Characterization of *Pasteurella multocida* Strains Isolated from Local Flocks in Egypt by Phenotypic and Genotypic Schemes

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Abstract

Pasteurella multocida has gained several mechanisms that developed antibiotic resistance due to the misuse and abuse of antibiotics.

Deep screening of bacterial molecular mechanisms and virulence factors will help find new threats for animal and human diseases. Therefore, this research aims to isolate and identify P. multocida from various affected Egyptian farms of layers and breeders and study phenotypic characterization, antimicrobial sensitivity, and PCR genotypic identification. Finally, the pathogenicity of isolated P. multocida strains was evaluated in SPF chickens. A total of 312 samples, from internal organs, were subjected to bacterial isolation. The isolated P. multocida strains were identified biochemically using API20NE and genetically by P. multocida-specificPCR. The antibacterial effect of some antibiotics was determined on the basis of the Clinical and Laboratory Standards Institute. In the pathogenicity test, the challenge dose contains 2.9x108 CFU/0.5mL via the IM route. The phenotypic and genotypic characterization revealed 47.7%(149 samples) positive samples for P. multocida. Genetic characterization confirmed that 5 out of 8 isolated strains were P. multocida type A by PCR. In the sensitivity test, the isolates were susceptible to ciprofloxacin and levofloxacin and resistant to clindamycin and erythromycin. Highly pathogenic strains inoculated into SPF chickens resulted in 84% (21 chickens from 25) morbidity with severe clinical signs. The results of this study indicate that P. multocida can potentially cause significant losses to flocks of layer and breeder chickens in Egypt due to being a contagious pathogen. The isolated strains were highly pathogenic to SPF chickens.

Keywords: *P. multocida*, Identification, Sensitivity, PCR, Pathogenicity.

Introduction

Fowl cholera (FC) is One of the most significant infectious diseases affecting chicken productivity and results in high mortality and morbidity or chronic illnesses ***(Furian et al., 2016)***. *P. multocida* causes the disease ***((WOAH), 2021),*** and several host species are affected by *P. multocida* subspecies *multocida*, contributing significantly to the development of certain economic diseases ***(Peng et al., 2018)***. Disease conditions include FC in chickens and wild birds, hemorrhagic septicemia in livestock animals, swine atrophic rhinitis, and rabbit hemorrhagic septicemia ***(Nuri et al., 2018)***. In addition, many human infections have been recorded worldwide, although they are rare and frequently associated with animal bites, such as cats and dogs **(*Weber et al., 1984*)**.

The *P. multocida* bacterium is an aerobic, gram-negative coccobacillus characterized by bipolarity, non-spore-forming, pleomorphic, and non-motile ***(Panna et al., 2015)***. The capsule and lipopolysaccharides (LPS) are the two main surface components that form the fundamental typing base of *P. multocida*. It consists of four serogroups: A, B, D, and F, and 16 somatic serotypes. The most common serotypes associated with FC outbreaks in most countries are A:1, A:3, A:3,4, and D **(*Rajeev et al., 2011, Zahoor et al., 2014*)**.

P. multocida LPS plays a crucial role in pathogenicity. Monoclonal antibodies synthesized from a serogroup A strain against LPS were bactericidal, and mice were immunized against homologous challenges ***(Wijewardana and Sutherland, 1990*)**. Acute FC symptoms include anorexia, ruffled feathers, fever, mucus discharge from the mouth, heavy breathing, and watery diarrhea with yellow color initially, then greenish mucus later on ***(Rhoades and Rimler, 1990*)**. Hemorrhagic serosa is one of the most common lesions usually observed, with several small necrotic foci in the liver and spleen, enlargement of the liver and spleen, pneumonia, moderate ascites, and pericardial edema.

Chronic FC occurs in birds infected with low-virulent strains of *P. multocida* or those that survive the acute infection stage and usually develop localized infections. As a result of these infections, the sternal bursa, foot pads, tendon sheaths, wattles, conjunctiva, lungs, air sacs, bone marrow, and meninges are often affected. In addition, bacterial colonization and necrosis are typically present in lesions ***(AZIZ, 2015, Ievy et al., 2013)***. Clinical, pathological, and biochemical identifications of FC are still required ***(Mutters et al., 1985*)**. Additionally, serologic tests were developed to diagnose infections ***(Rimler, 1987)***.

