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Genomic identification of Pasteurella multocida isolated from turkey flock in Egypt early 2008

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Molecular detection and differentiation of *Pasteurella multocida* strain involved in a separate fowl cholera outbreak in a turkey flock farm located in El-Menofia Governorate, Egypt early 2008 was investigated. The isolated strain was compared with an Egyptian *Pasteurella multocida* isolate that was previously isolated from turkey flock during last decade besides four vaccinal strain (A:5, A:8, A:9 and D:2) on phenotypic and genotypic characterization basis. Phenotypically all the strains were similar as all the strains produce non haemolytic colonies on blood agar, and all the strains revealed similar biochemial behaviour. On the other hand, the genomic typing of all the stains using rep-PCR techniques [repetitive BOX elements, enterobacterial repetitive intergenic consensus (ERIC) and repetitive extragenic palindromic (REP) polymerase chain reaction (PCR)] differentiated the six *Pasteurella multocida* strains into six different profiles. The molecular identity between the *Pasteurella multocida* 2008 strain and the previously isolated strain was 76.6 % and were ranged from 65.2% to 79.2% with the 4 vaccinal strains. These results reported the continuous mutations of the field *Pasteurella multocida* strains among poultry flocks in Egypt indicating the urgent need for the frequent and continuous molecular epidemiological investigations of fowl cholera outbreaks in various poultry flocks to detect these new strains and update the fowl cholera vaccines.

Fowl cholera, caused by *Pasteurella multocida*, occurs sporadically or enzootically in most countries of the world wherever intensive poultry production occurs, and is known as a bacterial disease of major economic importance due to its high mortality (Rimler and Glisson, 1997). Currently, incidences of fowl cholera along with other bacterial diseases are on the increase despite vaccination and proper medication as a sequel of various incriminating factors (Jonas *et al.,* 2001). Conventional methods of disease diagnosis depend on isolation, identification and differentiation by employing bacteriological techniques, serotyping, pathogenicity tests, antibiotic sensitivity tests and biochemical tests (Rimler and Glisson, 1997). However, these techniques are laborious, time consuming and not free from certain limitations.The limitations of currently employed techniques have led to significant problems in understanding the disease outbreaks, origin and transmission of pathogens, the virulence characteristics of the organism and in determining disease incidence and economic importance. DNA-based techniques, which are rapid, specific and highly sensitive, are efficiently employed for early detection and differentiation of various strains of different micro-organisms from single/different outbreaks (Blackall and Miflin, 2000; Biswas *et al.,* 2004) The heterogeneous diversities of avian P. multocida strains can be well differentiated with the use of molecular biological techniques such as restriction endonuclease analysis (REA)(Wilson *et al.,* 1993), single primer PCR (Dabo *et al.,* 2000), enterobacterial repetitive intergenic consensus (ERIC -PCR) (Loubinoux *et al.,* 1999), and repetitive extragenic palindromic (REP -PCR) (Townsend *et al.,* 1997). In the present investigation of fowl cholera outbreak in a turkey flock, the applic-ability and efficiency of conventional as well as novel molecular methods for their ability to identify and characterize *P. multocida* strains were studied, compared with the standard *P.multocida* serotypes.

Materials and methods

Bacterial isolation. A fowl cholera outbreak that occurred within a 14 week old turkey flock in a single private turkey farm, at El-Menofia governorate during Jan .2008, was investigated Bacterial cultures isolated from the heart blood and bone marrow of dead birds were maintained

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on blood agar slants as well as freeze-dried ampoules for further studies.

Conventional identification. All strains [The recently isolated one which designated as T08 besides a previously isolated and identified *P . multocida* strain T90 and four standard *P.multocida* vaccinal strains (A:5,A:8,A:9 and D:2)] were subjected for identification based on cultural, morphological and biochemical characteristics as described in the standard bacteriological methods (Cruickshank *et al.,* 1975). The bacterial cultures identified as *P . multocida* were further classified as capsular and somatic serogroups based on an indirect haem-agglutination assay (Carter, 1955) and an agar gel precipitation test (Heddleston *et al.,* 1972), respectively.

Pathogenicity test. All strains of *P .multocida w*ere grown for 18 h in a shaker-come-incubator at 37ºC in brain heart infusion (BHI) broth. About 0.2 ml each culture containing approximately 2.4 x 10^8 colony forming units/ml was inoculated into each of three mice by the intra- peritoneal route and observed for 72 h to study the mortality pattern .Re-isolation of organisms were carried out on a blood agar plate using heart blood collected from dead mice, and an impression smear from the liver was prepared for observation following the Giemsa method of staining.

