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PHENOTYPIC, GENOTYPIC, MULTIDRUG RESISTANCE GENES AND DISINFECTANT BIOCIDAL EFFECT OF *PASTEURELLA MULTOCIDA* ISOLATED FROM CHICKENS

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ABSTRACT

Pasteurella multocida (*P. multocida)* type A is the etiologic agent of fowl cholera, a highly contagious and fatal disease of chickens. In this study 300 birds were examined for isolation of *P. multocida*. Isolation of P. *multocida* was attempted from the spleen, lung, trachea and bone marrow collected from chickens. The targeted bacteria from the samples were isolated, identified and characterized based on their morphology, Gram staining, cultural & biochemical characters and confirmed by pathogenicity test and Polymerase Chain Reaction (PCR). Among the examined chicken, 12 (4 %) *P. multocida* were isolated and identified (n= 12/300). The organisms were gram negative, non-spore forming rod, nonmotile, occurring singly or pairs by Gram staining, whereas in Leishman's stain, bipolarity were observed. All the isolates were positive for oxidase and catalase tests, produced indole. In type specific PCR reaction, the organisms were confirmed as *P. multocida* type A. Disinfectant suspension test used to determine the efficacy of three disinfectants on the isolated strain, only gluteraldehyde- QAC combination product could achieve standard log. reduction of *P.multocida* after 15 minutes.

Key word: *Pasteurella multocida*, Multidrug resistance genes, Chickens, Disinfectant

INTRODUCTION

Fowl cholera is contagious and economically important disease of poultry (chicken, ducks and geese). It is caused by Gram-negative rods *P. multocida* which inhabit the upper respiratory tract of many avian species as commensals and cause a severe disease (Rhoades & Rimler, 1989 and Rimler & Glisson, 1997). It usually occurs as primary disease that requires predisposing, factors but the factors may increase the severity of the disease (Aye *et al*., 2001). It may be occurs either as per-acute, acute or chronic forms, and the clinical signs vary depending on the form of the disease. Symptoms include depression, ruffled feathers, fever, and anorexia, mucous discharge from the mouth, diarrhea and an increased respiratory rate (Rhoades & Rimler, 1989). Carrier birds play a major role in the transmission of fowl cholera (Christensen & Bisgaard, 2000). Also, research shows that transmission can occur by bird-to-bird contact via aerosolized bacteria as well as through ingestion of bacteria in contaminated environments (Bodenstein *et al.*, 2015).

Five capsular serotypes(A, B, D, E, and F) are usually found in *P. multocida* and each is generally associated with a specific host, for example, Serotype A causes FC in avian species(Harper *et al.*, 2006), serogroup A strains cause the majority of fowl cholera cases (Marza *et al*., 2015). Virulence of P. multocida varies depending on the strain involved and factors host species (Glisson *et al*., 2008). The FC is mostly prevalent in winter and late summer (Heddleston and Rhoades, 1978). Laying flocks are mostly affected by FC because of their more susceptibility to the disease as compared with younger chickens (Choudhury *et al.*, 1985; Wang *et al.*, 2009).

The disease usually occurs as septicemia with high morbidity and mortality rates or chronic localized indication of the joints and sinuses (Rimler and Glisson, 1997). The infected birds remain carrier up to 9weeks after infection. It can affect birds at any age, but occurs rarely in commercial poultry of less than 8weeks of age (Rimler *et al*., 1998).

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The diagnosis of Pasteurellosis by conventional methods is not reliable and time consuming (Berge *et al.*, 2006; Bell, 2008; Rajeev *et al*., 2006). Therefore, the use of molecular techniques, specially the polymerase chain reaction (PCR) for molecular detection and characterization of the capsular antigens of the *Pasteurella* is very important for rapid and specific detection and characterization of the organism which play an important role in the control of the disease among the farms and reducing the economic losses (Rajeev *et al.*, 2006).

