Molecular Typing of *Pasteurella multocida* strains recently isolated from Poultry flocks

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1. Abstract

Pasteurella multocida (*P. multocida*) is a wide-range pathogen that infects poultry and animals. The objective of this study is to use molecular biology techniques to develop a fast characterization and genotyping strategy for *P. multocida*. Sixty bacterial isolates were isolated from suspected *Pasteurella* outbreaks in two chicken farms in El-Sharkia Governorate, Egypt, during April 2025. These 60 strains were subjected to chemical and bacteriological identification using traditional methods for both tests. Afterwards, the positive isolates were utilized in molecular identification of capsular typing using PCR targeting the universal primer (*KMT1* gene) and multiplexing PCR using primers targeting the different serogroups (A, B, D, E, and F). Out of 60 samples, only 45 isolates tested positive for *Pasteurella* both morphologically and biochemically. All isolates exhibited different levels of sensitivity to the tested antibiotics. The molecular identification showed that only 40 isolates showed the expected 460 bp targeting the (*KMT1* gene). Molecular capsular typing of positive PCR strains showed 1044 bp belonging to type A (22 strains), and 3 strains showed 760 bp corresponding to the B type (uncommon). Those 25 isolates were molecularly negative for the D, E, and F capsular antigens. Meanwhile, 15 PCR-positive isolates were untypable. In conclusion, the majority of the isolated *Pasteurella* strains were phenotypically and genotypically confirmed to be *P. multocida* type A from a local Egyptian poultry farm. Compared to biochemical analysis and traditional serotyping, which can take a lengthy period, PCR typing could allow for the quick and accurate characterization of *P. multocida* and confirm its identity. Since reference antisera are not accessible, it can shorten the time required to produce polyvalent vaccines. Moreover, the continuous identification of field strains of Pasteurella may provide better data to control *Pasteurellosis* in Egypt.

Key words: Capsular, Molecular, *Pasteurella multocida*, serotypes.

2. Introduction

The extremely contagious Gram-negative bacterium *Pasteurella multocida* (*P. multocida*) is the primary cause of fowl cholera, which poses a serious problem to the poultry industry [1, 2]. Due to mortality, weight loss, carcass condemnation, and higher medicine expenses, the disease leads to financial losses for chicken farmers [3]. According to estimates, fowl cholera led to decrease in egg production and kills between 40 and 60 percent of freshly hatched chicks, with rates of illness and mortality of 52 and 56 percent, respectively [4].

Various *Pasteurella* species continue to pose serious threats in Egypt, where they are linked to the potential causes of pneumonia in domestic sheep and goats and hemorrhagic septicemia (HS) in cattle, as well as fowl cholera (FC) in both domesticated and wild birds [5, 6]. FC infection is an endemic disease and mainly affects the respiratory tracts of chickens, turkeys, ducks, and geese [4, 7]. The serotyping of *P. multocida* isolates is based on two typing methods: the somatic typing, which uses 1-hour boiled supernatant agar gel precipitation, and capsular typing, which uses Carter's IHA system in which *P. multocida* serologically typed into five capsular serotypes (A, B, D, E, and F) as a heterogeneous species [8, 9].

It takes a lot of effort and time to use the phenotypic characterization approaches that include morphology, biochemistry, and serotyping [10]. Some isolates may react identically to both antigens, making differentiation impossible even after the identification of the capsular and somatic antigens [11]. Moreover, homologous antiserum agglutination may not succeed. One of the primary reasons for the decreased sensitivity in this phenotypic test is the inability of serogroups A, D, and F to agglutinate with homologous antisera [12].

P. multocida has demonstrated a propensity to become resistant to a number of antibiotics, including those often used in human and veterinary medicine. Antibiotic abuse or overuse, as well as the possibility of gene transfer from other bacteria, might cause this resistance [13]. So there is a great demand for regular screening for antibiotic sensitivity and adjusting treatment as necessary.

Various molecular methods have been established for *P. multocida* typing, including (PCR)-based fingerprinting [14, 15]. To get around the drawbacks of phenotyping, PCR-based techniques have offered substitute characterization methods [11]. The purpose of *P. multocida* primers was to identify a portion of the *KMT1* gene that codes for the outer membrane protein, resulting in an amplification product that is specific to each strain of *P. multocida* [16, 13].