DNA extraction. Genomic DNA of all strains was extracted following the method described by (Wilson, 1987). The concentration of DNA was measured spectrophotometrically at 260 nm.

Enterobacterial repetitive intergenic consensus ERIC-PCR. This PCR was conducted using primers targeting palindromic sequences of ERIC according to (Versalovic *et al.,* 1991) as shown in table 1.The ERIC-PCR components volumes and concentrations and the reaction conditions are shown in (Table 2, 3).

Repetitive extragenic palindromic (REP)-PCR. The assay was performed using oligonucleotide primers targeting REP sequences of bacteria according to (Versalovic *et al.,* 1991.) as shown in table 1.The REP-PCR components volumes and concentrations and the reaction conditions are shown in Table 2 , 3..

Repetitive BOX-PCR. The assay was performed using oligonucleotide primers targeting BOX sequences of bacteria according to (Versalovic *et al.,* 1991) as shown in Table 1. The BOX-PCR components volumes and concentrations and the reaction conditions are shown in Table 2, 3.

All three types of PCR-based typing methods were performed three times- using T-gradient Thermal cycler of Biometra co. - to ensure that strains were correctly assigned to respective patterns .Assigning of the profile produced by each strain was denoted as distinct when their PCR types differed by (1) band .PCR profiles generated by various typing tools were analyzed by composing a data matrix built on the presence (+) or absence (-) of any fragment appearing in each strain . The size of bands that differed by 5 % on the different gels were considered as the same band .Cluster analysis was performed using the un-weighed-pair-group method with arithmetic linkages, as per the method of Li (1981), to identify and establish the relationship among various strains, if any.

Results

The observed clinical signs in the affected turkey flock were anorexia, dullness and depression with ruffled feathers, shallow rapid respiration and diarrhea at later stages before death. The mortality rate was 1% daily with cumulative mortality rate of 10%.The flock was of 95 day old at the beginning of infection and was vaccinated once at the 65th day old with the local inactivated fowl cholera vaccine via subcutaneous route at a dose of 0.5 ml. The Postmortem examination of acutely dead birds revealed generalized septicemia, swollen liver with necrotic foci and petechial haemorrhages. Haemorrhages on subepicardial and subserosal areas were prominent. Congestion of lungs and mild enlargement of kidneys with distended tubules were also observed.

Conventional identification. Small glistening mucoid dew drop like colonies were found on blood agar plates after incubation at 37 °C for 18 h, and Gram-negative, cocco-bacilli appeared following Gram's stain and bipolar organisms in Giemsa staining. All strains produced indole, positive for catalase, oxidase and the nitrate reduction test. No reaction was seen with citrate, methyl red and Voges/Proskauer tests and gelatin liquefaction tests. The organisms did not grow on McConkey's agar and were found to be non-haemolytic on blood agar. Glucose, fructose, galactose, mannitol and sucrose were fermented and none of the strains reacted with salicin, raffinose, inositol and rhamnose. Conventional methods of capsular and somatic serotypingidentified the T08 isolate of turkey as well as the T90 as *P. multocida* serogroup A:1.

Table (1): List of primer sequences used in PCR techniques.

Serial number	Type of PCR	Primer sequences	Reference
1	REP-PCR	REP1R-IDt, 5'-NNN NCG NCG NCA TCN GGC-3' REP2-IDt, 5'-NCG NCT TAT CNG GCC TAC-3'	Versalovic *et al.,* (1991)
2	ERIC-PCR	ERIC1R,5'- ATG TAA GCT CCT GGG GAT CAC-3' ERIC2, 5'-AAG TAA GTG ACT GGG GTG AGC G-3'	Versalovic *et al.,* (1991)
3	BOX-PCR	BOX A1R 5'- CTA CGG CAA GGC GAC GCT GAC G -3'	Versalovic *et al.,* (1991)

Table (2): Concentration of various components used in rep-PCR (QIAGEN PCR kit).

Sr. No	Components	BOX-PCR	ERIC-PCR	REP-PCR
1.	PCR Master mix (2X)	12.5 µl	12.5 µl	12.5 µl
2	Primer (10 pmol/ µl)	1.0 µl	1.0 µl each	1.0 µl each
3	DNA Template (30 ng/ µl)	3.0 µl	3.0 µl	3.0 µl
4	Distilled Water	8.5 µl	7.5 µl	7.5 µl

Table (3): Thermal cycling protocol for rep-PCR.

