 3µl of template DNA (50ng/µl), 1µl of each primer and sterile deionized water for a final volume of 25µl. Amplification was performed in the thermal cycler 2720 (Applied Biosystems, USA) under the following reaction conditions: initial denaturation at 94ºC for 5 min followed by 30 cycles of denaturation (95ºC for 30 sec), annealing (42ºC for 30 sec), extension (72ºC for 30 sec) and a final extension step of 72ºC for 10 min.

Table 1. The sequence of primers that used in PCR and multiplex PCR capsular typing of *P. multocida* strains.

Serogroup	Gene	Primer	Primer sequence 5'-3'	Amplicons (bp)
A	*hyaD-hyaC*	capA-F capA-R	GATGCCAAAATCGCAGTCAG TGTTGCCATCATTGTCAGTG	1044
B	*bcbD*	capB-F capB-R	CATTTATCCAAGCTCCACC GCCCGAGAGTTTCAATCC	760
D	*dcbF*	capD-F capD-R	TTACAAAAGAAAGACTAGGAGCCC CATCTACCCACTCAACCATATCAG	657
E	*ecbJ*	capE-F capE-R	TCCGCAGAAAATTATTGACTC GCTTGCTGCTTGATTTTGTC	511
F	*fcbD*	capF-F capF-R	AATCGGAGAACGCAGAAATCAG TTCCGCCGTCAATTACTCTG	850

2.6 Agarose gel electrophoresis

All PCR products were analyzed using electrophoresis on a 1.2% agarose gel which stained with 2µl ethidium bromide (0.5µg/ml) and run at 90 V for 45 minutes. A 5µl of the DNA ladder 100bp (BioTeke Corporation) was used as marker and the gel was viewed under UV transilluminator.

3. Results and Discussion

3.1. Confirmation of bacterial strains by species-specific PCR (pm-PCR) assay

The presence of a DNA band approximately 460bp in size (amplification of a specific fragment of the *KMT1* gene) in all strains confirmed the identification of all strains as *P. multocida* (Fig. 1). The results of previous studies [13,24,25,26] using primers KMT1T7 and KMT1SP6 in agreement with these results. The PCR assay was shown to be species-specific, providing a valuable supplement to phenotypic identification of species within this group of bacteria [27]. Species-specific PCR (PM-PCR) assay was a suitable technique for specific detection of *P. multocida*.

3.2. Capsular typing by conventional methods

Capsular typing of *P. multocida* is useful for epidemiological evidence and has been assessed by conventional and genotyping assays. Fig. (2) show the results of acriflavine test, only one out of the ten strains (10%) which isolated from chicken was detected as capsular type D. The result of capsular typing by using hyaluronidase test did not detect the capsular type A for all the ten strains (Fig. 3).

Conventionally, an indirect haemagglutination test (IHA) for typing of specific capsule antigens was described [5]. To overcome the difficulties with IHA test, non-serologic tests were designed for recognition of types A [9], D [8] and F [28]. Hyaluronic acid is the main chemical component of the capsular type A structure, those colonies exhibiting growth inhibition in close proximity to the *S. aureus* streak were assigned to serogroup A [28]. Arumugam *et al.*, [19] identified capsular types A, B and D as the common capsular types among 114 *P. multocida* isolates in Malaysia.