Sequences in *hyaD, bcbD, dcbF, ecbJ,* and *fcbD* have been identified as a highly specific for their respective serogroups, according to a comparative study of the genetic organization of region 2 of the capsule biosynthetic locus of the five *P. multocida* capsular serogroups (serogroups A, B, D, E, and F) [17]. Since hyaluronic acid is a major component of Type A capsule, the *hyaD–hyaC* gene was the best amplification target for PCR amplification [18].

Despite being a very closely related group, hemorrhagic septicemia (HS)-associated *P. multocida* strains from capsular serogroup B can be distinguished using whole genome analysis. During detection of *P. multocida* in field, the primary challenge is the lack of antisera for capsular typing and the pressing requirement to characterize the field isolates of recent infections or prior to vaccine production. Additionally, field isolates must be identified and genotyped in the early phases of illness or prior to the

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development of effective polyvalent *Pasteurella* vaccines, which require significant resources [19].Since the importance of identifying the specific *P. multocida* type is important for understanding the epidemiology of the disease , developing an effective control strategies and assessing antimicrobial resistance so, the purpose of this work was to detect and molecularly characterize the different types of recently circulating *P. multocida* in chicken farms to solve the struggle in obtaining antisera through quick and alternate ways.

3. Materials and Methods

3. 1. Sampling

Sixty bacterial isolates were collected from two-layer chicken farms in the governorate of El-Sharkia in April 2025. For the first and second farms, the age and farm capacity were 5000 (26 weeks old) and 12000 (34 weeks old), respectively. Comb and wattle swelling and facial edema affected 5:6% of the birds. General drop in egg production of 8% below average. Bacterial isolation was performed on the hearts of recently dead cases (n = 20 and 40) from the first and second farms, respectively.

3.2. Bacterial isolation and identification of Pasteurella from diseased chickens

Bacterial isolates were identified by VSVRI aerobic bacterial vaccines department and subculture in Brain Heart Infusion broth (BHI) broth. Then they were inoculated on MacConkey's agar media and blood agar containing 5–10% defibrinated sheep blood, followed by incubation for 18–24 hours at 37° C. All isolates were identified morphologically and biochemically [10, 20]. In this investigation, two vaccinal strains served as positive control: (PM/VSVRI/1962) *P. multocida* Type B which is utilized in the Pneumobac® vaccine, and (PM/VSVRI/2004) which is utilized in the FC vaccine® was identified as *P. multocida* Type A.

3.3. Conventional biochemical tests

To ensure pure cultures of *P. multocida*, reactivation and preliminary testing were conducted in accordance with Glisson [20]. After the subculturing of samples in brain heart infusion broth (BHI-Oxoid; Cambridge, UK), the isolates were kept for 24 hours at 37° C. Following this time frame, the isolates were plated on MacConkey agar plates and blood agar plates (supplemented with 5% defibrinated sheep blood) to obtain pure cultures of *P. multocida*, accompanied by evaluation of the colonies on blood agar. Catalase and oxidase tests, beside biochemical identification tests, were used in addition to Giemsa staining to observe the characteristics of the bacterial bipolar cells [20].

3.4. Antimicrobial sensitivity test

According to CLSI and NCCLS [21, 22], all *P. multocida* isolates were tested using the disc diffusion method. The name and the concentrations of antibiotic discs utilized during this study are presented in Table (1).

3.5. Molecular detection of the capsular serogroups

A 1-mL aliquot of a BHI overnight culture for each positive *P. multocida* sample was prepared to extract DNA using the EasyPure Bacteria Genomic DNA Kit (EE161-01), Transgen, according to the manufacturer's protocol. Several sets of published primers (Table 2) were utilized during molecular characterization of *P. multocida*. According to Townsend et al. [16], PCR was performed targeting the *KMT* gene. Singleplex PCR for universal detection and multiplex PCR for capsular

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serogrouping of *P. multocida* were performed as following protocol: 25 µL 2× EasyTaq PCR Super Mix (+dye) (S10328), Transgen, 100 pmol (0.5 µL) of both primers [17], 5 µL of template DNA, and up to 50 µL of nuclease-free water. The SimpliAmp Thermal Cycler PCR system was used to carry out amplification reactions. Initials denature at 95° C for 5 minutes, then 30 cycles at 95° C for 1 minute, 55° C for 1 minute, and 72° C for 1 minute, and a final extension at 72° C for 7 minutes. The amplified products were electrophoresed and seen using a UV transilluminator. Thermo Scientific's Gene Ruler 1K bp DNA Ladder was used to measure the product size.