Compared with molecular methods for detecting *P. multocida*, conventional diagnostic techniques are time-consuming and less sensitive; accordingly, they are not always effective **(*Kamp et al., 1996*)**. For the detection of *P. multocida*, molecular techniques (such as PCR) are quicker and more specific ***(Zhangcheng et al., 2018)***. Although initial treatment with antibiotics and vaccinations has been effective, the prevalence of infection has continued to increase **(*Jonas et al., 2001*)**. The objective of this study was to define the phenotypic and genotypic criteria of *P. multocida* isolates from layers and breeder flocks in some governorates of Egypt and to determine their virulence.

Material and methods

1. Samples

A total of 75 chickens from 13 suspected chicken layer , breeder flocks and one broiler flock from Sharkia government (El Salhya El Gadeda, Minya El-Kamh and Abu-Kabir), El-Tal El-Kabeer Ismailia and Alexandria, at a period from December 2019 to December 2021, 312 samples were collected from internal infected organs lung, heart, liver, spleen, ova, ovary, bone marrow, intestine and pancreas of diseased and freshly dead chickens suspected to be suffered from Pasteurellosis (Fowl Cholera)Table (1). The broiler age ranges between 4-8 weeks, and the layer farms age ranges between 17-33 weeks. All samples were kept in ice bags and transferred as soon as possible to the laboratory.

2. Bacteriological isolation

Primarily collected samples were inoculated directly into brain heart broth, blood agar, and MacConkey agar and incubated at aerobic conditions (37°C for 24 hours), then examined for suspected *P. multocida* colonies as described in ***(WOAH, 2018)***.

3. Phenotypic identification of isolated *Pasteurella* spp.

Suspected colonies were examined for their colonial morphology (shape, color, size, arrangement, and elevation) by staining. Different films were prepared from pure cultures of isolated organisms and stained with Gram's stain, then examined microscopically under an oil immersion lens (X100). Using oxidase, catalase, Indole production, nitrate reduction, urease activity, and API® 20NE test biochemical identification was conducted ***(Brink, 2010,MacWilliams, 2012)***

4. Antimicrobial susceptibility profiles of bacterial isolates

The antibiotic sensitivity test protocol of *P. multocida* for selected antibiotics was determined according to **(*Finegold and Martin, 1982, Hudzicki, 2009*),** while the interpretation of the sensitivity test result is described in Table (2) **(*CLSI, 2015*)**.

5. Genotypic characterization and molecular identification by using Polymerase Chain Reaction (PCR).

By using PCR technology, a Molecular Characterization of *P. multocida* isolates from chickens has been performed ***((WOAH), 2021).***

Total DNA was extracted from the broth by using a DNA extraction Kit Patho Gene-spin^{TM} DNA/RNA Extraction Kit (iNtRON Biotechnology) according to Kit instructions. Specific oligonucleotide primer sequences (Table 3) **(*Townsend et al., 2001*)** were used according to the cycling conditions of *P. multocida* PCR (Table 4).

6. Pathogenicity of isolated *P. multocida* strains (chicken inoculation)

Chicken inoculation was used to determine the virulence of isolated strains ***(Panna et al., 2015)***. Twenty-five SPF chickens aged 6-week-old were divided into 5 groups (five chickens/group). Five groups with challenge dose containing $2.93x10^8$ CFU/0.5mL (by McFarland) that injected by I/M route. Inoculated chickens were observed for 5 days for morbidity and mortality. Reisolation of bacteria following challenge was performed according to the procedures suggested by **(*Matsumoto and Helfer, 1977*)**. Dead chickens were sacrificed, collection heart blood from them and take tissue swabs from heart, liver, lungs, and spleen and streaked on blood agar, incubated at 37º C overnight for examination of *P. multocida* organism. The positive cases will be reconfirmed by re-isolation of *P. multocida* by PCR following the standard procedures **(*Matsumoto and Helfer, 1977*)**.

