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Susceptibility of *Pasteurella multocida* isolated from cattle in Egypt to antibiotics, silver, chitosan and curcumin nanoparticles

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Abstract

Pasteurella multocida is a Gram-negative bacterium causing economically significant diseases in cattle. This study aimed to determine *P. multocida* susceptibility to different antibiotics and antibiotic alternatives. In this study, 246 samples (180 nasal swabs and 66 lung tissue specimens) were collected from cattle showing respiratory manifestations in Egypt. Suspected *P. multocida* colonies following culture were subjected to polymerase chain reaction (PCR) for molecular confirmation of the isolates. A multiplex PCR was employed to identify *P. multocida* capsular groups. Susceptibility of the isolated *P. multocida* to different antibiotics and nanoparticles as antibiotic alternatives including silver (AgNPs), chitosan (CNPs) and curcumin (CurNPs) were tested using broth microdilution method. Thirty-two *P. multocida* isolates were obtained, *kmt1* gene was detected in these isolates, and molecular capsular types classification revealed that all isolates were belonged to the capsular type A. Based on broth microdilution method findings, 20 (62.50%) isolates were considered as multi-drug resistant (MDR); the isolates were most sensitive to danofloxacin and kanamycin, whereas they were most resistant to doxycycline and tilmicosin. Antibiotic alternatives showed high anti-microbial activity against tested isolates with minimum inhibitory concentrations ranging from 1.56 - 6.25 µg mL^{-1}, 156 - 625 µg mL^{-1}, and 128 - 512 µg mL^{-1} for AgNPs, CNPs and CurNPs, respectively. Our finding demonstrated that MDR *P. multocida* was evident in cattle in Egypt. Although antibiotic alternatives showed promising *in vitro* anti-microbial effects against MDR isolates, additional studies are required to be actually applicable in veterinary practices.

Introduction

Pneumonic pasteurellosis is a common disease of the ruminants' respiratory system characterized by fibrinous inflammation of the pulmonary parenchyma.[1] It is a multi-factorial disease as ecological and managemental stressors, as well as concurrent or predisposing viral or bacterial diseases are related to the occurrence of *Pasteurella multocida*-induced pneumonia.[1,2] This disease exists across the world and is usually spread through inhalation of contaminated droplets.[3] Pneumonic pasteurellosis is caused by *P. multocida* and occasionally *Mannheimia haemolytica*.[4]

Pasteurella causes massive economic losses in livestock because of increased morbidity, mortality, and treatment costs.[5] *Pasteurella multocida* is the causative agent of several economically significant diseases in veterinary medicine.[6] It has three subspecies, five capsular serogroups, and sixteen somatic serotypes. Serogroup A isolates are bovine nasopharyngeal commensals, and common isolates from bovine respiratory disease and shipping fever in weaned stressed beef cattle.[2]

Using various anti-microbial agents for prophylactic and treatment purposes against *Pasteurella* in veterinary field is still the most effective and traditional method to control pasteurellosis.[7] The emergence of antibiotic resistance in *Pasteurella* against commonly used anti-biotics in veterinary practices necessitates the urgent use of antibiotic susceptibility test to select the most effective and convenient agent to be used in every situation.[8]

Different anti-microbial susceptibility tests are applied to detect the anti-microbial sensitivity of each bacterial agent, including disc diffusion assay and broth microdilution method, the latter is used to obtain the minimum inhibitory concentration (MIC). Measuring MIC values helps to determine the degree of microbial resistance more accurately.[9]

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Continuous efforts are made to overcome antibiotic resistance pathogens by evaluating anti-microbial alternatives such as herbal compounds, plant extracts, and metals in combination with more progressed technologies such as nanotechnology.[10] Nanotechnology helps to increase the solubility and diffusion of used materials inside the cells, increasing the effectivity and decreasing the toxicity of these materials.[11] Nanomaterials were tested in many investigations as anti-microbial alternatives; metals such as silver and organic compounds such as chitosan and curcumin showed promising results against pathogenic microbes.[11-13]