4. Results

4.1. Examination of diseased chickens

The dead chickens showed congestion and cyanosis of the comb and wattle of layer chickens (Figure 1A). Postmortem examination of the diseased chickens showed echymotic hemorrhages scattered on visceral organs with rupture of ovarian follicles in the abdominal cavity (Figure 1B, 1C).

4.2. Bacterial isolation and identification of Pasteurella from diseased chickens

Pasteurella was detected in all 60 isolates using morphological and biochemical traits. As small Gram-negative rods, coccobacilli, oxidase, catalase, and ornithine decarboxylase positive, urease negative, and fermenting glucose, mannitol, and sucrose but not lactose, only 45 of the isolates (75%) were identified and characterized as *P. multocida*. They also showed no discernible growth on MacConkey agar and converted nitrate to nitrite (Table 3**,** Figure 2).

4.3. In vitro anti P. multocida sensitivity test

Isolates of *P. multocida* exhibited 95% resistance to trimethoprim, nitrofurantoin, oxacillin, and sulfamethoxazole, while exhibiting 78% resistance to ampicillin/sulbactam, penicillin, rifampicin, and chloramphenicol, and 20% resistance to oxytetracycline. However, 65% of the isolates were sensitive to streptomycin, ampicillin, kanamycin, erythromycin, cefoperazone, and amikacin, but 98% of the isolates demonstrated sensitivity to cephalexin, clindamycin, norfloxacin, cefotaxime, and polymyxin B (Table 4).

4.4. Molecular capsular typing:

PCR for all biochemically positive tested isolates (45 isolates) showed only 40 isolates with the expected PCR product (460 bp) targeting the universal primer (*KMT1* gene) (Figure 3A). Molecular capsular typing of positive PCR strains showed amplicon size 1044 bp that belonging to capsular type A (22 strains, 55%); on the other hand, 3 strains (7.5%) showed amplicon size 760 bp corresponding to the B type. Those 25 isolates (22A and 3B) were molecularly negative when tested with primers targeting the D, E, and F capsular antigens in a multiplex PCR reaction. Meanwhile, 15 PCR positive isolates (37.5%) were untypable since they showed negative for all tested capsular genes (A, B, D, E, and F), (Figure 3B).

5. Discussion

Pasteurellosis in birds and livestock has long been recognized as a disease of significant economic amplifications. Confirmation of *Pasteurellosis* appears to be challenging because of the numerous clinical signs and laborious laboratory investigations. Moreover, impurities and/or organism death make it challenging to obtain a

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clean culture of *P. multocida* from clinical isolates [23].

P. multocida infection in chickens leads to characteristic alterations observed in the spleen, liver, and heart, of birds that were experimentally inoculated with *P. multocida* isolates. After being challenged with *P. multocida*, birds died within 24 hours. During this study postmortem inspection showed clear septicemia, and hemorrhages were found in the heart, liver and spleen (Figure 1), as described by Zahoor and Siddique [24].

Every isolate exhibited the normal cultural traits of mucoid, dew drop, non-hemolytic on blood agar, Gram-negative coccobacillary by Gram staining, and distinctive bipolarity organism by Leishman's stain. These results are consistent with OIE and Quinn et al. [25, 26]. During this study, Indol generation, nitrate reduction, oxidase, and catalase production were all detected in 75% of the isolates that underwent biochemical testing; these results were consistent with a number of other studies [27, 28, 29], the same results were recorded by Mohamad and Mageed and Kwage et al. [30, 31] where 1.2% and 7.6% of their isolates were positive for *P. multocida* respectively.