Table 1. *Data of samples collected from local farms*

Type	Age (wks.)	Samples		Initial Isolation	Year
		No.	Type		
Layers	30	12	liver, heart, ovary, ova	Suspected *Pasteurella*	2019
Layers	17	8	liver, heart, bone marrow, ovary	Suspected *Pasteurella*	2020
Layers	4	80	liver, heart, lung, spleen	E. coli	2020
Layers	24	60	Heart, liver, spleen, lung, oviduct, kidney	Suspected *Pasteurella*	2020
Layers	27	12	Heart, liver, lung, spleen, kidney, oviduct	Suspected *Pasteurella*	2020
Layers Ducks	23	20	heart, liver, lung, spleen	Suspected *Pasteurella*	2020
Layers	27	25	heart, liver, spleen, ovary, oviduct	No bacterial growth	2021
Layers	27	25	heart, liver, spleen, ovary, oviduct	Suspected *Pasteurella*	2021
Layers	31	5	liver, heart, lung, intestine, oviduct	*E. coli*	2021
Broiler breeder	6	12	heart, liver, pancreas and kidney	*E. coli* and *Salmonella*	2021
Layers	33	12	heart, liver, spleen, lung, ovary, oviduct	Suspected *Pasteurella*	2021
Layers	30	20	Liver, heart	No bacterial growth	2021
Layers	31	9	Lungs, ova, ovary	*Salmonella* and *E. coli*	2021
Broiler	8	12	Liver, heart, spleen, lung	Suspected *Pasteurella*	2021
Total No.	312				

Table 2. *Interpretation of sensitivity test result* ***(CLSI, 2015)***

Antimicrobial drugs	Code / Disc Content in µg	Diameter of zone of inhibition		
		Resistant	Intermediate sensitivity	Sensitive
Amoxicillin/Clavulanic acid	AMC 30	≤13	14-17	≥18
Amikacin	AN 30	≤14	15-16	≥17
Ampicillin	AM 10	≤13	14-16	≥17
Ciprofloxacin	CIP 5	≤15	16-20	≥21
Colistin	CL 10	≤8	9-10	≥11
Doxycycline	D 30	≤12	13-15	≥16
Gentamicin	GM 120	≤12	13-14	≥15
Kanamycin	K 30	≤13	14-17	≥18
Neomycin	N 30	≤12	13-16	≥17
Norfloxacin	NOR 10	≤12	13-16	≥17
Oxytetracycline	T 10	≤15	16-18	≥19
Penicillin	P 10	-	-	≥25
Trimethoprim/Sulfamethoxazole	SXT	≤10	11-15	≥16
Tetracycline	TE 30	≤14	15-16	≥19
Trimethoprim	TMP 5	≤10	11-15	≥16

Table 3. *The Oligonucleotide primers sequences used in conventional PCR for gene expression*

Serogroup		Gene Name	Sequence (5′-3′)	size (bp)	Position
All	KMT1	*KMT1T7-F* *KMT1SP6-R*	5′ATCCGCTATTTACCCAGT3′ 3′GCTGTAAACGAACTCGCC5′	460	
A	*hyaD-hyaC*	*CAPA-FWD* *CAPA-REV*	5′TGCCAAAATCGCAGTCAG3′ 3′TTGCCATCATTGTCAGTG5′	1,044	8846–8863*a* 9890–9873
B	*bcbD*	*CAPB-FWD* *CAPB-REV*	5′CATTTATCCAAGCTCCACC3′ 3′GCCCGAGAGTTTCAATCC5′	760	13621–13603*b* 12863–12880
D	*dcbF*	*CAPD-FWD* *CAPD-REV*	5′TTACAAAAGAAAGACTAGGAGCCC3′ 3′CATCTACCCACTCAACCATATCAG5′	657	3142–3165*c* 3789–3766
E	*ecbJ*	*CAPE-FWD* *CAPE-REV*	5′TCCGCAGAAAATTATTGACTC3′ 3′GCTTGCTGCTTGATTTTGTC5′	511	4387–4408*d* 4899–4881
F	*fcbD*	*CAPF-FWD* *CAPF-REV*	5′AATCGGAGAACGCAGAAATCAG3′ 3′TTCCGCCGTCAATTACTCTG5′	851	2881–2896*e* 3733–3714

Table 4. *The cycling conditions used during PCR*

Stage	Temperature	Time	Cycles
Primary denaturation	95° C	2 min	1
Denaturation	95° C	30 sec	40
Annealing	56° C	45 sec	
Extension	72° C	1 min	
Final extension	72 ° C	10 min	1