Indiscriminate use of anti-biotic resulted in the emergence of multidrug resistant strains of Pasteurella (Rimler &Glisson, 1997). Fowl cholera is one of important bacterial diseases representing a major threat to the poultry industry (OIE, 2008). Effective cleaning and disinfection of poultry facilities is an important step to reduce microbial loads in the poultry farm environment, equipment and surfaces to break disease cycles. *P. multocida* is an example of pathogenic environmental bacteria that can survive for long periods under various conditions in the environment and act as a source of infection. (Chima *et al*., 2011). Many disinfectant classes are present of various modes of action but unfortunately, disinfectants may fail to achieve the purpose for which they are used (Onah, 2004). So, disinfectant choice must depend on data about efficacy against the target pathogen and the conditions under which they will be used in the field, such as contact time, organic matter and water hardness (Thomson *et al.*, 2007). Disinfectant efficacy is often tested against laboratory bacterial suspensions to detect their efficacy against specific pathogens in presence of interfering substances like organic matter and hard water (Bloomfield *et al.*, 1991).

Therefore, the present study aims were; isolation, identification, detection of antimicrobial sensitivity of *P.multocida* isolated from poultry and evaluating the efficacy of some disinfectants commonly used for poultry farm disinfection against the field isolated *P. multocida* under conditions simulating field application.

MATERIALS AND METHODS

A total of 300 chickens were collected as dead, affected and apparently healthy from different farms in Dakahlia province and examined for presence of *Pasteurella multocida*. Samples were immediately transferred to the laboratory in sterile plastic bags.

Isolation and identification of *Pasteurella multocida*:

Samples (heart blood & tissue pieces from spleen, liver, lung and bone marrow) were inoculated onto blood agar supplemented with 7% sheep blood. The plates were incubated for 24-48 hrs. at 37 °C. The agar plates were checked every day for suspected colonies. Identification or confirmation of isolated bacterial species was assessed by observation of the colonial morphology, Gram staining and biochemical tests which included catalase, nitrate reduction, H_2S production in triple sugar iron (TSI), growth on MacConky`s agar, indol production, urease activity, methyl red production, Voges Proskauer test reaction, oxidase reaction, coagulase, motility, citrate, carbohydrate fermentation from glucose, trehalose, xylose, arabinose, fructose, galactose, maltose, mannose, sucrose, lactose, dulcitol, inocitol, salicin (Quinn *et al*., 1994). Heart blood smears and tissue impression smears were prepared and subjected to Leishman's staining.

Pathogenicity test:

The pure cultures of *P.multocida* isolates in the present study were subjected to pathogenicity studies. One hundred microliter of broth culture were injected intraperitoneal in mice (16 -20g weight) and observed for 48 hrs. dead one were subjected to P.M. Examination and reisolution of *P.multocida* by streaking onto blood agar and incubated at 37º C for 24hrs impression smears from heart blood, liver, spleen and lung from dead mice were stained with Leishmans stain and examined for bipolarity. Each isolate was frozen at -80 °C in a nutrient broth with 10% of glycerol for further analysis.

***P.multocida* species specific PCR (PM-PCR):**

The PCR of *P.multocida* (PM-PCR) analysis was performed according to Townsend *et al.* (1998) and the instructor manual provided with kits (Qiagen) with some modification. A PCR reaction mixture (20µ total volume) was prepared as follows: 3µ nuclease free water, 10µ Hot Start Taq plus master mix (2 xs), 2 µ Coral Load concentrate (10 xs optional), 1µ forward species specific primer KMT1T7 (5′-ATCCGCTATTTACCCAGTGG-3′), 1µ reverse primer KMT1SP6 (5′-GCTGTAAACGAACTCGCCAC-3′) and 3µ Extracted DNA. The PCR reaction mixture and the thermal cycle protocol were as follows. Initial denaturation at 94˚C for 5 min, followed by 30 cycles, each cycle consisting of 3 steps- denaturation at 95˚C for 1 min, annealing at 55˚C for 1 min, Extension at 72˚C for 1 min. Final Extension was carried out at 72˚C for 9 min. Then, the PCR products were visualized by agarose gel electrophoresis.