Anti-*Pasteurella* sensitivity testing of all tested isolates showed that the organisms were 95% resistant to nitrofurantoin, oxacillin, and sulfamethoxazole/trimethoprim, the same findings reported by Kwaga et al. and Elalamy et al. [31, 32] and closely parallel to results recorded by Balakrishnan and Roy and Dashe et al. [13, 33]. On the other hand, isolates showed 78% resistance to rifampicin and penicillin, ampicillin-sulbactam, and chloramphenicol, results almost similar to the findings described by Balakrishnan and Roy and Dashe et al. [13, 33]; however, Elalamy et al. and Victor et al. [32, 34] recorded 100% resistance.

Only 25% of the tested isolates showed resistance to oxytetracycline, which is consistent with the findings of Kamruzzaman et al. [35]; nevertheless, 50% of isolates were shown to be sensitive by Balakrishnan and Roy [13]. However, the tested isolates exhibited 65% sensitivity to other tested antibiotics, as shown in table 4. Those results are roughly comparable to previously published ones [13, 35]. Given the emergence of drug resistance in *P. multocida*, all the studies suggested that doing antibiotic sensitivity testing is crucial to control chicken cholera [36].

The presence of *P. multocida* capsule, which is considered as an important virulence factor, enhances the ability of *P. multocida* to attack and proliferate within the host [37]. Furthermore, there are contradictory observations in the studies about the potential contribution of the capsule to the adherence to host tissues and cells [38]. The strain and host cell type of *P. multocida* may influence the capsule's significance in adhesion [39].

Important arguments for using the molecular approach include the difficulty in obtaining antisera specific to a particular capsular type and the need to identify and type field isolates early in the infection process or before either fowl cholera vaccination or challenging [40,41]. Furthermore, Perry et al. [42] stated that the multiplex assay techniques minimize the needed reagents and time to attain results.

As advised by OIE [25], PCR was successfully performed during this investigation using primers specific for *P. multocida* to confirm the isolate's identity; the universal *KMT1* gene was

amplified in the current PCR approach to identify any *P. multocida* strain. Only 40 of the 60 (66.6%) field isolates were positive, exhibiting the distinct 460 bp of *P. multocida* universal *KMT1* gene amplification, even though all 60 isolates were recognized as *Pasteurella* visually or biochemically.

The capsular loci at *hyaD* (serotype A), *bcbD* (serotype B), *dcbF* (serotype D), *ecbJ* (serotype E), and *fcbD* (serotype F) are essential for *P. multocida* genotyping [17]; hence, during this investigation, all genes belonging to types A, B, D, E, and F were evaluated using multiplexing PCR reaction. Results showed that most of the PCR-positive isolates were type A (55%) with specific band (1044 bp), and the rare results of type B (7.5%) was recorded with the specific band (760 bp), whereas the remaining strains were untypable (negative for all tested serotypes). These results are corroborated by earlier research [17, 43].

Multiplex PCR revealed that serogroup A was present in 22 out of 40 isolates (55 %), which agrees with Leotta et al. and Shivachandra et al. [40, 44] where type A was isolated from 8 out of 9 strains and 92 out of 94 samples from Argentina and India, respectively. According to Rhodes and Rimler [45], the predominant serogroup seen in avian isolates is type A. The current study is different from those by Kumar et al. and Chawak et al. [46, 47], which showed that capsular groups D:3 and F:3 is responsible for fowl cholera in birds. During the study of Davies et al. [48], serogroup B was only detected in 8% of samples; serogroup B was found during this study in 3 out of 40 isolates (7.5 %). On the other hand, during this study the multiplex PCR method failed to identify fifteen strains (37.5%), alike to Townseed et al. [17] and Leotta et al. [44], where 2-5% and 2-9% of samples, respectively, couldn't be classified. And this agrees with Moemen et al. [49], which found that, the other serotypes B, D, and F are less commonly attendant with the infection in poultry. The presence of serotype B during this study is considered a uncommon case, since its prevalence in poultry is very rare, and this can be clarified due to the mode of transmission of *P.maltocida* through direct contact with contaminated feed and water sources .These results are align with Rhoades and Rimler [50] where capsular B group was isolated from turkey poults although its uncommon prevalence**.** On the other hand during the study of Abood et al [51] and Townseed et al [16] they reported that all isolated and identified *Pasteurella* isolates in Egypt are related to *P.maltocida* serotype A which considered the most prevalent serotype.