Sr.No.	PCR Category	Initial denaturation	Denaturation	Annealing	Extension	Final extension
1	BOX-PCR	94º C, 5 min	94 º C, 1min	40º C, 2 min	72º C, 2 min	72 º C, 10 min
			Repeated for 35 cycles			
2	ERIC-PCR	94º C, 5 min	94 º C, 1 min	52º C, 1.5 min	65º C, 8 min	65 º C, 16 min
			Repeated for 40 cycles			
3	REP-PCR	94º C, 5 min	94 º C, 1 min	52º C, 1 min	65º C, 8 min	65 º C, 16 min
			Repeated for 40 cycles			

Pathogenicity test. Each examined strain of *P.multocida* was found to be pathogenic to mice, causing mortality within 24 h. No significant variation in mortality pattern was observed. Re-isolated colonies showed similar characteristics of *P. multocida*, and impression smears revealed typical bipolarity of the organism.

ERIC –PCR. Generated amplified products of 13 bands, which were in the range of 2.225 kb to 389 bp, categorized the 6 strains into six different profiles (Figure 1 and table 4) The number of bands amplified in different samples by ERIC-PCR were varied from 10 to 12 with the band frequency ranging from 0.167 to 1.000 . The bands of ERIC-PCR were 15.4% polymorphic, 15.4% unique and 69.2% monomorphic.

REP- PCR. Generated amplified products of 12 bands, which were in the range of 1.870 kb to 235 bp, categorized the 6 strains into six different profiles (Figure 2 and table 5) The number of bands amplified in different samples by REP-PCR were varied from 6 to 10 with the band frequency ranging from 0.167 to 1.000 . The bands of REP-PCR were 58.3% polymorphic, 16.7% unique and 25% monomorphic.

BOX- PCR. Generated amplified products of 12 bands, which were in the range of 1.315 kb to 155 bp, categorized the 6 strains into six different profiles (Figure 3 and table 6) The number of bands amplified in different samples by BOX-PCR were varied from 6 to 7 with the band frequency ranging from 0.167 to 0.833. The bands of BOX-PCR were 83.3% polymorphic and 16.7% unique.

Table (4): Plus / Minus data for ERIC- PCR analysis gel image.

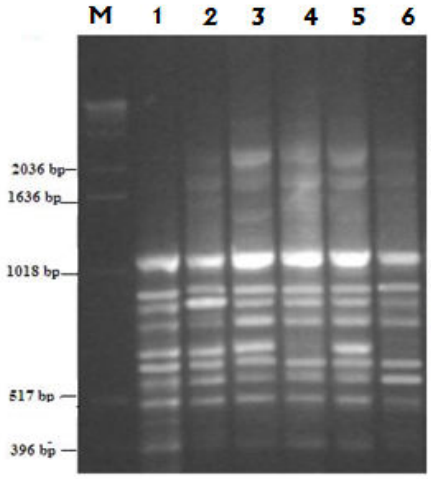
MW	Lane1	Lane2	Lane3	Lane4	Lane5	Lane6	Frequency	Polymorphism
2225	-	-	+	+	+	+	0.667	Polymorphic
1870	+	+	+	+	+	+	1.0	Monomorphic
1356	-	-	+	-	-	-	0.167	Unique
1130	+	+	+	+	+	+	1.0	Monomorphic
950	+	+	+	+	+	+	1.0	Monomorphic
920	+	+	+	+	+	+	1.0	Monomorphic
860	+	+	+	+	+	+	1.0	Monomorphic
670	+	+	+	-	+	-	0.667	Polymorphic
630	+	+	+	+	+	+	1.0	Monomorphic
580	+	+	+	+	+	+	1.0	Monomorphic
510	+	+	+	+	+	+	1.0	Monomorphic
430	+	-	-	-	-	-	0.167	Unique
389	+	+	+	+	+	+	1.0	Monomorphic
	11	10	12	10	11	10		

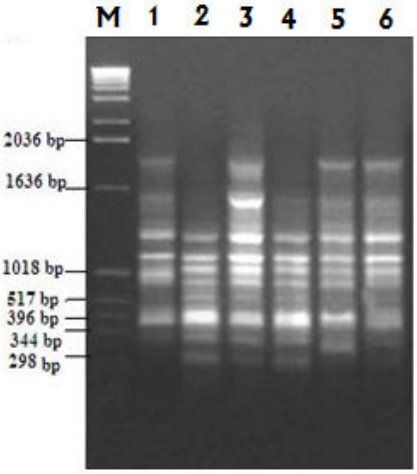
Table (5): Plus / Minus data for REP- PCR analysis gel image.

