

# ***Pasteurella multocida* in backyard chickens in Upper Egypt: incidence with polymerase chain reaction analysis for capsule type, virulence in chicken embryos and antimicrobial resistance**

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## **Summary**

The prevalence of *Pasteurella multocida* strains among 275 backyard chickens from different regions of Upper Egypt was studied. A total of 21 isolates of *P. multocida* were recovered in 21 out of 275 chickens tested (7.6%) and were confirmed using phenotypic characterisation. Somatic serotyping of the 21 isolates resulted in 12 isolates being classed as serotype A:1 (57.14%), 4 as serotype A:3 (19.05%) and 5 could not be typed (23.8%). Capsular typing, using multiplex polymerase chain reaction (PCR), demonstrated that 18 strains were capsular type A (85.7%), and 3 were type D (14.3%). The present findings suggest that a multiplex capsular PCR could be valuable for the rapid identification of *P. multocida* in cases of fowl cholera infection. A total of 5 isolates of *P. multocida* were selected to study their pathogenicity in embryonated chicken eggs instead of conducting a study in mature chickens. The results showed a variation in pathogenicity between the strains tested, namely: serotype A:1 strains caused 80% mortality, in contrast to 20% mortality by type D strains. Pathological findings included severe congestion of the entire embryo,

haemorrhaging of the skin, feather follicles and toe, and ecchymotic haemorrhages on the liver of the inoculated embryos. The observations in this study indicate that *P. multocida* serogroup A could be highly pathogenic for mature chickens and therefore might be a cause of considerable economic losses in commercial production. A total of 10 isolates were subjected to antimicrobial susceptibility to determine the minimal inhibitory concentration of 7 antimicrobials. All isolates were susceptible to ciprofloxacin, florfenicol, streptomycin and sulphamethoxazol with trimethoprim and with varying degrees of sensitivity to the other agents.

## **Keywords**

Antimicrobial, Chicken, Egypt, Embryo, Fowl cholera, *Pasteurella multocida*, Pasteurellosis, Polymerase chain reaction, Susceptibility.

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## ***Pasteurella multocida* in polli da cortile nell'Alto Egitto: analisi PCR del tipo capsulare per stabilire incidenza, virulenza verso gli embrioni e resistenza antimicrobica**

### **Riassunto**

È stata studiata la prevalenza di ceppi di *Pasteurella multocida* in 275 polli da cortile provenienti da diverse regioni dell'Alto Egitto. Su 275 (7,6%) campioni, sono stati individuati in 21 polli altrettanti isolati, confermati mediante caratterizzazione fenotipica. In seguito a sierotipizzazione somatica, 12 isolati sono stati classificati come sierotipo A:1 (57,14%) e 4 come sierotipo A:3 (19,05%). Per 5 isolati non è stato possibile eseguire la sierotipizzazione (23,8%). La tipizzazione capsulare mediante PCR multiplex ha evidenziato 18 ceppi di tipo A (85,7%) e 3 di tipo D (14,3%). I dati ottenuti suggeriscono che questa metodica potrebbe essere utile per l'identificazione rapida di *P. multocida* durante l'infezione da colera aviario. Per studiare la patogenicità nelle uova embrionate, anziché in esemplari vecchi, sono stati selezionati 5 isolati di *P. multocida*. I risultati hanno evidenziato una differenza tra i ceppi sottoposti ad analisi, al sierotipo A:1 è risultata associata la mortalità dell'80% rispetto al 20% associata al tipo D. I reperti patologici hanno evidenziato: grave congestione dell'intero embrione, emorragia cutanea, alterazioni dei follicoli e delle piume, ecchimosi epatiche. Dieci isolati, inoltre, sono stati sottoposti a test di suscettibilità antimicrobica per determinare la concentrazione inibitoria minima (MIC) di 7 antimicrobici. Tutti gli isolati sono risultati sensibili a ciprofloxacina, florfenicolo, streptomicina e sulfametoxazolo più trimetoprim e, in vari gradi, ad altri agenti. Quanto emerso dallo studio permette di ipotizzare che il sierogruppo A di *P. multocida* potrebbe essere altamente patogeno per gli esemplari anziani, causando notevoli perdite economiche nella produzione commerciale.