From the above mentioned, using both phenotypic and molecular techniques are still essential in launching an ultimate detection of *Pasteurellosis*. And the multiplexing PCR is considered as a replacement tool for phenotypic testing during the detection of *Pasteurellosis* as it allows for rapid gene detection and affords a higher incidence for typing.

6. Conclusions

The majority of the isolated *Pasteurella* strains were phenotypically and genotypically confirmed to be *P. multocida* type A from local Egyptian poultry farm. The presence of serotype B during this study is considered as uncommon case, since its prevalence in poultry is very rare. Compared to biochemical analysis and traditional serotyping, which can take a lengthy period, PCR typing could allow for the quick and accurate characterization of *P. multocida* and confirm its identity. Since

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reference antisera are not accessible, they can shorten the time required to produce polyvalent vaccines. Moreover, the continuous identification of field strains of *Pasteurella* may provide better data to control *Pasteurellosis* in Egypt.

Conflict of interest: Nothing to declare

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Table 1: Antibiotic discs and its concentrations (µg / ml)

Antimicrobial agent	Disc concentra	Antimicrobial agent	Disc concentra
Ampicillin/Sulbactam	10/10 µg	Clindamycin	2 µg
Cefoperazone	75 µg	Norfloxacin	10 µg
Penicillin	10 IU	Sulfamethoxazole/trimethoprim	1.25/23.75 µg
Cephalexin	30 µg	Erythromycin	15 µg
Ampicillin	10 µg	Oxytetracycline	30 µg
Cefotaxime	30 µg	Chloramphenicol	30 µg
Oxacillin	1 µg	Nitrofurantoin	300 µg
Amikacin	30 µg	Rifampicin	5 µg
Kanamycin	30 µg	Polymyxin B	300 µg
Streptomycin	30 µg		

Table 2: Sequences of the oligonucleotides used in the *P. multocida* multiplex capsular PCR typing assay.

Serogroup /Gene	Primer name	Sequence	Annealing Temp.	Product Size (bp)
ALL KMT1	KMT1T7-F KMT1SP6-R	ATC-CGC-TAT-TTA-CCC-AGT-GG GCT-GTA-AAC-GAA-CTC-GCC-AC	55^{0}c	460
A *hyaD-hyaC*	CAPA-F CAPA-R	TGC-CAA-AAT-CGC-AGT-CAG TTG-CCA-TCA-TTG-TCA-GTG		1044
B *bcbD*	CAPB-F CAPB-R	CAT-TTA-TCC-AAG-CTC-CAC-C GCC-CGA-GAG-TTT-CAA-TCC		760
D *dcbF*	CAPD-F CAPD-R	TTA-CAA-AAG-AAA-GAC-TAG-GAG CCC CAT-CTA-CCC-ACT-CAA-CCA-TAT-CAG		657
E *ecbJ*	CAPE-F CAPE-F	TCCGCAGAAAATTATTGACTC GCTTGCTGCTTGATTTTGTC		511
F *fcbD*	CAPF-F CAPF-R	AATCGGAGAACGCAGAAATCAG TTCCGCCGTCAATTACTCTG		851

Table 3: Biochemical tests for isolated *P. multocida* strains

Test	*Pasteurella multocida*
Hemolysis on blood agar	-
Growth on MacConkeys agar	-
Indole production	+
Oxidase production	+
Catalase production	+
Urease production	-
Ornithine decarboxylase	+
Lactose fermentation	-
Sucrose fermentation	+
Glucose fermentation	+

Table 4: The distribution percentage of antibiotics sensitivity against the *P. multocida* isolates