MW	Lane1	Lane2	Lane3	Lane4	Lane5	Lane6	Frequency	Polymorphism
1870	+	-	+	-	+	+	0.667	Polymorphic
1568	+	-	+	-	+	+	0.667	Polymorphic
1350	+	+	+	+	+	+	1.0	Monomorphic
1270	+	+	+	+	+	+	1.0	Monomorphic
1150	-	+	+	+	+	+	0.833	Polymorphic
1028	+	-	-	-	-	-	0.167	Unique
895	-	+	+	+	+	+	0.833	Polymorphic
520	-	+	+	+	-	-	0.500	Polymorphic
365	+	+	+	+	+	+	1.0	Monomorphic
335	-	+	+	+	-	+	0.667	Polymorphic
300	-	-	-	-	+	-	0.167	Unique
235	-	+	+	+	-	-	0.500	Polymorphic
	6	8	10	8	8	8		

Table (6): Plus/ Minus data for BOX - PCR analysis gel image.

MW	Lane1	Lane2	Lane3	Lane4	Lane5	Lane6	Frequency	Polymorphism
1315	-	-	-	-	+	-	0.167	Unique
1056	+	+	-	+	-	+	0.667	Polymorphic
980	-	-	+	-	+	-	0.333	Polymorphic
878	+	-	-	+	-	+	.500	Polymorphic
870	+	+	+	-	-	-	.500	Polymorphic
750	-	-	-	-	+	-	.167	Unique
645	-	-	-	+	+	+	.500	Polymorphic
360	+	+	+	+	-	+	0.833	Polymorphic
343	+	+	+	+	-	+	.833	Polymorphic
310	-	-	-	+	-	+	0.333	Polymorphic
292	+	+	+	+	-	+	0.833	Polymorphic
155	-	+	+	-	-	-	0.333	Polymorphic
	6	6	6	7	4	7		





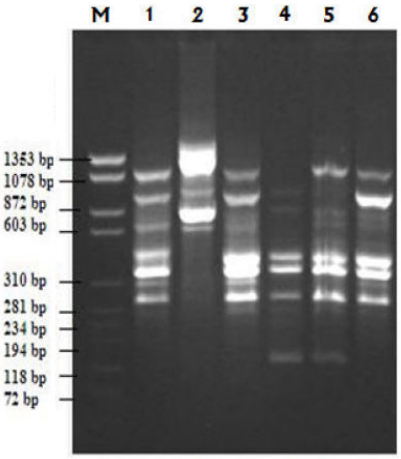


Fig. 1 **Fig.2** **Fig.3**

Fig (1): ERIC-PCR fingerprinting profiles of *P.multocida* strains Lane M, DNA molecular weight marker X of Roche Applied Science; lane 1 represent the recent 2008 *P.multocida* strain; Lane 2 represent the previously isolated *P.multocida* strain ;Lanes 3 to 6 represent the vaccinal *P.multocida* strains.

Fig (2): REP-PCR fingerprinting profiles of *P. multocida* strains Lane M, DNA molecular weight marker X of Roche Applied Science; lane 1 represent the recent 2008 *P.multocida* strain; Lane 2 represent the previously isolated *P.multocida* strain; Lanes 3 to 6 represent the vaccinal *P. multocida* strains.

Fig (3): BOX –PCR fingerprinting profiles of *P. multocida* strains Lane M, DNA molecular weight marker IX of Roche Applied Science Lane 1 represent the recent 2008 *P. multocida* strain; Lane 2 represent the previously isolated *P.multocida* strain; And Lanes 3 to 6 represent the vaccinal *P.multocida* strains.