### **Parole chiave**

Antimicrobico, Colera aviario, Egitto, Embrione, *Pasteurella multocida*, Pasteurellosi, Pollo, Reazione a catena della polimerasi, Suscettibilità.

## **Introduction**

Fowl cholera is a highly contagious disease caused by *Pasteurella multocida* that affects a broad host range of birds and causes high mortality that incur significant economic losses in commercial and backyard poultry production (6). The incidence of fowl cholera, along with other bacterial diseases, is on the increase, despite vaccination and proper medication and can be attributed to various incriminating factors (14, 15).

Backyard chickens are an important livestock species for many rural families in Egypt and in other countries across the globe. In spite of this, little information is available on the presence of fowl cholera among family raised chickens. This may be due to not receiving the dead birds from villages for diagnostic examination. Most dead birds are eaten by dogs and cats, or are discarded, which means that most cases go unreported, and some sick birds undergo emergency slaughter for human consumption (18).

Four capsular serogroups are recognised among the avian strains of *P. multocida*, namely: A, B, D and F. Strains of serogroup A are recognised as being the primary cause of fowl cholera. Sixteen somatic serotypes (1 to 16) are recognised in *P. multocida* and most of these have been demonstrated in avian capsular serogroup A strains. *P. multocida* serotypes A:1, A:3 and A:3,4 are widely recognised as the causative agent of most fowl cholera outbreaks in poultry flocks (8).

There are complexities associated with the diagnosis of fowl cholera by conventional methods, using capsular serotyping. Some avian strains of *P. multocida* are non-encapsulated and cannot be classed into a serological group (31). In addition, indirect methods could fail due to the difficulty in producing high titre antibodies against serogroups A, D and F; this is mainly due to the presence of inert capsule materials, such as hyaluronic acid and muco-polysaccharides concealed beneath the serogroup antigens (4). There is an urgent need to establish reliable and rapid methods to identify and type field isolates of *P. multocida* before an effective

vaccine against fowl cholera can be developed in Egypt. In recent years, genotypic methods for bacterial identification have proved beneficial in overcoming limitations of traditional phenotypic procedures (1). A polymerase chain reaction (PCR) assay has been developed for capsular typing of *P. multocida* strains. This assay represents a rapid and reproducible alternative to serological methods (23, 28).

*P. multocida* is a heterogeneous species in which the pathogenicity of individual strains is highly variable and susceptibility of the host to these bacterial strains varies considerably among avian species (5). Experimental mouse and chicken inoculation has been commonly used to test *P. multocida* pathogenicity; this method may not be feasible when screening large numbers of strains. An alternative approach is needed to avoid the tedious and expensive use of chicken and mouse inoculation. The procedure for testing pathogenicity of *P. multocida* in inoculated embryos of chickens needs to be investigated as it is both inexpensive and easy to perform.

Control of fowl cholera is primarily ensured by good management practices and treatment with antimicrobial agents. Antimicrobial susceptibility tests play a crucial role in the selection of the most efficacious antimicrobial therapy against *P. multocida* infection (4, 12, 27). However, the prolonged and imprudent use of antimicrobials has resulted in the development of resistance among various strains of the organism and multi-drug resistant (MDR) forms of *P. multocida* have emerged (25). This has resulted in a reduction in the efficacy of the antimicrobial agents that are currently available for the treatment of infections in poultry infected with *P. multocida*.

The distribution and prevalence of serotypes, pathogenicity and antimicrobial resistance profiles can vary considerably from region to region and over time in a given region. Due to the scant literature available on the subject among backyard chicken flocks in Egypt, we decided to focus the objective of the present work on covering the above objectives.

