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Antibiotic resistance pattern of Pasteurella multocida isolates from broiler chickens by phenotypic method in Babylon region of Iraq

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Abstract

Pasteurella multocida is responsible for infectious diseases across various animal species. It causes fowl cholera in birds and acts as a secondary contributor to respiratory syndromes. Antimicrobial therapy remains an effective treatment option. Generally, isolates of *P. multocida* are responsive to most commonly used medicinal antibiotics; however, their overuse and misuse result in the development of resistant strains. We characterized the antimicrobial sensitivity of 89 *P. multocida* strains obtained from poultry against 14 widely used antibiotics—ampicillin, colistin, azithromycin, enrofloxacin, erythromycin, florfenicol, flumequine, neomycin, fosphomycin, penicillin, trimethoprim + sulfamethoxazole, tetracycline, streptomycin, and gentamicin—using the disk diffusion technique. Most of the strains tested were vulnerable to most antimicrobial agents. Nevertheless, notable resistance was observed against ampicillin, making these compound unsuitable for treating infections caused by *P. multocida*. Conversely, the antimicrobial effectiveness of classical florfenicle, gentamycin, and fosphomycin were identified as effective agents for this bacterium. Fowl cholera has led to substantial economic losses in numerous poultry-producing nations around the world. In Iraq, outbreaks of fowl cholera are often reported across various types of poultry production. The antimicrobial susceptibility testing revealed several multidrug-resistant strains among the *P. multocida* isolates. All isolates were high resistant to penicillin (50%), streptomycin (13%), tetracycline (10%), enrofloxacin (6%), florfenicol (11%), Erythromycin (14%), gentamicin (16%), and amoxicillin (14%). This study offers valuable epidemiological insights into *P. multocida* and its role in fowl cholera outbreaks in broilers in the central region of Iraq, particularly in Babylon

Keywords: *Pasteurella multocida*, poultry, antibiotic resistance, Iraq

1. Introduction

Pasteurella multocida naturally resides on of the mucosal surfaces of vertebrates, capable of residing in the upper respiratory tracts of clinically healthy animals, where it may occasionally induce disease. Notably, *P. multocida* causes fowl cholera in poultry, atrophic rhinitis in pigs (1, 2), and hemorrhagic septicemia in cattle and buffaloes (3, 4). As a secondary pathogen, it often contributes significantly to the progression of severe pleuropneumonia in both poultry and ruminants (5, 6). Poultry that are infected with P‹ multocida can develop fowl cholera, this poses a significant economic problem in the commercial industry. The disease can exhibit in multiple varieties, including peracute, acute, and chronic infections (7). The morphological features, symptomatology, and macroscopic findings associated with P. *multocida* infections in poultry are not distinctive and can be confused with other respiratory infections characterized by upper respiratory tract inflammation, pneumonia, airsacculitis, polyserositis, and septicemia (8). Thus, accurate identification of the causative organism is essential for making a proper diagnosis.

The illness can manifest as a localized chronic infection or as an acute septicemia, with severe morbidity and fatality rates (up to 100%) (9). Acute poultry cholera usually manifests as sudden mortality in a considerable proportion of birds (10). There are now four subspecies of Pasteurella multocida: subspecies multocida, subspecies gallicida, subspecies septica, and subspecies tigris. Every subspecies has been isolated from poultry cholera epidemics, with the exception of tigris (11). Serotype A of P multocida is the predominant serotype that causes cholera in poultry, but serotypes B, D, and F have not been as frequently identified to cause illness in poultry (12). Globally and in Asia, outbreaks have been documented (13, 14). Antibiotic resistance has increased as a result of the widespread use of antibiotics to treat P. multocida infections in poultry (15).

Antibiotic therapy is still the most effective method for dealing with diseases that are caused by P. multocida (16). To ensure effective treatment, it's essential to isolate the organism that causes the disease and determine its sensitivity to in vitro antibiotics (17). However, because these laboratory studies take a lot of time, clinicians base antibiotic therapy as soon as symptoms appear on the hypothesized origin of the illness (17). Even when the selection of antibiotics is based on experience or consensus, the effectiveness of these pre-diagnostic therapy is questionable due to the widespread nature of the antibiotic resistance issue. (18). The excessive and misguided use of antibiotics results in a selective pressure on the genes associated with resistance to antibiotics. As a result, the data from national programs monitoring antimicrobial resistance is essential (19). Unfortunately, these programs that focus on the genus Pasteurella are only implemented in a limited number of countries (20, 21). In this research, we studied the antibacterial properties of *P. multocida* strains that were derived from poultry in Iraq. Understanding the antibiotic sensitivity patterns of this bacterium is crucial to its general description and includes the assessment of several antibiotics that are not appropriate for treating diseases caused by *P. multocida*.