Results and Discussion

P. multocida is a Gram-negative pathogenic bacterium that can infect poultry and other farm animals, as well as humans **(*Wilkie et al., 2012*)**. *P. multocida* causes fowl cholera in chickens, a bacterial disease that affects both domestic and wild birds and causes severe financial losses ***(Pedersen et al., 2003, Biswas et al., 2005, Chrzastek et al., 2012, Singh et al., 2014)***. *P. multocida* infection affects hens of all ages; however, death losses in laying flocks are more common **(*Rhoades et al., 1992*)**. FC is diagnosed by isolating suspicious birds that have clinical symptoms and lesions consistent with the disease and identifying the causal agent ***(El-Ghany et al., 2018)***. In this *study* a total of 312 chicken organs that were represented as heart blood, lung, liver, spleen, oviduct, and ovary were examined. From 312 tested samples 139 samples (44.5%) were positive for *P. multocida*, while 166 samples (53.2%) were negative for by cultural examination and biochemical characters (Table 5). Identification is based primarily on the results of API20NE and biochemical tests. Isolates fermented sucrose, glucose, fructose, and mannose; maltose is not fermented, and no liquefaction of gelatin. Triple sugar iron produced acid/acid and indole ring. It was catalase and oxidase positive. On blood agar, there was no hemolysis. Although, there was no reaction on methyl red and citrate test as in (Table 6). These results showed a strong correlation with **(Lukert and Saif, 1997, Shivachandra et al., 2005, Kiran et al., 2012),** where a Giemsa stain examination of blood agar cultured samples revealed tiny glistening non-hemolytic mucoid dew drop-like colonies containing Gram-negative coccobacilli. All strains were biochemically identified as *Pasteurella* spp based on indole production, nitrate reduction, positive catalase and oxidase tests, and glucose fermentation (glucose, fructose, galactose, mannitol, and sucrose). In contrast, citrate, methyl red, Voges/ Proskauer, and gelatin liquefaction tests were negative.

Microscopically, isolates showed the typical bipolarity of the *P. multocida* ***(Barrow and Feltham, 2009, Cheesbrough, 1981*)** After confirmation with PCR, Five out of eight flocks of layers and breeders of chickens tested were positive for *P. multocida* (62.5%). The *P. multocida* prevalence in different flocks is illustrated in (Table 1) and (Figure 1). These results were higher than the value reported in previous studies, where *P. multocida* was reported from chicken with a prevalence of 21 out of 300 (7%) **(*Abdelmalek, 2020*)** and 21 out of 275 chicken samples tested (7.6%) **(*Mohamed and Ahmed, 2012*)**. The differences in results could be

attributable to age and breed differences in the chickens, as well as the commercial chicken's increased resistance power as a result of better management, vaccination, and nutrition.

The sensitivity test results of isolated *P. multocida* from chickens revealed that all isolates were sensitive to ciprofloxacin, levofloxacin, and tetracycline. Meanwhile, all strains were intermediate to colistin sulfate and resistant to clindamycin and erythromycin (Table 7). These results agree with previous reports, which indicated that ciprofloxacin and levofloxacin were the most active drugs against *P. multocida* compared to clindamycin and erythromycin **(*Yoshimura et al., 2001*)**.

However, different sensitivity profiles of *P. multocida* were reported, including susceptibility to erythromycin **(*Furian et al., 2016)*** and high levels of resistance to 7 types of antimicrobial drugs (kanamycin, neomycin, oxytetracycline, tetracycline, tobramycin, doxycycline, and gentamicin **(*Woo and Kim, 2006*).** The use of antibiotic additives in animal feed and the widespread use of antimicrobial drugs in veterinary care contribute to the rise of multidrug resistance ***(Tang et al., 2009*)**.

In this work, PCR proved to be an ideal approach for detecting *P. multocida* in a timely, sensitive, and accurate manner. Five out of eight tested isolates from layer and breeder chicken flocks identified by culture characters and biochemical testing as *P. multocida* using species-specific KMT1SP6 and KMT1T7 PCR primers exhibited amplification of 460bp. The PCR with CAPA-FWD primers identified 5 *P. multocida* Type A (type-specific), showing amplification of 1044bp PCR product (Figure 2) ***(Jabbari et al., 2002, Karthik et al., 2018, El-Ghany et al., 2018*)**.