## Materials and methods

### Recovery of *Pasteurella multocida* from backyard chickens

A total of 275 family chickens were sampled from Upper Egypt. Bacterial cultures were attempted from heart blood and bone marrow of dead birds and from tracheal swabs of live chickens.

The samples were inoculated in brain heart infusion (BHI) broth and incubated at 37°C for 18h-24 h then subcultured on sheep blood agar to isolate the organism. All isolates were subsequently characterised biochemically for the identification of *P. multocida* (2). All isolates were freeze-dried and kept at -80°C.

### Mouse bioassay

All strains of *P. multocida* were grown for 18 h in a shaker/incubator at 37°C in BH) broth. Approximately 0.2 ml of each culture, containing approximately  $2.4 \times 10^8$  colony forming units (cfu)/ml was inoculated into each of three mice by the intraperitoneal route, and observed for 72 h, to study the mortality pattern. Organisms were re-isolated on a blood agar plate using heart blood collected from dead mice, and an impression smear from the liver was prepared on microscopic slides for bacterial observation, using the Giemsa staining method.

### Somatic typing

All the isolates that were initially identified as *Pasteurella* by extended phenotypic methods as described by Blackall and Mifflin (2) and were subjected to somatic serotyping using the agar gel diffusion precipitation test (AGDPT) described by Heddleston *et al.* (10).

### Multiplex polymerase chain reaction analysis of capsule type

To identify the serotype of *P. multocida* strains collected from the chickens, capsular typing was conducted using multiplex PCR.

### Preparation of template DNA

Isolated bacterial cultures were inoculated into 2 ml BHI broth. After 18 h of incubation at 37°C, cultures were taken in an Eppendorf tube for centrifugation at 10 000 rpm for

10 min. The pellet was resuspended in 400 ml Tris-ethylenediaminetetraacetic acid (EDTA) buffer and boiled for 10 min, followed by immediate chilling. Cell debris were removed by centrifugation at 1 000 rpm for 5 min. The supernatant was used as the template DNA for the PCR reaction (30).

### Polymerase chain reaction typing

A multiplex PCR assay was performed using six primer sets, as described by Townsend *et al.* (28); this is a species-specific PCR assay used for capsular typing. A list of various primers used in the study is presented in Table I. The multiplex PCR mixture of 25 µl contained each primer within the six primer sets (*capA*, *capB*, *capD*, *capE* and *capF*) at a concentration of 3.2 µM, each dNTP at a concentration of 200 µM, 1× PCR buffer, 2 µM MgCl<sub>2</sub> and 1 µl Taq DNA polymerase and 30 ng template DNA (1 µl). The following cycle procedure was used to amplify all capsular serogroup-specific products: an initial denaturation was performed at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 5 min.

Amplified products were separated by agarose gel electrophoresis (1% agarose in 0.5× Trisborate-EDTA) at 100 V for 1 h and stained with ethidium bromide (0.5 mg/ml). A standard molecular size marker (1 kb plus DNA ladder) was included in each gel. DNA

fragments were observed by ultraviolet transilluminator and photographed.

### Virulence of isolated *Pasteurella multocida* strains in chicken embryos

The virulence of the isolated *P. multocida* was investigated by inoculating 11-day-old embryonated eggs through the chorio-allantoic sac with 0.1 ml of broth culture containing 10<sup>5</sup> cfu that was used in the inoculum (13), followed by incubation at 37°C in a humidified atmosphere. The inoculated embryos were candled twice daily. Mortalities and times of death were recorded every 12 h up to 72 h post inoculation.

All dead or killed (control) embryos were examined for the presence and severity of lesions of the embryo, chorioallantoic membrane and yolk sac. Embryonic fluids were subjected to bacterial culture and Gram staining. Control embryos, inoculated with sterile BHI broth, were also subjected to cultural examination.