2. Materials and methods

2.1. Study areas

Iraq geographical coordinates are between 29 and 38 °C north in latitude and 39 and 49 °C east in longitude, with a small portion extending westward into the latter zone. With the exception of the northern regions, the climate of the country is predominantly arid, characterized by cool to cold winter temperatures and dry, warm and sunny summers. During the summer months, temperatures in most regions of the country reach 40 °C, with some areas experiencing temperatures as high as 48 °C. The temperature rarely surpasses 21 degrees Celsius during the

winter season, with the most frigid conditions reaching between 15 and 19°C. Nighttime temperatures typically range between 2 and 5 °C.

2.2. Collection of samples

This descriptive study of the cross section took place in 2023، a total of 260 samples from the serum and 175 samples from the liver were gathered from 10 different broiler farms in Iraq, in the following step, samples were transported to the microbiology laboratory of the Faculty of Veterinary Medicine in a cold environment.

2.3. Isolation of *Pasteurella multicida*

The samples were cultured on Blood Agar medium with 7% defibrinated sheep blood (Merck, Germany) and incubated for 24 hours at 37°C. Subsequently, the colonies that developed in the BA medium were transferred to MacConkey agar medium. Non-growing bacteria on MacConkey agar, were selected for further analysis. Gram staining was performed to confirm the presence of Gram-negative bacilli. These isolates were then followed to determine biochemical reaction by the performance of the IMViC tests, which include Indole, Methyl Red, Voges-Proskauer, and Citrate tests. Colonies exhibiting across the IMViC tests, along with growth on blood agar medium without hemolysis and a negative MR-VP and Citrate test result, but positive for catalase, oxidase, and indole production tests were identified as ***Pasteurella multicida***. The selected isolates were subsequently cultured in Nutrient broth medium and stored alongside glycerol for future studies.

2.4. DNA extraction

DNA was extracted from the isolated bacteria by a DNA extraction kit (Pooya Gen Azma, Iran). Initially, 100 ml of the bacterial culture was transferred into a 1.5 ml micro-tube. Then, 200 µl of MR buffer was added to the sample, after which the mixture was vortexed twice. The tubes were then incubated at 0°C for 2–3 minutes. After that, the samples were centrifuged at 9,000 RPM for 4–5 minutes. The supernatant was discarded, and the remaining sediment was saved for the subsequent steps of the extraction process.Another 200 µl of MR buffer was added to the pellet, and centrifugation was repeated at 9,000 RPM for 4-5 minutes. After centrifugation, the pellet was redispersed in 200 µl of MI buffer. Then, 50 µl of MII buffer was added to it, and the tubes were gently inverted twice and laid down to be incubated at room temperature for 20-30 minutes until the solution became totally clear.Later, 100 µl of MIII Buffer was added to the tubes. They were inverted 10 times until the buffer turned white. The reaction volume was subjected to micro-sonication for 10 minutes at 13,000 RPM. The supernatant was transferred to a new micro-tube, and 96% ethanol was then added. The samples were centrifuged at 13,000 RPM for 5 minutes using a cylinder device.Then, 200-500 µl of ethanol was added to the micro-tubes after the cells had adhered to the bottom, and the tubes were centrifuged at 13,000 RPM for 2-3 minutes. The supernatant was discarded, and the sediment in the micro-tube was allowed to air dry at room temperature for 2-3 minutes. Add 20-50 µl of buffer that solubilizes the sediment to dissolute it fully. The DNA that results is now stored at –20°C until further processing by PCR. The extractive method was verified by measuring absorbance using a Nanodrop at a wavelength of 260 nm for 10 ex-tractions and calculating the 260/280 ratio. This confirms whether the manner of extraction is good and whether proteins are contaminated with DNA. Absence/presence and amount of protein affect the result of DNA analysis and reaction conditions in further steps; if there are proteins or too many proteins, the analysis of DNA will be false because they might give wrong absorption results. The higher numbers

reflect that there are more proteins/more something else in the sample than DNA. After that, DNA concentration was measured using only one light beam.