Capsular PCR typing:

The *P.multocida* capsular serogroup specific primers designed by Townsend *et al.* (2001) were used for capsular PCR typing. The serogroup specific primers hya D and hya C were used to amplify capsule biosynthetic loci of serogroup "A" The thermal cycle protocol was as follows. Initial denaturation at 95°C for 5 min, followed by 30 cycles, each cycle consisting of 3 steps- denaturation at 95ºC for 30 sec, annealing at 49ºC for 30 sec, Extension at 72ºC for 80

sec. Final Extension was carried out at 72˚C for 5 min (Sambrook *et al*., 1989)

Antibiotic sensitivity:

The antibiotic sensitivity test of the isolated *P.multocida* in vitro was conducted on Muller-Hinton agar plates according to (Bauer *et al*., 1966) using 15 antibiotic discs (Ciprofloxacin, Enrofloxacin, Ofloxacin, Oxytetracycline, Doxycycline, Trimethoprim, Ampicillin, Penicillin, Chloramphenicol, Gentamycin, Amikain, Erythromycin, Triple sulpha and Polymyxin as supplied by M/s. Hi-Media Laboratory, and the antibiotic sensitivity plates were incubated at 37^{o}c for 24-48 h.

Disinfectant suspension test:

Test disinfectants:

Three commercial disinfectants were selected for determination of their efficacy against the isolated *P. multocida* for 5- and 15-minutes contact times. Disinfectants were diluted according to manufacturer recommended concentrations. All dilutions were freshly prepared on the day of the test, using sterilized standardized 400 ppm hard water.

Table 1: Disinfectants used in quantitative suspension test.

Disinfectant	Used concentration	Composition	Manufacture
Virukill®	0.5%	Potassium peroxymonosulfate 50%, NaCL 3%	UBM (Egypt)
Pi Quat 20®	1:200	20% dual chain quaternary ammonium compound	Neogen (USA /Canada)
Synergize®	1:256	Alkyl dimethyl benzyl ammonium chloride 26% and Gluteraldehyde 7%	Neogen (USA /Canada)

Laboratory suspension test according to PrEN1276 CEN (2004) was used with some modifications to evaluate the efficacy of test disinfectants on the isolated *P.multocida* strain. In brief; 1ml of the bacterial suspension adjusted to approximately 10^8 cfu. /ml using McFarland standard was added to 1ml of yeast extract 5% sterile solution as a source of organic matter. The mixture was left for 2 min. after that 8 ml of the diluted test disinfectant added and the mixture maintained for 5 and 15 minutes. After the previously given contact times 1 ml of the mixture was received into a tube containing 8 ml of the neutralizing solution (polysorbate 80 (30 g/L, lecithin 3g/L, sodium thiosulphate 5g/L, L-histidine 1g/L and saponin 30g/L) dissolved in Tryptone Soya Broth and 1 ml of water to stop the disinfectant activity. Tubes then were mixed and left for neutralization for 5minutes. Following neutralization, viable bacterial counts were determined by spread plating of serially diluted samples in duplicates with plates incubated at 37 °C for 24 hours. The test compound was effective against the organism at the stated dilution if it achieved five log reductions in viable counts.

RESULTS

The collected samples revealed positive isolation of 12 strains (4%) of *P. multocida* isolated from lung, spleen and trachea of birds as shown in tables. No growth was observed on Mac Conky agar, Gram staining show gram negative coccobacillus and on Leishman`s revealed characteristic bipolar organism suggestive of *P.multocida*.

Table 2: Occurrence of *P. multocida* in birds.