### Antimicrobial susceptibility testing

Ten isolated strains of *P. multocida* were tested for their susceptibility to seven antimicrobial agents, namely: amoxicillin, florfenicol, tetracycline, ciprofloxacin, trimethoprim-sulfamethoxazole, streptomycin and doxycycline. The antibiogram was determined using the broth microdilution methods (27).

Determination and evaluation of the MICs were performed by using an interpretive criterion from the National Committee for

Table I  
Sequence and specificity of primers applied for the detection of capsular-type genes in *Pasteurella multocida* strains

| Target identified           | Primers sequence (5-3')                                  | Amplicon size (bp) |
|-----------------------------|--|--------------------|
| Serogroup A <i>cap</i> gene | F GATGCCAAAATCGCAGTCAG<br>R TGTTGCCATCATTGTCAGTG         | 1 048              |
| Serogroup B <i>cap</i> gene | F CATTATCCAAGCTCCACC<br>R GCCCGAGAGTTTCAATCC             | 758                |
| Serogroup D <i>cap</i> gene | F TTACAAAAGAAAGACTAGGAGCCC<br>R CATCTACCCACTCAACCATATCAG | 647                |
| Serogroup E <i>cap</i> gene | F TCCGCAGAAAATTATTGACTC<br>R GCTTGCTGCTTGATTTTGTG        | 512                |
| Serogroup F <i>cap</i> gene | F AATCGGAGAACGCAGAAATCAG<br>R TTCCGCCGTCAATTACTCTG       | 852                |

F forward  
R reverse

Clinical Laboratory Standards (NCCLS) (19). The MIC was defined as the lowest concentration of the antimicrobial that prevented visible growth.

## Results

### Prevalence of *Pasteurella multocida* in samples

Strains of *P. multocida* were detected in 21 of the 275 cases investigated (7.6%). Extended phenotypic characterisation confirmed the isolates as *P. multocida* subspecies *multocida*.

### Mouse pathogenicity

Isolated strains of *P. multocida* were found to be virulent with death times recorded at 18 h and 24 h. Heart blood smear, liver and spleen impression smears revealed characteristic bipolar organisms using Giemsa staining. The isolates showed typical cultural characteristics of dew drop, mucoid, non-haemolytic colonies in blood agar. No growth was observed in MacConkey agar, these observations were concurred with those reported previously (31).

### Antigenic type of *Pasteurella multocida*

#### Somatic serotyping

Serotyping of 21 *P. multocida* isolates were performed by using *in vitro* antisera against two control reference strains X-73 (serotype 1) and P-1059 (serotype 3) and results revealed that the 12 isolates belonged to serotype A:1 (57.14 %), while 4 isolates of *P. multocida* belonged to serotype A:3 (19.05%) and the remaining isolates gave negative results to both serotype 1 and serotype 3 antisera. Due to the unavailability of all reference antisera for all serotypes, 5 isolates were reported as 'untypeable' (23.81%).

#### Capsular genotyping

The M-PCR demonstrated that 18 *P. multocida* strains had an amplicon size of 1 044 bp (belonging to type A), and three isolates gave an amplicon at 0.657 kb (belonging to type D) Figure 1.

### Pathogenicity of chicken embryos

A variation in the pathogenicity of isolated strains to chicken embryos was observed by the mortality rate that ranged from 20% to 80%, and the time to death for the five isolates examined ranged from 30 h to 124 h post inoculation (Table II). The pathological lesions were severe congestion of the entire embryo, oedema of the head and haemorrhages of the feather follicles and toes. The liver was enlarged, severely congested and sometimes of mottled appearance. The heart showed petechial haemorrhages and the lung was severely congested. The yolk sac was congested and the allantoic fluid was stained with blood (Figs 2 and 3). Control embryos showed neither mortality nor lesions. *Pasteurella* were recovered from all dead embryos.