2.5. Molecular confirmation of *Pasteurella* spp.

The *16S rRNA* gene was used to identify the *Pasturella* spp. genus. The primers were designed using Amplifix version 1.5.4 software. The list of primers and temperature protocols can be found in Table1.

Table 1: The primer list and temperature programs for amplifying the *16S rRNA* gene of the *Pasturella* spp.

Protocol	Target gene	Primer Name	Sequence 5'----3'	PCR condition	PCR product size (bp)
PCR	*16S rRNA*	*Pasturella* spp.-F	TAGCGGTGAAATGCGTAGAG	95c for 30m, 95 c for 30s, 58 c for 30s, 72c for 45s and 72c for 7 m. (38)	353
		Pasturella spp.-R	TAAGTTCCCGAAGGCACAAG		

2.6. Antibiotic susceptibility test (Antibiogram)

To determine the bacterial susceptibility pattern to various antibiotic groups, the antibiogram test was performed using the desired antibiotic discs through the disk diffusion method based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI). In this method, after inocul ating the bacteria in BA medium and incubating at 37°C for 18 to 24 hours alongside a standard 0.5 McFarland suspension (containing 1.5 × 10^8 bacteria), a bacterial dilution was prepared. Us ing a swab, a lawn culture was performed on Mueller Hinton Agar. The antibiotic discs were pla

ced using sterile forceps, 2.4 cm apart from each other and 1.5 cm from the edge in the plate cont aining the cultured bacteria, and were incubated for 18 hours at 37°C (8).

Antibiogram discs include Gentamicin (GM10µg), Tetracycline (TE30 µg), Ceftriaxone (CRO30 µg), Ampicillin (AM10 µg), Neomycin (N30 µg), Azithromycin (AZM15 µg), Erythromycin (E 15 µg), Enrofloxacin (EFX5 µg), Colistin (CL10 µg), Florfenicol (FF30 µg), Streptomycin (S10 µg), Flumequine (FM30 µg), Fosfomycin (FOS200 µg), and Trimethoprim-Sulfamethoxazole (STX25-23 µg) (Padten Teb, Iran).

3. Results

3.1. Bacterial culture results of broiler chicken samples

A total of 260 serum samples and 175 liver samples from broiler chickens were collected from farms in Babylon, Iraq. Following culture and biochemical testing, 63 serum samples and 26 liver samples were identified as infected with *Pasteurella*. Subsequently, the presence of the *Pasteurella* species in all samples was confirmed through polymerase chain reaction (PCR) targeting the *16S rRNA* gene.

PCR amplification of *16S rRNA* gene

The PCR reaction was made up of 25 µl, which was divided into 4 µl of the template DNA, 1 µl of each primer, and 12.5 µl of the master mix. The remainder of the volume was filled with sterile water. The cycling conditions and program for PCR were implemented in a thermal cycler (Quanta Biotech, UK) as described in Table 1. Following amplification, the PCR products were separated by electrophoresis on a 2% agarose gel containing Safe Stain (Labnet, ENDURO, USA) and visualized using the Genius Gel Documentation system (Syngene Bio-Imaging, UK) (Figure 1).