Antimicrobial agent	Resistant %	Sensitive %	Antimicrobial agent	Resistant %	Sensitive %
Ampicillin/Sulbactam	78%	22%	Clindamycin	2%	98%
Cefoperazone	35%	65%	Norfloxacin	2%	98%
Penicillin	78%	22%	Sulfamethoxazole/ trimethoprim	95%	5%
Cephalexin	2%	98%	Erythromycin	35%	65%
Ampicillin	35%	65%	Oxytetracycline	25%	75%
Cefotaxime	2%	98%	Chloramphenicol	78%	22%
Oxacillin	95%	5%	Nitrofurantoin	95%	5%
Amikacin	35%	65%	Rifampicin	78%	22%
Kanamycin	35%	65%	Polymyxin B	2%	98%
Streptomycin	35%	65%			

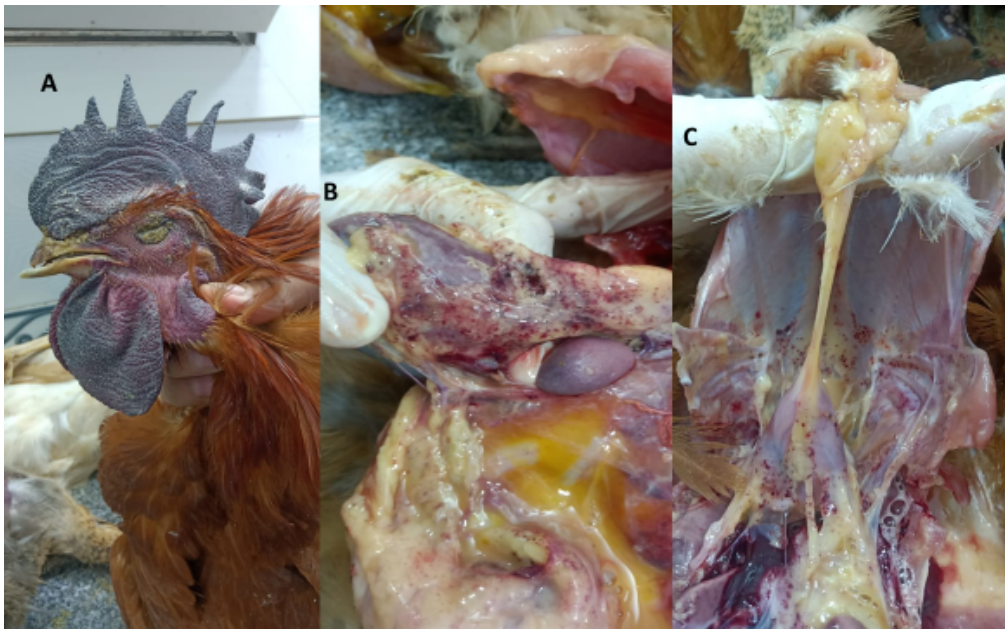

Fig (1): Clinical signs appeared on chicken suffered from *P. multocida* infection. A). Congestion and cyanosis of the comb and wattle of adult chicken. B, C). Echymotic hemorrhages scattered on visceral organs with rapture of ovarian follicle in the abdominal cavity.