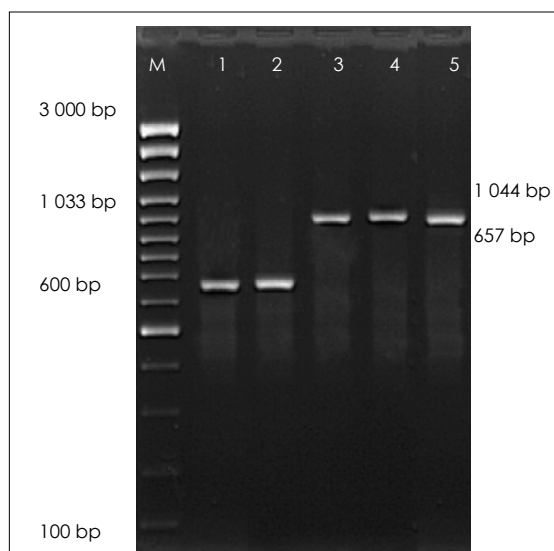


Figure 1  
Multiplex capsular polymerase chain reaction typing

Lane M: marker, 100 bp DNA ladder  
Lanes 1 and 2: strains belong to capsular D type  
Lanes 3-5: strains belong to capsular A type

### Antimicrobial susceptibility

The results of the antimicrobial drug susceptibility analysis are presented in Table III which shows that 100% of isolates were resistant to tetracycline and amoxicillin and 40% were resistant to doxycycline. No resistance to florfenicol, ciprofloxacin and trimethoprim-sulfamethoxazole was observed.

Table II  
Time-related numbers of dead and surviving embryos after challenge with *Pasteurella multocida*\*

| Isolate/<br>capsule<br>type | Time to death (hours) |    |    |    |    |    |    |    |    |    |    |    |    |     |     |     | No.<br>dead | Dead<br>(%) | Time to<br>death<br>(h) |        |
|-----------------------------|-----------------------|----|----|----|----|----|----|----|----|----|----|----|----|-----|-----|-----|-------------|-------------|-------------------------|--------|
|                             | 24                    | 30 | 36 | 42 | 48 | 54 | 60 | 66 | 72 | 78 | 84 | 90 | 96 | 102 | 108 | 114 |             |             |                         | 120    |
| 1/A:1                       | -                     | -  | -  | 1  | 1  | -  | -  | -  | -  | -  | -  | -  | -  | -   | -   | 1   | -           | 3           | 40                      | 42-48  |
| 2/A:1                       | -                     | -  | -  | -  | -  | -  | -  | 1  | -  | -  | -  | -  | 1  | 1   | 1   | -   | -           | 4           | 80                      | 66-108 |
| 3/A:3                       | -                     | 2  | 1  | -  | 1  | -  | -  | -  | -  | -  | -  | -  | -  | -   | -   | -   | -           | 4           | 80                      | 30-48  |
| 4/D                         | -                     | 2  | -  | -  | 1  | -  | -  | -  | -  | -  | -  | -  | -  | -   | -   | -   | -           | 3           | 60                      | 30-48  |
| 5/A:3                       | -                     | 1  | -  | -  | -  | -  | -  | -  | -  | -  | 1  | -  | -  | -   | -   | -   | -           | 2           | 20                      | 30-84  |
| Control                     | -                     | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -   | -   | -   | -           | -           | 0                       | -      |