The negatively charged silver nanoparticles (AgNPs) react with positively charged compounds at the bacterial surface, leading to increased bacterial cell permeability; inside the bacteria cell, metals increase oxidative stress and interact with cellular components such as nucleic acid and proteins.[11]

Chitosan has anti-microbial properties; its amino groups can combine with metal ions on the bacterial cell surface and interfere with different cell functions.[13] Curcumin acts by inhibiting biofilms formed by bacteria and interfering with the regulation of bacterial cell density and microbial behavior. Moreover, it disrupts cell function *via* damaging nucleic acid and proteins.[14]

On that account, the current study aimed to determine the susceptibility of *P. multocida* strains isolated from Cattle in Egypt to different antibiotics, detect multidrug-resistant strains, and evaluate the *in vitro* efficiency of AgNPs, CNPs, and curcumin nanoparticles (CurNPs) as antibiotic alternatives against isolated strains.

Materials and Methods

Sample collection and preparation. This study was carried out in Egypt from 2022 to 2023. A total of 246 samples were collected from different cattle herds located in Giza Governorate, Egypt, including 180 nasal swabs from pneumonic animals showing respiratory manifestations and 66 lung tissue specimens from dead animals exhibiting pulmonary macroscopic alterations. Approval was obtained from the Ethical Committee of the Faculty of Veterinary Medicine, Cairo University, Giza, Egypt (Vet CU 09092023803).

Nasal swabs. After disinfecting the outer surface of the nostril using 70.00% alcohol, a sterilized cotton swab was introduced and rotated along the inner surface of the nasal cavity. The swabs were maintained in Amies transport medium (Oxoid, Hampshire, UK) and then, placed in an ice box until they were transported to the laboratory.[15] Because the organism is sensitive, the samples were delivered to the laboratory within 5 - 6 hr of being collected.[4]

Lung samples. Upon the animal's slaughtering, lung tissues with macroscopic alterations (congestion, edema, multi-focal red, and grey hepatization of lungs and fibrin deposits on lung and pleural surfaces) were taken in ice-filled containers for transporting of the samples to the laboratory. Before cutting the inner surface of the pneumonic lungs, the outer surface was cleaned in the laboratory by touching the lung surface with a heated spatula. Each animal's inner lung tissue part was taken out using aseptic techniques. The lung tissue samples were treated aseptically in a laminar airflow cabinet near a flame by homogenizing 20.00% w/v tissue homogenate in a sterilized phosphate-buffered saline.[3]

Isolation and identification of *Pasteurella*. At the laboratory, nasal swabs and tissue homogenates were immersed in brain heart infusion (BHI) broth (Oxoid). After 24 hr incubation at 37.00˚C, a loopful of each broth culture was streaked onto blood agar base supplemented with 7.00% sheep blood and MacConkey agar (Oxoid), aerobically incubated at 37.00˚C for 24 hr and then, *Pasteurella*-like colonies were stained with Gram's and Leishman's stains. Suspected colonies were then collected and kept at – 80.00˚C in BHI supplemented with 20.00% glycerol (HiMedia, Maharashtra, India).[16]

Molecular confirmation and capsular typing of *P. multocida* isolates by polymerase chain reaction (PCR). Bacteria were cultivated overnight in BHI at 37.00 ˚C, and bacterial DNA was extracted from overnight cultures using the QIAamp® DNA Extraction Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's directions. The extracted DNA was kept at – 20.00 ˚C until it was used in PCR amplification.

***Pasteurella multocida*-specific PCR (PM-PCR).** It was performed on all suspected *P. multocida* isolates using primer sets designed from the *kmt1* sequence.[17]

Capsular grouping by multiplex PCR. *Pasteurella multocida* strains were screened for the capsule biosynthesis genes (*Cap A*, *B*, *D*, *E*, and *F*) using multiplex PCR.[18] The positive control used was *P. multocida* type A. Primer sequences and amplified products for the targeted genes for *P. multocida* isolates were used according to Townsend *et al.*[17,18] and are presented in Table 1. The PCR amplified products were separated by agarose gel electrophoresis (1.50% agarose in 1X TAE (Thermo Fisher Scientific, Waltham, USA) at 4.00 V cm^{-1} for 1 hr and stained with ethidium bromide. Ultraviolet illumination was used to view DNA fragments, being imaged using a GelDoc 1,000 fluorescent imaging system (Bio-Rad, Hercules, USA).