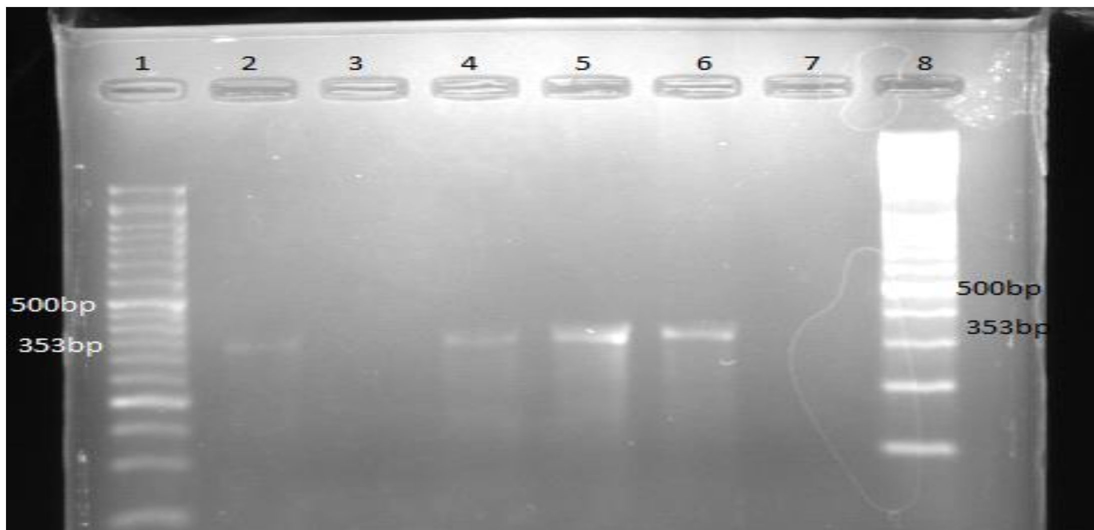

Figure 1. Agarose gel image PCR test on leaver and surem to identify *Pasteurella* spp*,* with *16srRNA* gene (353bp), which (Lane M-marker 100 and 50 bp DNA (Smobio Technology Inc., Taiwan), (Lanes 2,4,5 and 6 positive samples and 3 negative samples, 7 Lane of negative control).

3.2. Antibiogram results

The sensitivity of *P. multocida* isolates from broiler chickens to various antibiotics was assessed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Figure 2). Based on this evaluation, the bacteria were classified into three categories: sensitive (characterized by a growth inhibition zone diameter exceeding 17 mm), intermediate (with a diameter ranging from 11 mm to 17 mm), and resistant (defined by a growth inhibition zone diameter of less than 11 mm). The results are presented in Table 2.

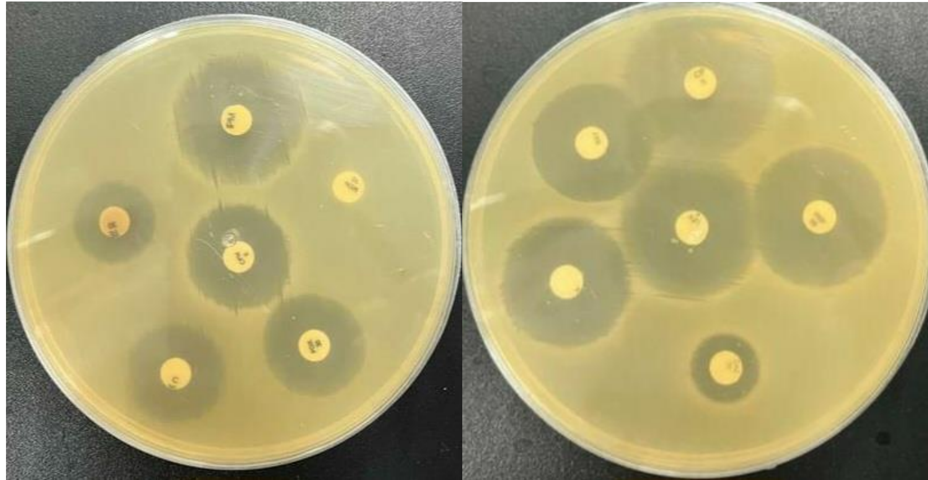

Figure 2. Antibiogram test of *P. multicida* in Mueller Hinton agar culture medium

Table 2. Susceptibility to antibiotics used in *P. multicida* isolated from broiler chickens

Sample		AM	FF	E	GM	SXT	AZM	CRO	TE	FOS	N	NFX	S	CL	FE
Liver (26)	S (%)	24	71	67	66	61	65	60	52	65	51	61	60	55	76
	R (%)	51	12	13	15	11	3	7	8	8	17	6	14	14	10
	I (%)	25	17	20	19	28	32	33	40	27	22	33	26	31	14
Serum (63)	S (%)	23	72	66	67	62	65	59	54	66	50	62	63	51	78
	R (%)	49	10	14	14	13	5	9	11	5	8	6	11	11	6
	I (%)	28	18	30	19	25	30	32	35	29	42	32	26	33	16

R= Resistance, S=Sensitive, I= Intermediate

4. Discussion

"Multiple resistance to drugs in *P. multocida* was documented by Chang and Carter (1976) and Hirsh et al. (1992). (1985). This resistance was caused by plasmids that possessed resistance to Streptomycin, sulfonamides, and tetracycline (22, 23). The longstanding use of these antibiotics for decades increased the prevalence of resistant bacteria, particularly in residential settings. At the start of the 90s, the frequency of resistance in *P. multocida* strains derived from animals