Status of birds		No. of samples	Occurrence of *P.multocida*	%
Diseased	Layers (3-12month	**150**	**9**	**6**
	Broiler (6-8 week)	**50**	**1**	**2**
Apparently healthy	Layers (3-12month)	**70**	**2**	**2.8**
	Broiler (6-8 week)	**30**	**--**	**0**
	Total	**300**	**12**	**4**

Table 3: Occurrence of *P. multocida* in diseased and apparently healthy chickens.

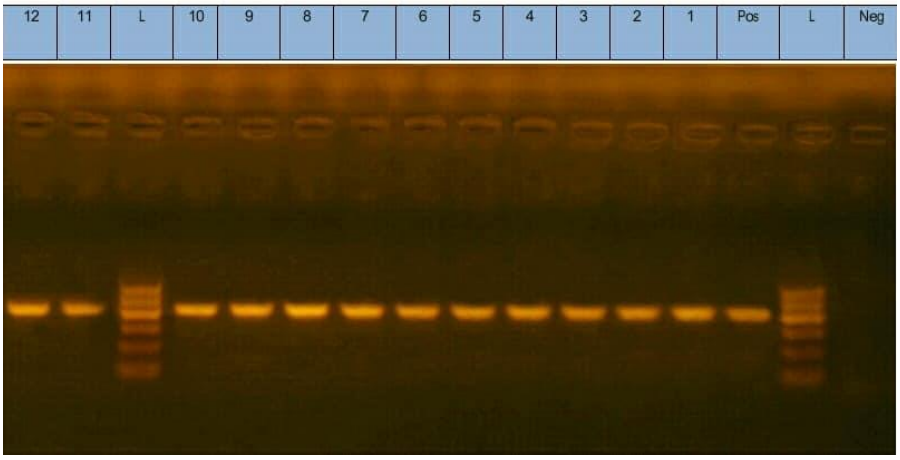
Tissue	Number of *P. multocida*			
	Diseased		**Apparently healthy**	
	broiler	**layer**	**broiler**	**Layer**
Bone marrow		**2**	_	_
Lung	**1**	**2**	_	**1**
Trachea	**2**	**2**	_	_
Spleen		**1**	_	**1**
Total	**10**		**2**	

Table 4: Seasonal variation of *P. multocida* in tissues of diseased and apparently healthy chickens.

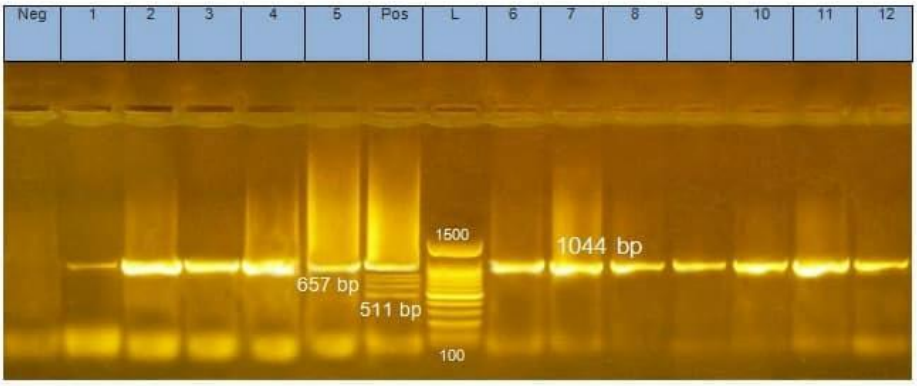
Season	Diseased	Apparently healthy
Autumn	**2**	_
Summer	**1**	_
Winter	**5**	**2**
Spring	**2**	_
Total	**10**	**2**

All isolates subjected to mice inoculation test killed the mice within 24 to 48 h. post inoculation. On Leishman's staining, Heart blood smears, tissue impression smears prepared from liver, spleen and lung revealed characteristic bipolar organisms suggestive of *P. multocida*.