Antibiotic susceptibility testing using micro-dilution method. Minimum inhibitory concentration for all the isolated strains was assessed using a broth microdilution method in a microtiter plate containing 96 wells.[19] Six antibiotic agents from different antibiotic classes were tested according to the Clinical and Laboratory Standards Institute (CLSI),[20] with the antibiotic dilution range being illustrated in Table 2. To obtain a

bacterial inoculum with a concentration of 5.00×10^5 colony forming unit (CFU) mL^{-1}, a suspension of each tested strain in saline was matched with 0.5 McFarland suspension ($1.00 - 2.00 \times 10^8$ CFU mL^{-1}), and the matched suspension was further diluted into Mueller Hinton broth (HiMedia) to reach the aimed bacterial concentration. The inoculum (10.00 µL) was added into wells containing 100 µL of diluted antibiotics. Reference strains *E. coli* American Type Culture Collection (ATCC) 25922 and *Staphylococcus aureus* ATCC 29213 were used as quality control organisms. The MIC was calculated as the lowest concentration of antibiotic (µg mL^{-1}) entirely inhibiting visible growth of the test strain of bacteria *in vitro*. *Pasteurella multocida* isolates were considered as multi-drug resistant (MDR) in case they were resistant to ≥ three various antibiotic classes.[24]

Silver nanoparticles. The AgNPs were synthesized by NanoTech (Cairo, Egypt) with some modifications in previously described method. [25] Silver nitrate (≥ 99.00%) and trisodium citrate dehydrate (TSC; ≥ 99.00%) powder were obtained from Sigma-Aldrich (St. Louis, USA). To prepare 100 mL of a solution (1.00 mM of $AgNO_3$), 17.00 mg of $AgNO_3$ powder was dissolved in distilled water. After the solid was completely dissolved, the solution was diluted to a final volume of 100 mL with distilled water. A 100 mL of 1.00 mM silver nitrate solution was boiled for 5 min using a water bath, 12.50 mL of (TSC 1.00%) solution was added dropwise with continuous stirring and then, the two solutions were mixed and heated at 50.00˚C till their color changed to pale yellow; the solution was then cooled and stored at 4.00˚C.

Chitosan nanoparticles. Chitosan of low molecular weight, sodium tripolyphosphate, and glacial acetic acid were obtained from Sigma-Aldrich. The CNPs (NanoTech) were prepared using the ionotropic gelation method according to the former method with some adjustments.[13] Briefly, 50.00 mg of chitosan was dissolved in 10.00 mL of 2.00% acetic acid, and 1.00 mL of sodium tripoly-phosphate (0.10%) solution was mixed with 4.00 mL of chitosan-prepared solution under continuous stirring at 1,000 rpm for one hr. Then, the prepared solution was centrifuged at 10,000 rpm for 30 min and suspended in distilled water.

Curcumin nanoparticles. For the preparation of CurNPs (NanoTech), curcumin (≥ 80.00% purity) was acquired from Sigma-Aldrich. Briefly, 100mg of curcumin powder was mixed with 20.00 mL of dichloromethane (Sigma-Aldrich) and then, 1.00 mL from the prepared mixture was added to 50.00 mL boiling water drop by drop under ultrasonic conditions. After adding 1.00 mL of the mixture, the content was sonicated for 20 min and then, stirred at 400, 600, and 800 rpm for 20 min at room temperature to get a clear, yellow-orange-colored product being dried to obtain an orange powder.[12]

Determination of the size, shape and optical properties of nanoparticles. Transmission electron microscopy (TEM; JOEL JEM-2100; Akishima, Tokyo, Japan) was used for scanning the surface of the prepared AgNPs, CNPs and CurNPs to determine their average size and shape. The TEM was performed at an accelerating voltage of 200 kV. The UV - VIS absorption spectra were accurately determined by an Ocean Optics USB 2,000 + VIS - NIR fiber optics spectrophotometer (Ocean Optics Inc., Orlando, USA). The size, shape and optical properties of prepared antibiotic alternatives are illustrated in Table 3 and Fig. 1.