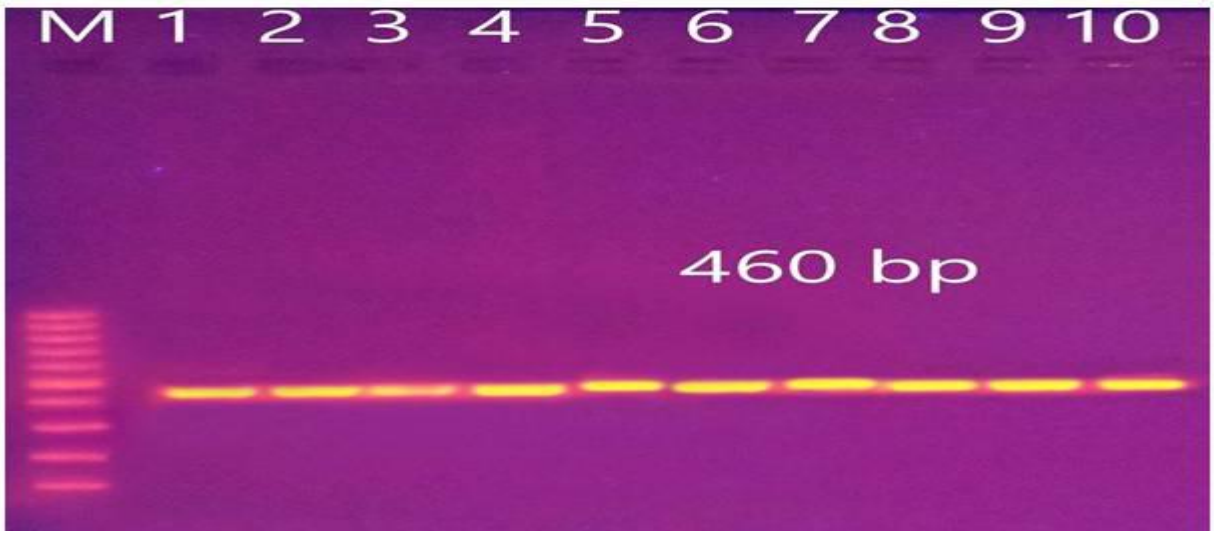

Fig. 1. PCR amplification of *KMT1* gene for identification of *P. multocida* strains.
Lane M: 100bp DNA Ladder, Lanes 1-10: ten *P. multocida* strains.

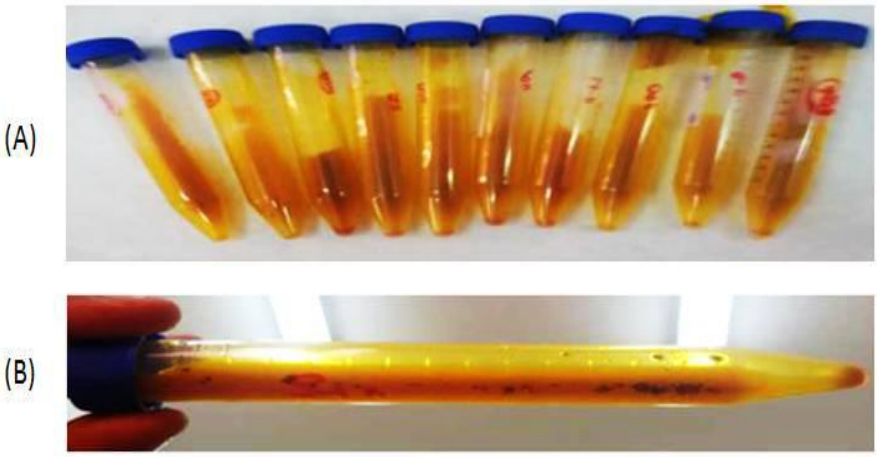

Fig. 2. Acriflavine test: (A) negative reaction. (B) Positive reaction between the inoculum of *P. multocida* type D and acriflavine solution.

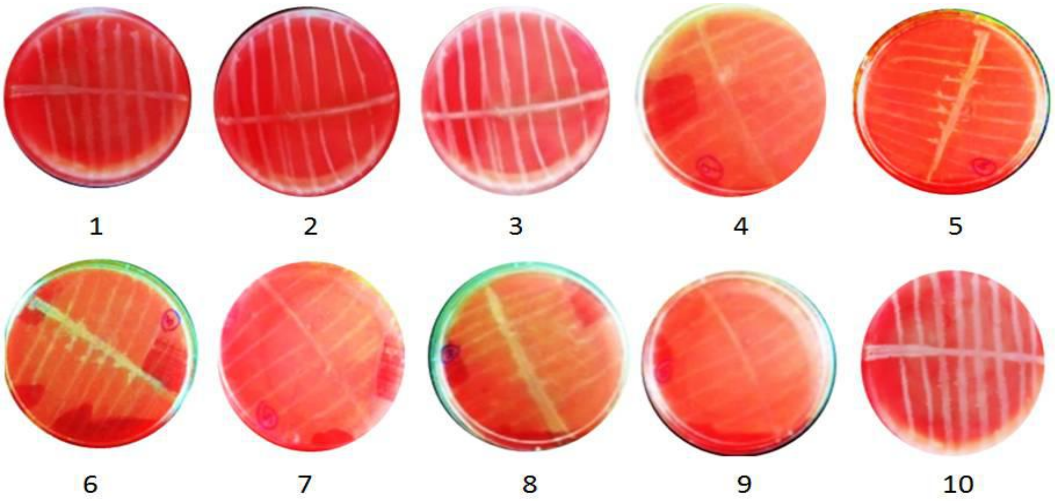

Fig. 3. Hyaluronidase test: colonies of *P. multocida* lack growth inhibition in the vicinity of the staphylococcal colony.