Chickens were experimentally challenged with *P. multocida* isolates (challenge dose containing $2.9x10^8$ CFU/0.5 mL) to determine their pathogenicity. FC acute clinical signs were observed after 5 days of challenge 100% morbidity in two isolates (Table 8), including greenish diarrhea, increasing respiratory rate, off food, emaciation, ruffled feather, and depression. Up on necropsy, septicemia was observed especially in the heart, liver, lungs, and spleen. Clinical signs and mortality were previously reported as early as 24 hrs after challenge with *P. multocida* serotype (A) due to, and lesions consisted of septicemic hemorrhagic liver, heart, and spleen lesions with necrotic foci ***(Panna et al., 2015)***. While only two from thirteen isolates (Bali&BP6) were pathogenic to challenged birds (3 birds dead from 3) meaning 100% mortality but after 10 days of injection ***(Mariana and Hirst, 2000)*** In conclusion, the results of this study further confirm the spread of *P. multocida* in flocks of layer and

breeder chickens in Egypt and potentially cause significant losses due to its contagious nature. Additionally, it was determined that the isolated strains were highly pathogenic to SPF chickens.

In conclusion, the experiment was undertaken to identify and characterize *P. multocida* that isolated from poultry by phenotypic and genotypic approaches and determine its pathogenicity for detection the virulence strains. Various bacteriological techniques, sensitivity test and PCR were used to identify and describe the bacterium.

Table 5. *Percentage of isolated P. multocida from collected samples in cultural examination and biochemical tests.*

Type of samples	Total No.	No. of positive samples	No. of negative samples	% Of positive samples	% Of negative samples
Heart blood	71	29	45	41%	63%
Lungs	46	19	27	44%	63%
Liver	71	29	42	43%	62%
bone marrow	2	2	0	100%	0%
Spleen	52	24	28	49%	57%
Ova	6	3	3	50%	50%
Ovary	20	12	8	60%	40%
Oviduct	25	19	6	76%	24%
Kidneys	15	12	3	80%	20%
Pancreas	3	0	3	0%	100%
Intestine	1	0	1	0%	100%
Total	312	149	166	47.7%	53.2%

Table 6. *Biochemical results of P. multocida isolates*

Test	Indole	Oxidase	Catalase	Gelatin liquefaction	Nitrate reduction	Urease	Citrate	H2S	Triple sugar iron
Result	+	+	+	-	+	-	-	-	Acid / Acid
Test	Voges Proskauer	Glucose	Sucrose	Maltose	Galactose	Mannose	Fructose	Lactose	
Result	-	+	+	+	+	+	+	-	

.

Table 7. *sensitivity testing results of Pasteurella isolates for selected antibiotics.*

Antimicrobial drugs	Code / Disc	Diameter of inhibition zone with mm				
	content in µg	**PA1**	**PA2**	**PA3**	**PA4**	**PA5**
Amoxicillin + Clavulanic acid	AMC 30	(s)	(s)	(s)	(s)	(R)
Erythromycin	E 15	(R)	(R)	(R)	(R)	(R)
Gentamicin	CN 10	(s)	(s)	(s)	(I)	(s)
Amikacin	AK 30	(I)	(s)	(s)	(I)	(s)
Ceftazidime	CAZ	(R)	(I)	(I)	(s)	(R)
Clindamycin	DA 2	(R)	(R)	(R)	(R)	(R)
Cefotaxime	CTX	(s)	(I)	(s)	(I)	(I)
Ampicillin	AMP10	(R)	(R)	(I)	(R)	(I)
Ciprofloxacin	CIP 5	(s)	(s)	(s)	(s)	(s)
Colistin sulphate	CT 10	(I)	(I)	(I)	(I)	(I)
Neomycin	N 30	(I)	(I)	(I)	(I)	(I)
Penicillin	P 10	(s)	(I)	(s)	(R)	(s)
Trimethoprim+ Sulfamethoxazole	SXT 25	(s)	(I)	(s)	(I)	(s)
Tetracycline	TE 30	(s)	(s)	(s)	(s)	(s)
Levofloxacin	LEV 5	(s)	(s)	(s)	(s)	(s)