Microdilution method for nanoparticles. The anti-bacterial effect of AgNPs, CNPs and CurNPs was tested using the broth microdilution method, similar to that used for antibiotic agents, and the MICs were recorded according to the CLSI.[20] Formation of working dilutions from the prepared antibiotic alternatives was done by double-fold serial dilution in a dilution range of 0.10 to 200 µg mL^{-1}, 1.20 to 2,500 µg mL^{-1} and 0.50 to 1,024 µg mL^{-1} for AgNPs, CNPs, and CurNPs, respectively. Distilled water, acetic acid (1.00%), and dimethylsulfoxide (1.00%) were used as diluent solutions for AgNPs, CNPs and CurNPs, respectively. Danofloxacin and kanamycin were used as control positives according to the results of the microdilution broth method for the tested isolates against antibiotic alternatives.

Table 1. Sequences of the oligonucleotides used in the *Pasteurella multocida*-specific polymerase chain reaction and multiplex capsular PCR typing assay.

Serogroups	Sequences (5' - 3')	Amplified size (bp)	References
All (PM-PCR)	F: ATCCGCTATTTACCCAGTGG R: GCTGTAAACGAACTCGCCAC	456	17
A	F: TGCCAAAATCGCAGTCAG R: TTGCCATCATTGTCAGTG	1044	18
B	F: CATTTATCCAAGCTCCACC R: GCCCGAGAGTTTCAATCC	760	
D	F:TTACAAAAGAAAGACTAGGAGCCC R: CATCTACCCACTCAACCATATCAG	657	
E	F: TCCGCAGAAAATTATTGACTC R: GCTTGCTGCTTGATTTTGTC	511	
F	F: AATCGGAGAACGCAGAAATCAG R: TTCCGCCGTCAATTACTCTG	851	

Table 2. Antibiotic agents' classes, dilution range and breakpoints.

Antibiotic (class)	Dilution range (µg mL^{-1})	Breakpoint (µg mL^{-1})			References
		S	I	R	
Danofloxacin (fluroquinolones)*	0.008 - 16.00	≤ 0.25	0.50	≥ 1.00	21
Doxycycline (tetracycline)†	0.0625 - 128	≤ 0.25	0.50	≥ 1.00	22
Kanamycin (aminoglycoside)‡	0.125 - 256	≤ 8.00	16.00	≥ 32.00	23
Florfenicol (amphenicols) †	0.03125 - 64.00	≤ 2.00	4.00	≥ 8.00	24
Spectinomycin (aminocyclitol) *	0.50 - 1,024	≤ 32.00	64.00	≥ 128.00	24
Tilmicosin (macrolide) §	0.125 - 256	≤ 8.00	16.00	≥ 32.00	24

* ADWIA Co., Cairo, Egypt; † PHARMA SWEDE, Ramadan City, Egypt; ‡ Alfasan, GmbH, Dreieich, Germany; and § Elanco, Greenfield, USA.
S: Sensitive, I: Intermediate, and R: Resistant.

Table 3. Average Size, shape and optical properties of prepared nanoparticles.

Parameters	AgNPs	CNPs	CurNPs
Average size (nm)	45.00 ± 5.00	40.00 ± 5.00	50.00 ± 5.50
Shape	Spherical-like	Quasi-spherical	Spherical-like
Absorption peak (nm)	410	270 - 290	425

AgNPs: Silver nanoparticles, CNPs: Chitosan nanoparticles, and CurNPs: Curcumin nanoparticles.