PCR has been proved to be useful in the detection of DNA of *P.multocida*. *P.multocida* species specific PCR (PM-PCR) was used in this study to identify *P.multocida* isolates. The molecular weight of the PCR products of all isolates were found to be 460bp specific for *P.multocida*.



Agarose gel electrophoresis showing amplification of 460 bp fragments specific for *P. multocida* field isolates.



Agarose gel electrophoresis showing amplification of 1044 bp fragments specific to the capsular antigen type A**.**

Results of phenotypic antibiotic susceptibility testing

P.multocida were multidrug resistant in which antibiotic sensitivity tests revealed that all the isolates were resistant to gentamycin, ampicillin, erythromycin, penicillin, tobramycin, chloramphenicol, doxycycline, cephotaxime, colistin (100%) followed by apramycin and ciprofloxacin (83.33%) then, tetracycline, amoxicillin, clavulenic acid (66.7%) vancomycin and neomycin (50%) and ofloxacin (16.6%).

Results of disinfectant quantitative suspension test:

Table 5: log reductions of *P.multocida* disinfectant suspension test after 5 and 15 min.

Disinfectant	Log reduction	
	5 minutes	15 minutes
Virukill®	1.9	3.1
Pi Quat 20®	2.2	4.4
Synergize®	3.8	7.5

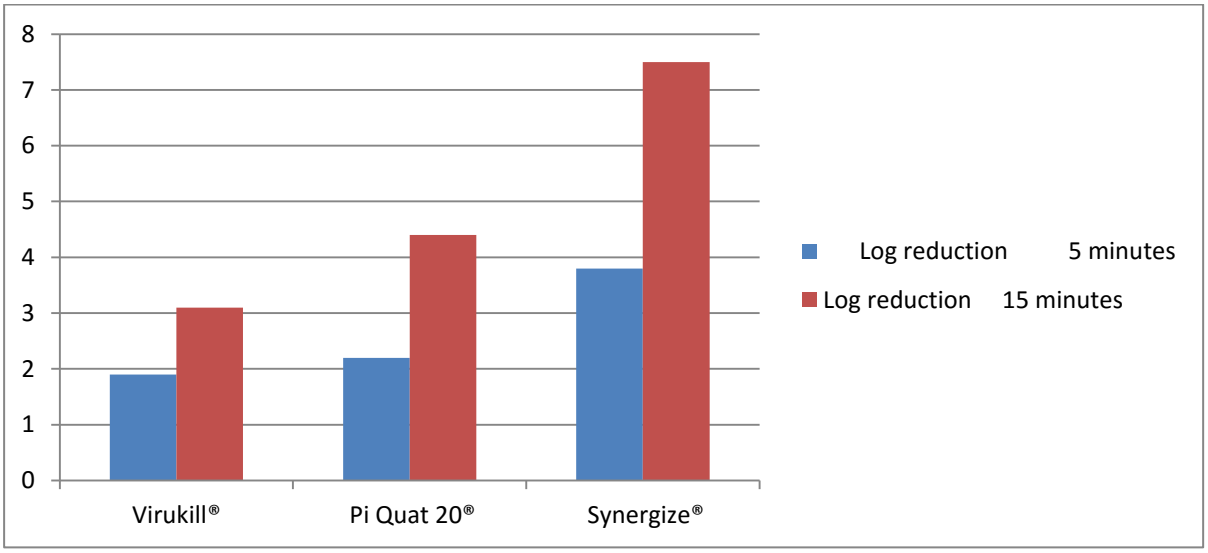


Fig. (1): Reductions of disinfectant suspension test after 5 and 15 minutes.