\* number of challenged embryos by isolate/capsule type and in control group = 5

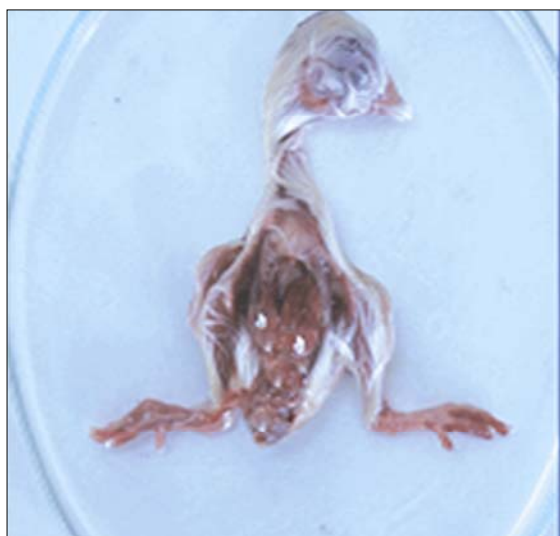


Figure 2  
Congestion of liver and heart



Figure 3  
Congestion and haemorrhages on the feather follicles and toe

Different distributions of MICs were recorded for resistant strains. The MICs of tetracycline ranged from 32 µg to 128 µg/ml). The MICs of doxycycline were (50%) with 16 µg/ml and 40% with 32 µg/ml, while those of amoxycillin were distinctly higher, with MICs of 512 µg/ml for all isolates. In contrast to sensitive strains, the MICs of florfenicol were 1 µg for 50% of isolates and 2 µg/ml for the remaining isolates, while the higher MIC noted for trimethoprim-sulfamethoxazole and streptomycin were 2 µg/ml and 16 µg/ml, respectively.

## Discussion

The occurrence of fowl cholera in commercial layers and breeder flocks has been reported as the major concern in the poultry industry by other workers (16, 17, 18). The epidemiology of fowl cholera outbreaks is complex (5). Most of the works published have focused on prevalence of *P. multocida* in commercial breeds, and not on backyard chickens.

In this study, the 7.6% isolation rate of *P. multocida* was higher than the level reported by Mbuthia *et al.* (17) who recorded a rate of 6.2%. This difference may be due to the number of samples, method of isolation, presence of stress and age of birds sampled.

Table III  
Minimum inhibitory concentrations of seven anti-microbial agents against examined *Pasteurella multocida* isolates

| Anti-microbial | ND | 0.125 | 0.25 | 0.5 | No. of isolates with MIC (µg/ml) |   |   |   |    |    |    |     |     |     | MIC break-point* (µg/ml) | No. of resistant strains | Percentage |
|----------------|----|-------|------|-----|----------------------------------|---|---|---|----|----|----|-----|-----|-----|--------------------------|--------------------------|------------|
|                |    |       |      |     | 1                                | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 |                          |                          |            |
| TET            |    |       |      |     |                                  |   |   |   |    | 1  | 1  | 8   |     |     | ≥16                      | 10                       | 100        |
| DO             |    |       |      |     |                                  |   | 1 |   |    | 5  | 4  |     |     |     | ≥16                      | 4                        | 40         |
| FFC            |    |       |      |     | 5                                | 5 |   |   |    |    |    |     |     |     | ≥8                       | 0                        | 0          |
| SXT            |    |       |      | 1   | 6                                | 3 |   |   |    |    |    |     |     |     | ≥512                     | 0                        | 0          |
| ST             |    |       |      |     |                                  |   | 3 | 3 | 4  |    |    |     |     |     | ≥128                     | 0                        | 0          |
| CIP            | 8  | 1     |      | 1   |                                  |   |   |   |    |    |    |     |     |     | ≥4                       | 0                        | 0          |
| AMX            |    |       |      |     |                                  |   |   |   |    |    |    |     | 10  | ≥32 | 10                       | 100                      |            |

MIC minimum inhibitory concentration  
TET tetracycline  
DO doxycycline  
FFC florfenicol  
SXT trimethoprim-sulfamethoxazole  
CIP ciprofloxacin  
ST streptomycin  
AMX amoxicillin  
ND not determined

\* values based on National Committee for Clinical Laboratory Standards (NCCLS) standards (19)

The gross lesions observed in our study were similar to the lesions observed during an acute form of fowl cholera described by others (8, 17, 32).