Table 8. *Pathogenicity test results*

Days post infection	**Morbidity***				
	PA1	PA2	PA3	PA4	PA5
1	2/5	1/5	0/5	0/5	0/5
2	3/5	1/5	0/5	0/5	0/5
3	3/5	3/5	2/5	0/5	1/5
4	4/5	4/5	2/5	3/5	2/5
5	5/5	5/5	3/5	3/5	2/5
6	5/5	5/5	3/5	4/5	3/5

7	5/5	5/5	4/5	4/5	3/5

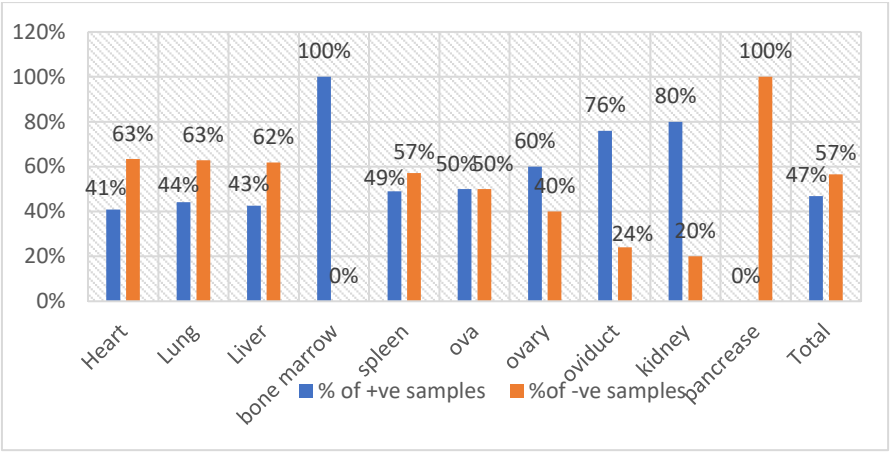

Figure 1. Percentage of isolated P. multocida from collected samples in cultural examination and biochemical tests.

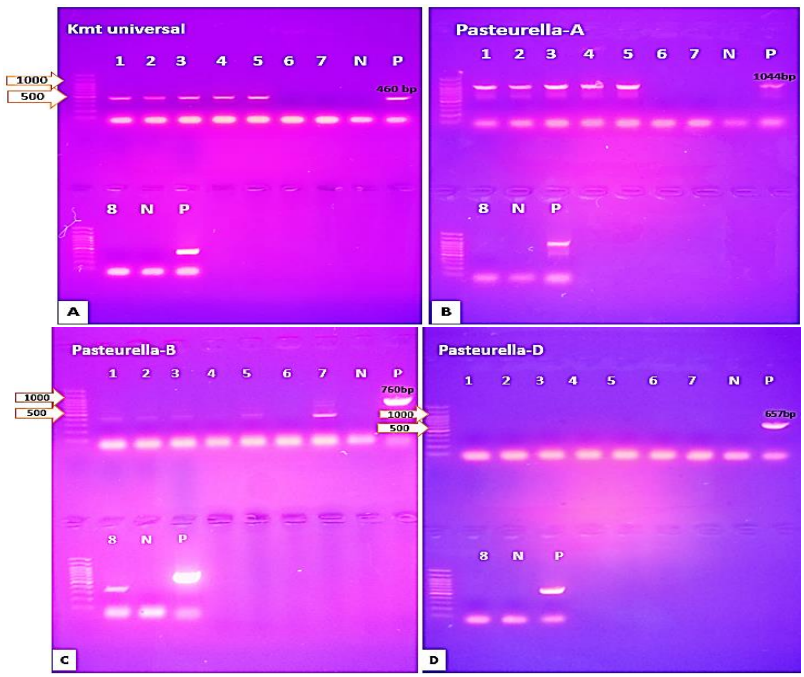

Figure 2. ***Agarose gel electrophoresis for PCR,*** results representing amplification of 460bp for general *P. multocida* (A), and 1044bp for *P. multocida* serotype A for 8 isolated samples (B), while no bands at 760bp for

P. multocida serotype B as shown in (C), and no bands at 657bp for *P. multocida* serotype D as shown in (D).
Note:(P) in the figure referred to control positive and (N) referred to control negative.