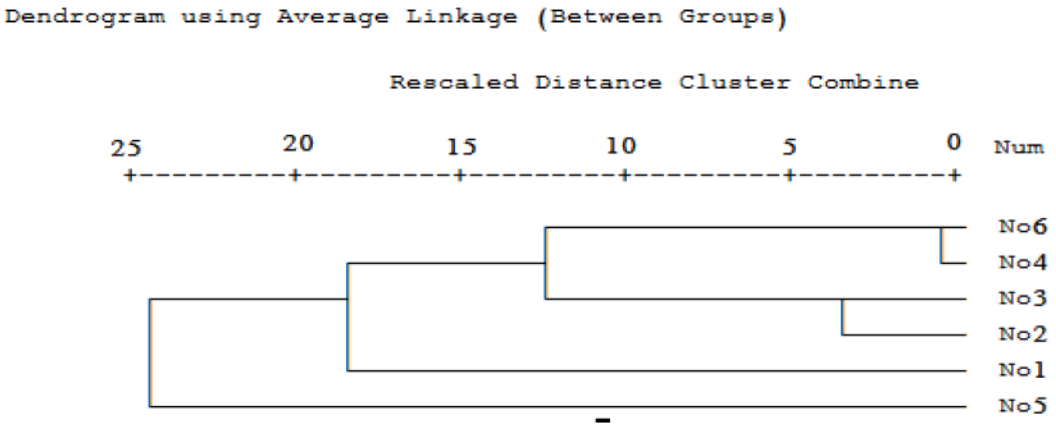


Fig (4): Combined cluster analysis of ERIC-PCR. REP-PCR and BOX- PCR

Table (7): The Proximity Matrix.

Case	Matrix					
	No 6	**No 5**	**No 4**	**No 3**	**No 2**	**No 1**
No 6 (A:5)	1.000	.750	.920	.792	.776	.792
No 5 (D:2)	.750	1.000	.667	.745	.638	.652
No 4 (A:9)	.920	.667	1.000	.792	.857	.708
No3 (A:8)	.792	.745	.792	1.000	.885	.745
No 2 (T90)	.776	.638	.857	.885	1.000	.766
No 1 (T08)	.792	.652	.708	.745	.766	1.000

Overall Cluster Analysis. Data from all the three rep-PCR fingerprints were pooled together to generate consensus tree. Dendogram generated by combining results obtained from all these primers showed large degree of genetic heterogeneity among the studied *P.multocida* strains (Fig 4 and Table 7). The recent 2008 *P.multocida* strain (T 08) showed 76.6 % similarity with the previously isolated *P.mulocida* strain (T90) and 65.2%,70.8%,74.5% and 79.2% with the *P.multocida* vaccinal strains D:2, A:9, A:8 and A:5; respectively.

Discussion

The epidemiology of fowl cholera outbreaks is complex (Christensen and Bisgaard, 2000). Traditional methods are only of limited use in studies of such outbreaks especially where there is an involvement of multiple strains of *P.multocida.* In the present investigation, isolation of *P.multocida* by standard bacteriological methods from an outbreak in a turkey flock, despite prophylactic vaccination practices with a single dose of 0.5 ml/bird -at the 65th day old ie since one month ago-with the local inactivated fowl cholera vaccine. These results are in agreement with that obtained by (Jonas *et al.,* 2001) as they revealed presence of fowl cholera outbreaks among poultry flocks previously vaccinated with killed fowl cholera vaccines. The isolated T08 strain was compared with an Egyptian *P.multocida* isolate (T90) that was previously isolated from turkey flock during last decade besides 4 vaccinal strain (A:5, A:8, A:9, D:2) on phenotypic and genotypic characterization basis.

Phenotypically, the two *P.multocida* field strains besides the 4 *P.multocida* vaccinal strains had similar cultural and morphological characteristics. Moreover, biochemical and pathogenicity tests have not yet been found to be useful in differentiation between the examined strains. As the two field strains shared similar capsular and somatic antigens (serogroup A:1), we could not differentiate them by the conventional methods . This result agreed with that noticed earlier that serotyping is not a useful means of establishing the relationship between strains of *P multocida* (Blackall *et al.,* 1995). ,