DISCUSSION

In the present investigation, as shown in tables, 12 isolates of *P.multocida* were isolated out of a total 300 samples with an isolation rate 4 %. All the isolates showed typical cultural characteristics of dew drop, mucoid, non-hemolytic on blood agar, Gram negative coccobacillary by gram staining and Leishman`s stain revealed characteristic bipolarity organism. These findings are in accordance with Quinn (1994) and *OIE* (2004). The isolates subjected to biochemical tests were positive for indol production ,nitrate reduction, oxidase and catalase production these findings were in agreement with several other studies (Shivachandra *et al.*, 2005; Ashraf *et al.*, 2011; Manasa, 2012; Ievy *et al.*, 2013; Akhtar, 2013). These results agree with those obtained by Kwage *et al.* (2013) who found that out of 512 poultry were cultured at necropsy for the organism from the liver, spleen, lungs and heart blood only 6 (1.2%) were positive for *P. multocida*. Also Mohamad *et al.* (2012) who revealed that a total of 21 isolates *P.multocida* were recovered in 21 out of 275 backyard chickens from different regions of Upper Egypt tested (7.6%) and were confirmed using phenotypic characterization.

The prevalence of *P.multocida* (4 %; n=12/300) in this study was lower than the value reported in the earlier studies Hassan *et al.* (2010) reported 12.05% prevalence in layer chicken and 4.25% in broiler chicken, whereas Hossain *et al.* (2013) found 13.04% prevalence in chicken and Belal (2013) found 59.72% in backyard poultry. All the isolates subjected to mice inoculation tests killed the mice in 24 - 48h. These results are in agreement with the findings of Kumar (1998) and Balakrishnan and Mini (2001).

PCR technology can be applied for rapid, sensitive and specific detection of *P. multocida* Townsend *et al*. (1998) and *OIE* (2008). The 12 isolates showed corresponding amplicons of 460 base pairs which is specific for *P. multocida* this result was in agreement with those reported by Townsend *et al*., 2001, Kumar *et al.,* 2009, Ranjan *et al*., 2011, Manasa, 2012, and Akhtar, 2013). It was noticed that there was homogeneity in the distinct banding patterns of all the 12 *P. multocida* isolates that were amplified.

The findings of this work showed that all the 12 isolates of *P. multocida* gave a uniform amplicons size corresponding to 1,044 base pairs indicating that they all belong to capsular group A. Similar results have been observed by Chung *et al.* (2001) and Jaglic *et al.* (2005). This report has lent credence to the reports of Glisson *et al*. (2003) and Kumar *et al.* (2004) who reported that fowl cholera is mainly caused by *P. multocida* belonging to capsular group A. Also Jabbari *et al*. (2003) stated that all *P.multocida* isolates identified as capsular type A. The present finding differs from reports of Kumar *et al*. (1996) and Chawak *et al*. (2000) who documented that fowl cholera in avian species, such as chickens, ducks, turkeys, quails, geese, pigeons and cage birds are caused by capsular group D:3 and F:3.

P.multocida were multidrug resistant in which antibiotic sensitivity tests revealed that all the isolates were resistant to gentamycin, ampicillin, erythromycin, penicillin, tobramycin, chloramphenicol, doxycycline, cephotaxime and colistin (100%) followed by apramycin and ciprofloxacin (83.33%) then, tetracycline, amoxicillin and clavulenic acid (66.7) vancomycin and neomycin (50%) and ofloxacin (16.6%).