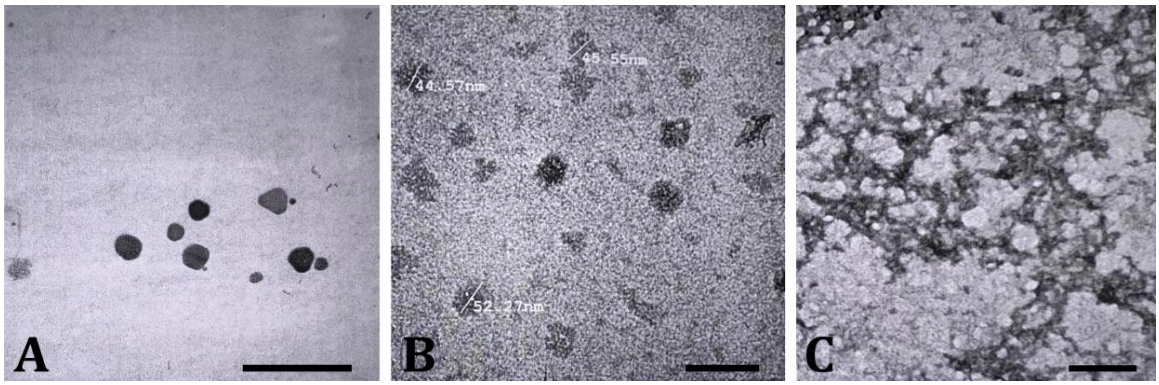

Fig. 1. Transmission electron microscopy of prepared nanoparticles. **A**) Silver nanoparticles (bar = 200 µm); **B)** Chitosan nanoparticles (bar = 100 µm); and **C)** Curcumin nanoparticles (bar = 200 µm).

Results

Isolation rate of *P. multocida* from collected samples. Out of 246 samples (180 nasal swabs and 66 lung tissues) collected and cultured, only 13.00% (32/246) were positive for *P. multocida*. Out of 32, 24 samples were from nasal cavities, whereas, eight samples were from the lungs.

Bacterial isolation and biochemical identification. The isolates exhibited typical morphological and cultural characteristics of small, glistening, dewdrop, mucoid, and non-hemolytic colonies in blood agar with a sweetish odor. In MacConkey agar, no growth was observed. Culture smears displayed characteristic Gram-negative cocco-bacillary organisms with bipolar staining features in Leishman's-stained smears.

Molecular confirmation of *P. multocida* isolates by PCR. Thirty-two *P. multocida* isolates were found to be positive by amplifying a 460 bp DNA fragment within the *kmt1* universal gene using the primers *KMTISP6* and *KMTIT7* (Fig. 2A).

Distribution of capsule biosynthesis genes among *P. multocida* isolates. The molecular classification of the *P. multocida* capsular types in multiplex PCR indicated that all isolates belonged to capsular type A by amplification of the *P. multocida hyaD-hyaC* gene, with an amplicon size of 1,044 bp (Fig. 2B).

Microdilution broth method for anti-microbial susceptibility testing. According to MIC results (Fig. 3), twenty isolates (62.50%) were considered MDR strains. The isolates were most sensitive to danofloxacin (62.50%), and kanamycin (62.50%), followed by spectinomycin, and florphenicol. The isolates were most resistant to doxycycline and tilmicosin.

Microdilution broth method for nanoparticles. The results of anti-bacterial activity of antibiotic alternatives are illustrated in Table 4. The tested antibiotic alternatives demonstrated anti-microbial activity against the isolated strains. The MICs of AgNPs, CNPs and CurNPs were ranged respectively from 1.56 - 6.25 µg mL^{-1}, 156 - 625 µg mL^{-1}, and 128 - 512 µg mL^{-1} depending on the tested strains.