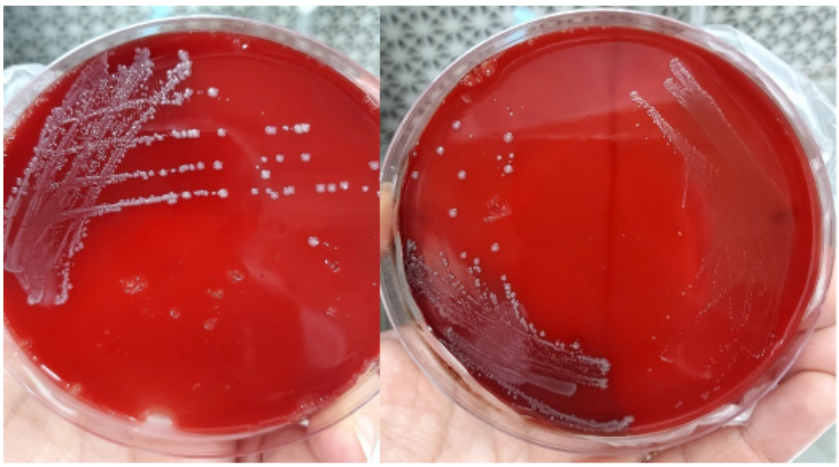

Fig (2): Growth of *P. multocida* on blood agar. Gray, non-hemolytic colonies

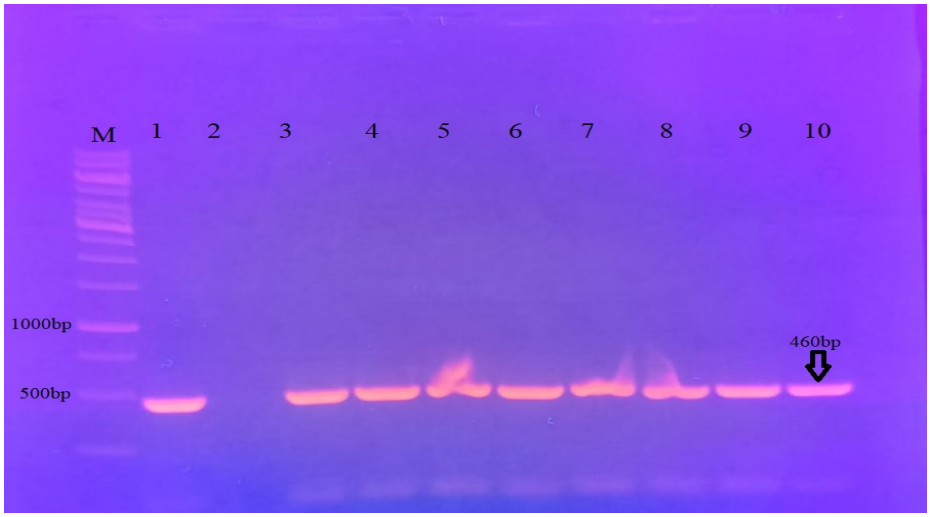

Fig (3-A): *Pasteurella multocida* PCR. All tested isolates lanes (3–10) shared the same band at 460 bp of *KMT1* gene general for all *P. multocida serotypes*. M: 1K bp DNA ladder. Lane (1) +Ve control. Lane (2) -Ve control.

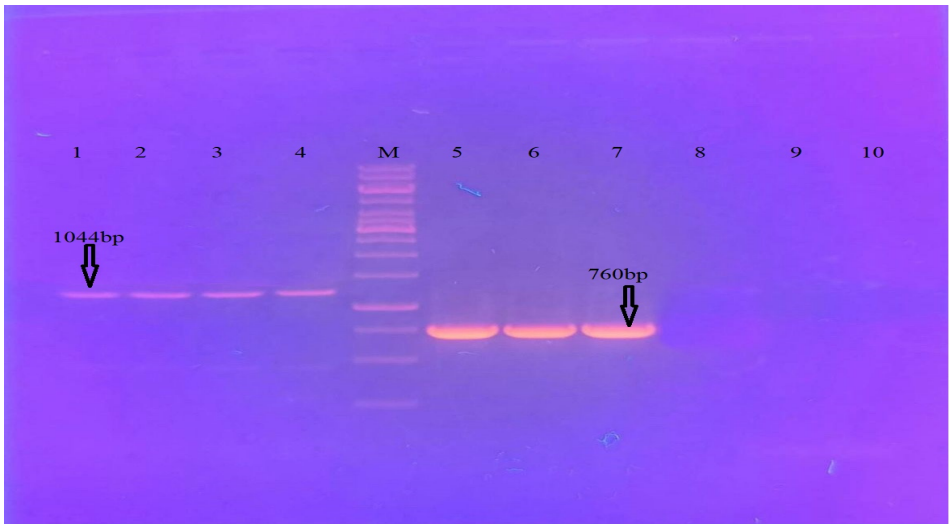

Fig (3-B): *Pasteurella multocida* multiplex capsular PCR typing system. M: 1K bp DNA ladder. Lane (4): PM/VSVRI/2004 vaccine strain type A as +ve control of type A. Lane (1-3): Field isolates showing specific band for type A at 1044 bp of *hyaD-hyaC* gene and Lane (5): PM/VSVRI/1962 vaccine strain type B as +ve control of type B. Lane (6, 7): Field isolates showing specific band for type B at 760 bp of *bcbD* gene. Lane (8 -10): un-typable isolates