Results of disinfectant suspension test revealed that logarithmic reductions of *P.multocida* required according to the used standard (5 log. reductions)was obtained after exposure to Synergize (gluteraldehyde-QAC combination) for 15 minutes as it achieved 7.5 log. reductions. However, it showed resistance to the same product at 5 minutes and to the other two products; Pi- Quat 20(QAC) and Virukill (potassium peroxymonosulfate- NaCl combination) after 5 or 15 minutes. Researchers recommended evaluation of disinfectants using locally isolated strains rather than laboratory ATCC strains due to the possibility of development of bacterial resistance following exposure to environmental factors or frequent disinfectant application particularly of quaternary ammonium compounds (Langsrud *et al.,* 2003, Chima *et al.,* 2012). In a study applied by In-Soo *et al.* (2014) the environmentally isolated *P.multocida* showed higher disinfectant resistance than ATCC strains. According to CDC, (2008), gluteraldehyde-based disinfectants are characterized by excellent biocidal properties even in presence of high organic matter. However, QACs potency impaired by hardness and gram-negative bacteria can survive or grow in them. Thomson *et al.* (2007) found that Gluteraldehyde plus quaternary ammonium1:50 – 1:190 and Quaternary ammonium 1:50–1:100 showed efficacy against *P.multocida* under low organic matter conditions. In a study applied on *Pseudomonas aeruginosa, Escherichia coli and Salmonella typhimurium* obtained from commercial poultry facilities using qualitative suspension test, the selected bacteria were resistant to QAC and virkon-s after 10 minutes. However, Gluteraldehyde + QAC achieved high level of antimicrobial activity even in the presence of organic matter (Gehan *et al.,* 2009). In another study Yan-Lin *et al.* (2008) stated that potassium monopersulfate 1250 mg/L for 2.5 min could achieve 5 logs. reductions of *P. multocida*. Sonthipet *et al.* (2018) examined the efficacy of potassium monopersulfate on E. coli and S. infantis in presence and absence of 5% FBS. Results showed that 312.5 ppm, in presence of organic materials could not inactivate E. coli within 15 min. and 625 and 312.5 ppm, could not inactivate S. infantis. Ruano *et al.* (2001) concluded the need of most microbial to increased contact times with the disinfectant or higher concentration.

CONCLUSION

Twelve isolates of *P. multocida* were isolated from filed cases of fowl cholera of chicken. In addition to conventional methods such as staining, cultural and biochemical test, identification of the isolated organisms as *P. multocida* was confirmed by molecular approach i.e., PCR using the primers specific for *P. multocida.* Antibiotic susceptibility was applied using isolated *P.multocida* for detection of most effective antibiotics. Disinfectant quantitative suspension test was applied against the isolated *P. multocida* using three disinfectants; Gluteraldehyde-QAC combination product was bactericidal after 15 minutes of contact with recommendations for increasing contact time and concentration.

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الخصائص المظهرية والجينية ودرجة مقاومة الباستيريلا مالتوسيدا المعزولة من الدجاج للأدوية وتأثير بعض المطهرات عليها

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يعتبر ميكروب الباستيريلا مالتوسيدا من النوع (أ) العامل المسبب لمرض كوليرا الطيور ، وهو مرض شديد العدوى ومميت فى الدجاج. في هذا البحث قمنا باختبار ٣٠٠ طائر لمحاولة عزل الميكروب من الطحال, الرئتين, القصبة الهوائية والنخاع العظمى للدجاج. تم عزل وتعريف الميكروب من العينات عن طريق شكل المستعمرات البكتيرية وخصائص الميكروب تحت الميكروسكوب والخصائص البيوكيميائيةله. بعد ذلك تم التأكيد باستخدام العدوى المعملية لحيوانات التجارب واختبار البلمرة المتسلسل. تم عزل الميكروب من ١٢ طائر من بين ال٣٠٠ طائر الخاضعين للاختبار بنسبة ٤%. بفحص الميكروب تحت الميكروسكوب تبين انه عصيات سالبة الجرام, غير متحوصلة , غير متحركة تتواجد امامفردة او فى شكل ازواج. وباستخدام صبغة الليشمان تبين انها ثنائية القطب. باستخدام اختبار البلمرة المتسلسل تبين انها لميكروب الباستيريلا مالتوسيدا من النوعى (أ). تم اختبار الميكروب للتعرف علي درجة مقاومته للمضادات الحيوية. قمنا ايضا باختبار الميكروب لمعرفة التأثير الكمي لثلاثة من المطهرات وتبين ان المنتج الذي يتكون من الجلوترالدهايد ومركب الامونيا الرباعي هو الذى تمكن من تحقيق التأثير النموذجى المذكور في الاختبار الاصلى بعد مرور ١٥ دقيقة من التعرض للمطهر.