(pigs) was 25%, which increased to 50% by the end of the decade (24). In addition to being highly resistant to sulphonamides, our strains of poultry origin had a low percentage of resistance to the combination of sulphamethoxazole and trimethoprim (SXT), this suggests that the efficacy of this treatment isn't decreasing with the passage of time. And also, the percentage of SXT resistance was same (11%) in liver isolates than the percentage of resistance (13%) in the serum isolated bacterium. During the 1990s, SXT was beneficial in treating infections caused by *P. multocida*; however, the efficacy of this treatment began to decline rapidly as the strains of *P. multocida* became more resistant to SXT (25, 26). Attempts to regulate the usage of antibiotics with a therapeutic purpose are intended to slow the progression of bacterial resistance; this may explain the minor increase in tetracycline resistance (25). The resistance to Azithromycin is uncommon in *P. multocida*. Among the liver's isolates, only 3% were resistant to liver isolates and5% to serum isolates. Chloramphenicol was historically used in the veterinary field for over three decades. Prior to its formal prohibition in 1994, it was used in animals that were food producing. At that point, the frequency of chloramphenicol-resistant strains was approximately 10-15% (27, 28). Its fluorinated counterpart, florfenicol, has been sanctioned in Europe to regulate the course of respiratory diseases in cattle and pigs (29). During that time, the majority of strains were affected by this antibiotic (30). No strains of *P. multocida* have demonstrated resistance to either chloramphenicol or florfenicol since 2005 (29). In this study, 12% of isolates were resistant to florfenicol. The increasing prevalence of resistance to sulfones, tetracyclines, and chloramphenicol has led to a greater reliance on quinolones, which are a class of antibiotics with a new composition. This class encompasses nalidixic acid and its synthetic counterparts, as well as various quinolones and their fluorinated derivatives. However, there is limited information about the resistance of *P. multocida* to quinolones. Few instances of quinolone-

resistant bacteria have been documented that exhibited a variety of levels of resistance to nalidixic acid (8-17%) or flumequine(6%). Enrofloxacin has been recognized as the most effective fluoroquinolone against *P. multocida*, with only 0.02-0.3% resistance as a whole (33). However, strains of poultry had a higher resistance rate of 43 percent (34) and 29 percent (35). This pattern was also observed in our strains, which had a majority of poultry serum and liver isolates, except for one that was resistant to flumequine (12%). Conversely, avian isolates had a high degree of resistance to oxolinic acid (40%) and flumequine (40%), but they still were susceptible to enrofloxacin. Aminoglycosides are typically effective against bacteria that are Gram-negative, but their effectiveness against *P. multocida* is limited, the intermediate vulnerability is high (36). In our research, many strains demonstrated either partial resistance to aminoglycosides like gentamycin and neomycin. The distribution of resistance was not consistent, as many strains (17%) had a high degree of resistance to neomycin. Among the isolated strains of poultry, the percentage of resistance to both gentamycin and neomycin was 8%. Also, the percentage of intermediate susceptibility to neomycin was significant in strains that were isolated from poultry serum (42%) and liver (27%). Only 19% of the strains demonstrated a moderate degree of resistance to gentamycin. Macrolide antibiotics were originally available in the 1950s; the most common use of this class of antibiotics was with erythromycin. *P ،multocida* strains are typically susceptible to erythromycin, and the resistance rate is between 2 and 4% (24). Many of our isolated strains were susceptible to erythromycin 67%; however, many strain (143%) was resistant to this antibiotic. However, the frequency of intermediate susceptibility to erythromycin was particularly high in poultry strains (40). Semisynthetic macrolide derivatives have the same antibacterial spectrum as erythromycin, the first member of this class of antibiotics, but they have been more effective against pathogenic *P.*

multocida, specifically in pigs (37). As such, semisynthetic macrolides have become commonly employed for the treatment of Dermatitis and Respiratory Tract Infection due to *P ،multocida*. "

The antibiotics that are still effective against *P. multocida* comprise a large pool. Although generally overall resistance to commonly used antibiotics is low, avian strains should not be treated with sulfonamides, SXT, tetracyclines, aminoglycosides, and first-generation quinolones. In conclusion, Most of the *P. multocida* strains tested were susceptible to the aminoglycosides gentamycin (67%) and neomycin (51%) and also to erythromycin (67%) but notably at a high percentage to the significant intermediate level: 19%, 42%, and 30%, respectively. florphenicol is still active against *P. multocida* (72%) although rare instances of resistance exist. With florphenicol, the newer macrolide tulathromycin, third-generation fluoroquinolone, enrofloxacin, and fourth-generation Ceftriaxone,Azitromycin, streptomycin and fosfomycin, our study identified the most potent agents against *P. multocida*.

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