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الملخص العربى

تعتبر كوليرا الطيور (FC) ، التي يسببها ميكروب الباستريللا مالتوسيدا، مرضًا معديًا لأنواع الطيور الداجنة والبرية التي تحدث في جميع أنحاء العالم ، لذلك تم إجراء هذه الدراسة لعزل وتحديد الباستريللا مالتوسيدا من قطعان الدجاج المصري المختلف من تسمين وبياض المصابة في الفترة من نوفمبر 2019 إلى نوفمبر 2021 ثم تم دراسة الخصائص المظهرية والوراثية للعامل الميكروبي وتم تحضير لقاح ميت من المعزولات المحلية محمل بالزيت و تحديد سلامته وفعاليته وفحص الأنسجة المرضية للأعضاء بعد اختبارالتحدي .

تم جمع 312 عينة دجاج من دجاج يعاني من اعراض مشتبه بها باستريللا مالتوسيدا .ثم تم الحصول على عينات من أعضاء محددة للاصابة بالمرض وغير محددة مثل القلب (عدد= 71) ، الرئة (عدد= 46) ، الكبد (عدد = 71) ، الطحال (عدد = 52) ، الكلى (عدد = 15) ، المبيض (عدد = 20) ، بويضة (عدد = 6) قناة البيض (عدد=25) ، البنكرياس (عدد=3) ، نخاع العظام (عدد=2) والأمعاء (عدد=1). وبعد ذلك تم تحديد جميع العينات بواسطة خصائص المستعمره البكتيرية على وسط أجار الدم والمظهر المجهري والاختبارات البيوكيميائية باستخدام API20NE. ثم استخلص الحمض النووي للتأكيد والتنميط المصلي للسلالات المعزولة بواسطة تفاعل البلمرة المتسلسل باستخدام بادئات متخصصة باستخدام تضاعف نسخ الماده الوراثية عند 460 قاعدة نيتروجينيه لتحديد الباستريللا مالتوسيدا عامة وعند 1044 قاعدة نيتروجينية للنمط المصلي A.

وفقا لنتيجة اختبار حساسية الدواء المذكورة في الجدول رقم (10) وجد أن الباستريلا مالتوسيدا حساسة للسيبروفلوكساسين والليفوفلوكساسين والتتراسيكلين بينما كانت متوسطة لكبريتات الكوليستين ولكنها مقاومة للكليندامايسين والإريثروميسين. أيضًا ، في اختبار ضراوة البكتريا وحدتها ، ظهرت علامات مرضية حادة على الطيور بعد 5 أيام من التحدي في PA1 و PA2 أكثر من المجموعات المتحداه الأخرى. تم تحضير لقاح كوليرا الطيور عن طريق إكثارالمستضد البكتيري للحصول على عدد 1.5 × 10^9 cfu/ مل لكل سلالة. ثم تثبيطه باستخدام الفورمالديهايد 0.25٪ مُحمل على Montanide ISA70 بنسبة 30:70 زيت / بكتيريا على التوالي.

تم استخدام 12 دجاجة خالية من اي ميكروبات او تحصينات لاختبار سلامة اللقاح الذي تم حقنه بـ 0.5 ملل (2.93 * $CFU10^8$) لكل جرعة تحت الجلد. بينما استخدمت 70 دجاجة في اختبار الفعالية عن طريق حقن 0.5 مل (2.93 × $CFU10^8$) تحت الجلد. تم جمع مصل الدم لتحديد استجابتها المناعية السائدة عند اليوم 7،14،21،28 و 35 بعد التطعيم عن طريق اختبار المناعة الدقيق.وتم الفحص التشريحي المرضي للدجاج المصاب بواسطة صبغة E&H

أظهرت خصائص المستعمرة البكتيرية والنتائج البيوكيميائية أن 47.7٪ من العينات المعزولة كانت موجبة لبكتريا الباستريللا مالتوسيدا بينما 53.2٪ كانت سلبية لوجود الباستريللا مالتوسيدا. جميع العينات الموجبة اظهرت ان الباستريللا مالتوسيدا جرثومة سالبة لصبغة الجرام تعطي خاصية ثنائية القطبية عصوية الشكل وتتميز بتركيب الصبغة عند طرفيه غير متحرك، مستدير ، وينمو جيدا بشكل مخاطي مائل للبياض على وسط

أجار الدم ولا يوجد نمو على أجار ماكونكي. ينتج حمض من الدكستروز والسكروز والمانيتول ولكن لا يتم تخميره من المالتوز أو اللاكتوز. لذلك تم التأكد ان خمسة من أصل ثمانية معزولات بنسبة عزل (62.5٪) مستعمرات معزولة صنفت علي انها بكتريا الباستريللا مالتوسيدا من النوع المصلي A.