3.3. Molecular capsular typing

PCR was applied to confirm the capsular typing using specific primers for each type. The findings of this study in Fig. (4) showed that a uniform amplicon size corresponding to 657bp, 851bp and 510bp indicating that it belongs to capsular type D (one strain which isolated from chicken), Type F (one strain which isolated from cattle) and type E (eight strains which isolated from sheep, cattle, buffalo and chicken), respectively (Table 2). Likewise, the data of multiplex PCR showed that one strain (10%) was detected as capsular type D and one strain (10%) was detected as capsular type F and eight out of ten bacterial strains (80%) were classified into capsular type E (Fig. 5 and Table 2). On the other hand, no strains gave any amplification with either the capsular type A or B. The capsular typing using PCR was successfully optimized and applied to confirm the capsular types of *P. multocida*. Multiplex PCR is an alternative to comparative conventional tests for the identification of capsular *P. multocida* because it allows for the simultaneous, rapid detection of genes and provides a greater capacity for strain typing.

The conventional methods used in this study were failed to identify the serogroups E and F whereas, all strains were initially tested with the multiplex PCR that involved identification at the species level and then assignment to serogroups D, E and F. These findings are in agreement with Shivachandra *et al*., [29] they found that a 16% of 123 isolates from different avian species were not identified by conventional tests, whereas all samples were typed by multiplex PCR. Similarly, Arumugam *et al*., [19] found that 48% of strains were nontypeable strains with the hyaluronidase and acriflavine test or through the use of specific antisera to identify the capsule types A, D and B, respectively. Diverging classifications for these two methodologies have been noted in other studies [30,19].

Table (2) show the serogroup D obtained from chicken (1/5); serogroup E obtained from sheep (1/1), from buffalo (2/2), from cattle (1/2) and from chicken (4/5) and serogroup F obtained from cattle (1/2). These results suggested that the serogroup E is common in

Egypt and has a wide range of host. Whereas, the results of previous studies have shown that capsular types A and D are common among isolates recovered from sheep and goats [31,32,33]. Differing frequencies of *P. multocida* serotypes have been observed in other studies. Values for *P. multocida* serotype D vary from 0% in rabbits in Brazil [34] to 82.6% in cattle in India and South Asia [35]. Similar results were obtained with *P. multocida* serotype A, which exhibit a prevalence ranging from 17.4% in cattle [35] to 95.24% in geese in Hungary [36]. These differences between serotypes of *P. multocida* may be due to several factors, including the prevalence of the microorganism in the breed and processing techniques.

The ability of *P. multocida* to invade and multiply within the host is enhanced by the presence of its capsule, a polysaccharide structure that is one of the most important virulence factors for this species [37]. Additionally, there are conflicting reports in the literature regarding the possible role of capsule in the adhesion to host cells and tissues [38]. The importance of the capsule in *P. multocida* adherence possibly depends on its strain and host cell type [39].

Table 2. The relationship between animal host and capsular type of 10 *P. multocida* strains based on molecular capsular typing methods.

Strain	Host	Serogroup	Genotype frequency
1	Sheep	E	1/1 (100%)
2	Buffalo	E	2/2 (100%)
3	Buffalo	E	
4	Cattle	E	1/2 (50%)
5	Cattle	F	1/2 (50%)
6	Chicken	E	4/5 (80%)
7	Chicken	E	
8	Chicken	E	
9	Chicken	E	
10	Chicken	D	1/5 (20%)
Total		E	8/10 (80%
		D	1/10 (10%)
		F	1/10 (10%)

4. Conclusion

We concluded that the multiplex PCR assay represents a rapid and reproducible alternative compared to conventional capsular typing currently used for the classification of *P. multocida*. The serogroup E of *P. multocida* is common in Egypt and has a wide host range.