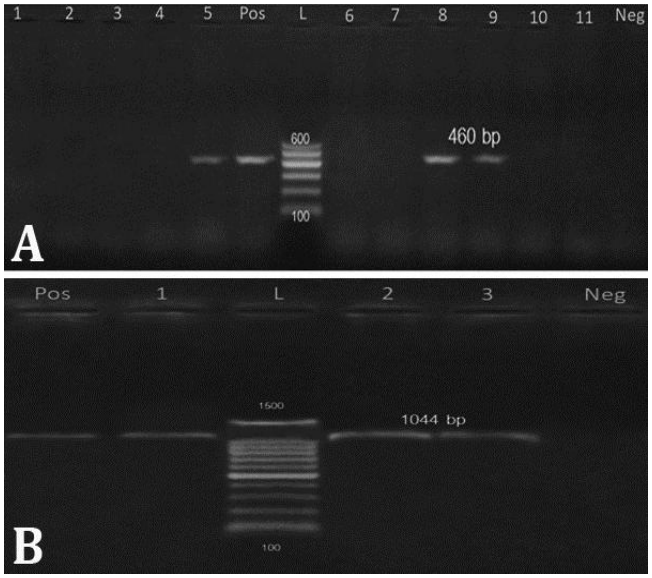

Fig. 2. A) Molecular confirmation of *Pasteurella multocida* isolates. Polymerase chain reaction for detection of *kmt1* gene in *P. multocida* isolates at amplicon size of 460 bp. Lane L: Molecular weight marker (100 - 600 bp, 100 bp plus DNA ladder; Vivantis, Shah Alam, Malaysia); Lane Pos: Positive control; Lanes 5, 8, and 9: Positive samples; Lanes 1 - 4, 6 - 7, and 10 - 11: Negative samples; Lane Neg: Negative control; **B)** Multiplex PCR showing *Cap A* (*hyaD-hyaC* gene) in *Pasteurella multocida* isolates at amplicon size of 1,044 bp. Lane L: Molecular weight marker (100 – 1,500 bp); Lanes 1, 2, and 3: Positive samples; Lane Pos: Positive control; Lane Neg: Negative control.

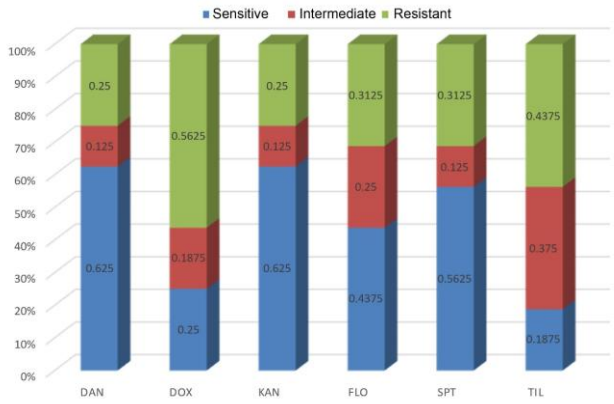

Fig. 3. Sensitive, intermediate and resistant isolates percentages for each antibiotics according to the minimum inhibitory concentration (MIC) results. DAN; Danofloxacin, DOX: Doxycycline, KAN: Kanamycin, FLO: Florfenicol, SPT: Spectinomycin, and TIL: Tilmicosin.

Discussion

Pasteurella species are thought to be the most common respiratory pathogens.[2] *Pasteurella multocida* causes enzootic bronchopneumonia in ruminants as well as hemorrhagic septicemia in cattle and buffalo.[6,8]

In the current investigation, the isolation rate of *P. multocida* was 13.00% being nearly similar to the former report detected *P. multocida* in 16.60% of pneumonic animals.[26]

Pasteurella multocida could be identified very quickly and accurately by PCR amplification carried out directly on bacterial colonies or cultures, allowing researchers to precisely estimate pasteurellosis consequences for the animal productions.[27] The *KMT1T7* and *KMT1SP6* primers yielded an amplified product being characteristic to all *P. multocida* isolates tested.[17]

All *P. multocida* isolates in this study were capsular type A, being comparable to the previous findings reported that all *P. multocida* isolates from cattle were belonged to this type.[28] Additionally, it was found that all isolates from sheep and goats were only capsular type A, indicating that type A is the predominant type of *P. multocida*.[26]

Since immunoprophylactic measures are frequently unsatisfactory or even ineffective, anti-microbials are widely used as prophylaxis for secondary bacterial infections, especially in case of viral diseases in cattle and therapy of diseases involving *Pasteurella* isolates; this accelerates the emergence of anti-microbial-resistant bacteria against recent antibiotics.[29,30] Observation of bacterial resistance from animals and its impact on public health status is one of the essential health threats of current time.[31]

This study used the broth microdilution method to examine the susceptibility of *P. multocida* isolates to various antibiotics and antibiotic alternatives. The results of MIC revealed that a high percentage (62.50%) of tested *P. multocida* isolates were MDR to various antibiotics; this finding is nearly similar to previous reports indicating a high percentage of MDR isolates in cattle in North America, Germany, and Switzerland.[32-34]

Our study showed a higher resistance percentage for different tested antibiotics as 56.25, 43.75, 31.25, 31.25, 25.00 and 25.00% for doxycycline, tilmicosin, florfenicol, spectinomycin, danofloxacin and kanamycin, respectively. These findings agree with reports recorded tetracycline resistance as 57.47%,[33] and tilmicosin resistance as 41.90%.[35]

Table 4. Number of isolates inhibited and their percentage with different dilutions of nanoparticles.