In the current study PCR-based typing techniques such as ERIC-PCR, REP-PCR and BOX- PCR are utilized to differentiate between the two field *P.mulocida* isolates and the 4 vaccinal strains included in the local inactivated oil adjuvenated fowl cholera vaccine, the results of each test revealed presence of significant differences between the recent field isolate T08 , the other field isolate T90 and the other 4 vaccinal strains. Data from all the three rep-PCR fingerprints were pooled together to generate consensus tree. Dendogram generated by combining results obtained from all the 3 PCR based techniques showed large degree of genetic heterogeneity among the studied *P.multocida* strains (Fig. 4 and Table 7). The recent T08 *P.multocida* strain showed 76.6 % similarity with the previously isolated *P.mulocida* T90 strain and 65.2%, 70.8%, 74.5% and 79.2% with the *P.multocida* vaccinal strains D:2, A:9 ,A:8 and A:5 ; respectively indicating the continual mutation of the *P.multocida* strains among the poultry flocks-including the turkey flock under study - under field condition despite the prophylactic vaccination with the local inactivated fowl cholera vaccine .These results proved that the diagnosed fowl cholera outbreak among turkey flock under study was due to infection with a mutant strain of *P.multocida* differed from those included in the vaccine .These results proved the urgent need for frequent and continuous molecular epidemiological investigations of fowl cholera outbreaks among poultry flocks in Egypt with continual isolation ,identification and genotyping of *P.multocida* strains. Then the collected *P.multocida* strains should always compared with the vaccinal strains with edition of the predominant mutant strains to the vaccine. On the other hand further studies should be conducted on the application of the current local inactivated fowl cholera vaccine in turkey flocks, ducks and other poultry species other than chickens to determine the optimal dose and regime of vaccination for each species. The importance of selecting the right molecular typing method, which seems to be very crucial in studying either single or multiple *P.multocida* outbreaks. In such cases, Shivachandra *et al*., (2005) reported that PCR-based typing methods, especially those that are based on repetitive consensus sequences, are found to be highly reproducible and discriminatory in identifying multiple strains and establishing relationship between outbreaks in a given region as well as between regions. ERIC-PCR, REP-PCR and BOX –PCR were found to have a high discriminatory ability (D_/0.89). In addition, Loubinoux *et al.,* 1999; Amonsin *et al.,* (2002) found that these methods are rapid, reproducible, and easy to perform. The present results also

recognized the ability of these tools to provide reproducible results with a satisfactory discriminatory power, as all strains in the current study were differentiated. Overall, the current results signify that PCR-based amplification of repetitive regions of the genome has greater utility for investigating the epidemiology of fowl cholera and conducting outbreak investigations. Moreover, our evaluations also suggests that future investigations of fowl cholera outbreaks could benefit from the use of both PCR-based identification and typing techniques, conducted in parallel with conventional methods of confirmatory diagnosis to study infectious agent in a disease outbreak.

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تعريف وتوصيف جينى لعترة الباستريلا ملتوسيدا المعزولة من قطيع رومى فى مصر فى أوائل عام ٢٠٠٨

باستخدام تقنيات البيولوجيا الجزئية تم الكشف عن وتشخيص وتحديث سلالة الباستريلا ملتوسيدا من قطيع رومي مصاب بكوليرا الطيور وذلك فى محافظة المنوفية بجمهورية مصر العربية أوائل عام ٢٠٠٨ وقد تمت مقارنة هذه السلالة المعزولة علي الأساسين المظهرى والجينى بسلالة باستريلا ملتوسيدا سبق عزلها من قطيع رومى فى العقد الماضى وكذا بأربعة سلالات من الباستريلا ملتوسيدا والتى يتضمنها لقاح الكوليرا المثبط (A:5, A:8 , A:9 &D:2) ، وأوضحت النتائج أن كل السلالات محل الدراسة متشابهة مظهرياً حيث أن كل السلالات كونت مستعمرات غير مسيلة للدم على أجار الدم كما أنها متشابهة فى نتائج الإختبارات البيوكيميائية. من ناحية أخرى وباستخدام إختبارات التوصيف الجينى تبين وجود اختلافات جينية بين الستة سلالات للباستريلا محل الدراسة وبتحليل هذه النتائج وجد أن مدى التطابق بين سلالة الباستريلا ملتوسيدا المعزولة عام ٢٠٠٨ وبين السلالة المعزولة فى العقد السابق ٧٦.٦% كما يتراوح مدى التطابق بينها وبين باقى عترات لقاح الباستريلا الأربعة من ٦٥,٢% الى ٧٩.٢% .وهذه النتائج تسجل حدوث طفرات مستمرة لسلالات الباستريلا ملتوسيدا الحقلية وسط قطعان الدواجن فى مصر مما يؤكد الحاجة الملحة لوجود نقص وبائى مستمر ومتجدد لأوبئة كوليرا الطيور فى قطعان الدواجن المختلفة للكشف عن وجود سلالات جديدة وذلك للتحديث المستمر للقاحات كوليرا الطيور.