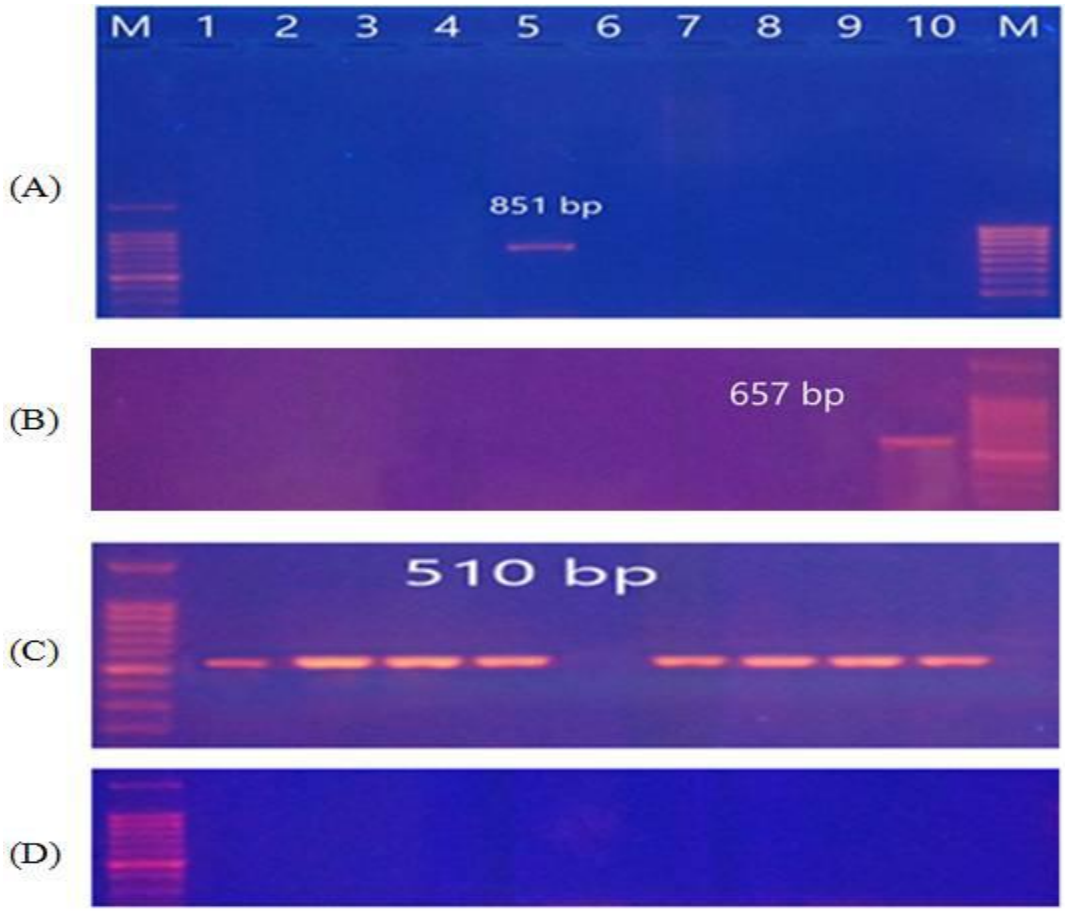

Fig. 4. PCR amplification of *fcbD*, *dcbF*, *ecbJ*, (*hyaD-hyaC*) and *bcbD* genes for detection capsular types: F, D, E, A and B, respectively.
Lane M: 100bp DNA Ladder, Lane 1-10: ten *P. multocida* strains. plate (A): Type F for strain No. 5 (851bp), plate (B): Type D for strain No.10 (657bp), plate (C): Type E for strains No. 1, 2, 3, 4, 6, 7, 8 and 9 (510bp) and plate (D): Type A or Type B, not found.

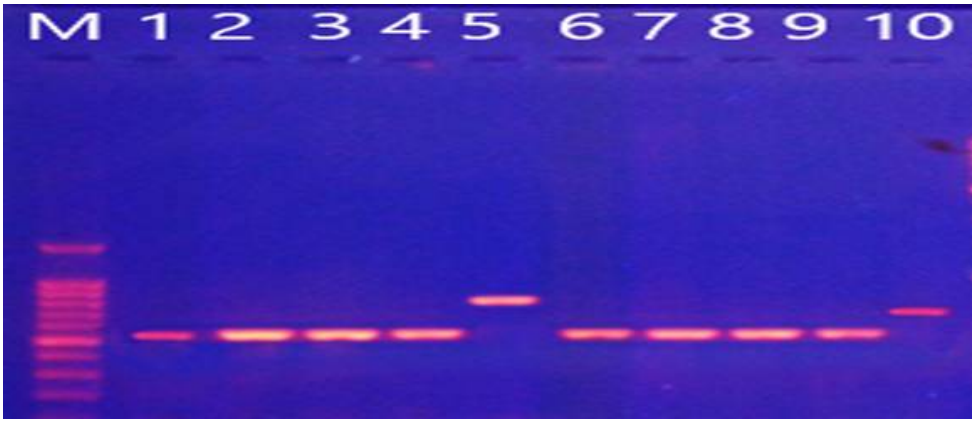

Fig. 5. Multiplex PCR amplification of *fcbD, dcbF* and *ecbJ* genes for detection capsular types: F, D and E, respectively.
Lane M: 100bp DNA Ladder, Lanes 1-10: ten *P. multocida* strains, Type F: strain 5 (851bp), Type D: strain 10 (657bp), Type E: strains 1, 2, 3, 4, 6, 7, 8 and 9 (510bp).

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