The phenotypic characterisations of the 21 isolates of *P. multocida* were confirmed as *P. multocida* subspecies *multocida* (2, 6, 7). This is consistent with other studies on fowl cholera cases in chickens and turkeys (25, 32, 33). The serotyping results revealed that most isolates belonged to serotype A:1 (57.14%), a serotype that has been reported to be a major cause of acute forms of fowl cholera (8, 25, 33, 35); the other four isolates belonged to serotype A:3 (19.05%). A total of 23% of the isolates (5/21) were untypable, due mainly to a lack of antibodies specific to the other serotypes (22).

Serological typing is a valuable diagnostic tool for avian pasteurellosis but technical problems and difficulties in interpreting results make it necessary to look for alternative methods. A particular problem in serological typing and diagnosis of *P. multocida* is the inability of serogroups A, D and F to agglutinate in homologous antisera. This inability to agglutinate of capsulated *P. multocida* is associated with a serologically inert substance that is a component of the bacterial capsule (hyaluronic acid) (20).

Capsular genotyping was performed on all the isolated strains of *P. multocida*, using six primer sets, as described by Townsend *et al.* (28). This was done using multiplex PCR. A total of 18 strains of 21 tested isolates were identified as capsular type A and 3 strains were type D. This data is similar to previous findings that recognised strains of serogroup A as the primary and dominant cause of fowl cholera, whereas isolates of serogroups B, D and F are less frequently associated with the disease (31, 32). No amplicons specific for capsular serogroups B, F and E were observed. The assay was found to be a reliable technique for reliable capsular serogrouping and could be useful in the detection of isolates that are difficult to type. Actually, the five strains that could not be typed by conventional serotyping were serogrouped under A and D in the multiplex capsular PCR assay (1, 24, 26, 28).

Pathogenicity or virulence of *P. multocida* is complex and variable (8), with endotoxins being produced by virulent and avirulent strains of *P. multocida* strains. Endotoxins, in addition to bacterial invasion and multiplication, may contribute to virulence (13). Our results demonstrated the septicaemic nature of inoculated embryos and showed differences in the severity of lesions and time

until death, revealing a level of virulence ranging from medium to high. Endotoxins may have been partly responsible for the rapid deaths (11). Heddleston and Rebers were able to induce signs of acute fowl cholera in chickens by injecting small amounts of endotoxin (11). Our observations in this study indicated that the strains tested can be highly pathogenic for layers and breeders and might be the cause of considerable economic losses in commercial production.

Antibacterial treatment is still commonly used to control fowl cholera but has been accompanied by the emergence of resistant strains. The resistant strains are a result of the widespread use of antimicrobials in feed, both for prophylaxis and for growth promotion. Subtherapeutic uses of antimicrobials in feed have caused the emergence of multi-antimicrobial resistance. Antimicrobial resistance can evolve in the strains by the molecular transmission of resistance mechanisms from other bacteria carried by mobile genetic elements (27). The results of our study indicated a prevalent resistance to tetracycline and amoxicillin that reached 100%, suggesting that extensive use of

antimicrobials on poultry farms might have contributed to this situation. Our findings related to susceptibility concurred with the findings in France (15, 21), in North America (34) and in Japan, where florfenicol, and fluoroquinolones (ciprofloxacin) were the most active drugs (12). The aminoglycoside antimicrobials usually showed poor activity against *P. multocida* (9). The higher resistance among strains to doxycycline was considered to be due to cross-resistance between oxytetracycline and doxycycline (3, 29, 32).

Since strains of *P. multocida* vary in susceptibility to chemotherapeutic agents and considering that resistance to treatment may develop, especially during prolonged use of these agents, high morbidity and mortality rates might increase in poultry. Consequently, it is necessary to pre-test antimicrobial efficacy against *Pasteurella* to identify the effective antimicrobial agents that should be used by poultry specialists for therapeutic purposes.

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