في اختبارضراوة البكتريا ، كانت السلالات الأكثر فعالية هي PA1 و PA2 حيث نفقت جميع الطيور في اليوم الخامس من الحقن بينما كان اللقاح آمنًا حيث لم يكن هناك نفوق أو التهاب في موقع الحقن بجرعة وحيدة أو متكررة. من ناحية أخرى ، تم وقاية الدجاج الملقح من التحدي بسلالة الباستريللا مالتوسيدا PA: 1 و PA: 2 حيث كانت معدلات الحماية 70٪ و 100٪ على التوالي بينما كانت 10٪ و 0٪ في الطيور الغير الملقحة على التوالي.

الفحص النسيجي لأنسجة القلب للدجاج المحصن بدت طبيعية حيث بدى غشاء التامور طبيعي وعضلات القلب والأوعية القلبية وكذلك تجمع الخلايا الليمفاوية. مجموعة الأنسجة الكبدية ظهرت مع خلايا الكبد والأوعية الكبدية وكذلك تجمعات الخلايا الليمفاوية بين الصفائح الكبدية. بينما تظهر أنسجة الرئة تنكس الشعيرات الدموية واحتقان الأوعية الرئوية وتكاثر النسيج الضام الليفي بين الفصيصات وحول الأوعية الرئوية وكذلك التجمع الهائل من قبل الخلايا المناعية والخلايا الليمفاوية. و أظهرت أنسجة الطحال بنية طبيعية حيث ظهر اللب الأبيض واللب الأحمر بشكل طبيعي مع عقد متطورة ومحتقنة بالخلايا الليمفاوية.

بينما في اختبار التحدي علي الطيور الغيرمحصنة ظهراحتقانًا شديدًا في الأوعية القلبية المحاطة بالوذمة والنخر التخثرى لعضلات القلب بالإضافة إلى تجمع هائل من الخلايا المناعية وكريات الدم البيضاء. وتراكم الإفرازات الليفية في الفراغ تحت النخاب وبين خلايا عضلة القلب. بينما تظهر أنسجة الكبد احتقان شديد في الأوردة المركزية ، ونخر تخثرى لخلايا الكبد مكونة بؤر نخرية وكذلك تجمع هائل من الخلايا المناعية والخلايا الليمفاوية. إلى جانب أنسجة الرئة تظهر تنكس الشعيرات الدموية واحتقان الأوعية الرئوية وتكاثر النسيج الضام الليفي بين الفصيصات وحول الأوعية الرئوية وكذلك التجمع الهائل من الخلايا المناعية والخلايا الليمفاوية. أخيرًا ، اظهرت أنسجة الكلي لب أبيض ولب أحمر يعاني من ضمور شديد للخلايا الليمفاوية بالإضافة إلى تسلل هائل من قبل الخلايا المناعية.

وفي الختام ، أجريت التجربة لعزل الباستريللا مالتوسيدا من الطيور الداجنة ، وتحديد ضراوتها، وتحضير اللقاح وتحديد فعاليته ، والفحص التشريحي للطيور النافقة المصابة حيث تم استخدام تقنيات بكتريولوجية مختلفة واختبار الحساسية و اختبار البلمرة لتحديد ووصف البكتيريا المعزوله، ثم تم تحديد مستويات المناعة المستحثة في كل مجموعة من الطيور المحصنة باستخدام اختبارقياس المناعة الدقيق لقياس نسبة الأجسام المضادة في الدم. كما تم تحديد الفعالية الوقائية للقاح كوليرا الطيور باستخدام تجربة التحدي لتقييم معدل بقاء الطيور المحصنة ومدى فعالية اللقاح.

أثبتت الدراسة أن لقاح كوليرا الطيور المحضر تجريبياً آمن وفعال ويوفر 90٪ حماية من تحصين الدجاج ضد كوليرا الطيور.