Concentration	AgNPs (µg mL^{-1})			CNPs (µg mL^{-1})			CurNPs (µg mL^{-1})		
	6.25	3.125	1.56	625	312	156	512	256	128
Number of inhibited isolates	4	20	8	6	18	8	18	10	4
Percentage	12.50	62.50	25.00	18.75	56.25	25.00	56.25	31.25	12.50

AgNPs: Silver nanoparticles; CNPs: Chitosan nanoparticles; and CurNPs: Curcumin nanoparticles.

Our findings disagree with reports found that 62.10% of isolates were not susceptible to danofloxacin in the USA,[36] and 100% of isolates were resistant to kanamycin in China.[23]

Time series analysis demonstrated ongoing increases in antibiotic resistance in *P. multocida* strains recovered from cattle, as being reported formerly that isolates from cattle exhibited the greatest percentage of resistance in comparison with other animals like sheep, rabbits, dogs, and cats.[37]

Despite several studies reporting a high percentage of non-susceptible bacteria to different antibiotics, great diversity in susceptibility of *Pasteurellaceae* family to the tested anti-microbial agents is evident, which may be due to the difference in the intensity, frequency, and commonly used antibiotics in veterinary practices from country to country and from period to another.[32]

Anti-microbial nanoparticles as metals and organic materials gained great importance in substituting conventionally used antibiotics with satisfactory effect against MDR bacteria.[10]

Our results indicated that antibiotic alternatives in combination with nanotechnology are effective against tested *P. multocida* strains. Considering AgNPs, most of the isolates (20; 62.50%) were inhibited at 3.125 μg mL^{-1}, this report is similar to previous one recorded an average MIC of 3.90 μg mL^{-1} against various tested Gram-negative bacteria[38], and nearly similar to another one recorded the range of MIC for MDR *P. aeruginosa* as 1.406 - 5.625 μg mL^{-1}. [39]

In agreement with the preceding findings, the current study reported that most of the tested isolates (18; 56.25%) were inhibited at a concentration of 312 μg mL^{-1} of CNPs.[13] Our findings disagree with preceding ones reported a higher MIC value of CNPs at 1,200 μg mL^{-1} for *Mycobacterium*. [40]

In the present study, the MIC value for most of the tested isolates (18; 56.25%) against CurNPs was 512 μg mL^{-1}. In comparison with previous studies, a great difference in MIC concentrations is noted ranging from 32.00 μg mL^{-1} to more than 1,200 μg mL^{-1}. [12,14,41]

Wide variations in MIC values of the used nanomaterial antibiotic alternatives are noted between different studies depending on the particle size, shape, and surface area of the used nanoparticles, type and concentration of the solvent used, species and isolates of the tested bacteria, environmental factors including pH, and laboratory variation.[10,11]

To the best of our knowledge, limited studies were interested in testing antibiotic alternatives against *P. multocida* isolated from animals showing respiratory manifestations.

In conclusion, MDR *P. multocida* is evident in Egypt. The findings highlight the importance of veterinarians and farmers to use antibiotics more prudently in treating pneumonia in cattle, as well as the rising threat of the spread of *P. multocida* strains resistant to most anti-microbials, which could complicate the future management of pneumonia in these animals. Antibiotic alternatives used in this study as nanoparticles were effective *in vitro* against MDR *P. multocida*. Antibiotic alternatives must have standard procedures to help obtain clear results, interpretation, and future practical application in the veterinary field.

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Conflict of interest

Authors declare no potential conflict of interest associated with this manuscript.

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