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Effect of Silver Nanoparticles on Biofilm Formation by *Clostridium perfringens* Isolated from Poultry and Molecular Typing of Strains by ERIC-PCR

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Abstract

The aim of this is study was to evaluate of antibacterial activity of silver nanoparticles (AgNPs) on multidrug resistant (MDR) *C. perfringens* strains isolated from broilers as an alternative to conventional antibiotics and Molecular typing of the *C. perfringens* strains using ERIC-PCR for assessment of genetic relationship between strains isolated from different organs. A total of 20 isolates of *C. perfringens* were isolated from necrotic enteritis affected poultry located in El-Behera Governorate, Egypt. Antimicrobial resistance pattern was evaluated. ERIC- PCR genotyping of *C. perfringens* isolates was performed to detect the genetic diversity and the degree of similarity between isolates. Antibacterial activity of AgNPs on MDR strains was evaluated by the minimum inhibitory concentration (MIC) and, minimum bactericidal concentration (MBC). Transmission Electron Microscopy (TEM) imaging was conducted to evaluate the effect of silver nanoparticles on *C. perfringens* cells. Scanning Electron Microscopy (SEM) imaging used to evaluate the effect of AgNPs on *C. perfringens* biofilm formed on glass cover slide for different periods of incubation with the same concentration of silver nanoparticles. Out of 85 collected samples from broiler chicken farms, 20 isolates of *C. perfringens* type A were isolated from broiler chickens at El-Behera governorate, Egypt. Most of isolates were MDR. ERIC PCR genotyping classified the isolates into 4 clusters (C1-C4). MBC was 30ug/ml, while MIC was 20ug/ml and the bacterial growth completely inhibited after 24 hours. TEM and SEM images showed that AgNPs has exerted broad-spectrum bactericidal activity against MDR *C. perfringens* isolate and against biofilm.

KEYWORDS
MDR *C. perfringens*, ERIC PCR, MIC, MBC, AgNPs.

INTRODUCTION

Necrotic enteritis (NE) is one of the most important disease in poultry industry caused by *C. perfringens*, which leads to economic losses in poultry industry between $2 and 6 billion USD worldwide annually (Wade and Keyburn, 2015). *C. perfringens* is Gram +ve bacilli, anaerobic and sporulated bacteria which habitat in the gastrointestinal tract (Ali *et al*., 2020). *C. perfringens* colonial morphology appear as blackish which in turn reacts with iron and forms a black iron sulfide precipitate and glistening colonies surrounded by double zone of hemolysis on sheep blood agar after 48 h of incubation at 37ºC under anaerobic condition (Craven, 2000; Craven *et al*., 2001).

C. perfringens not only the causative agent of NE disease, but there are predisposing factors as diets high in non-starch polysaccharide grains and fish meal protein as well as coccidia infection have been shown to influence the outbreak of NE (Moore, 2016).

C. perfringens produce various toxins and enzymes which play a role in its pathogenicity (Kiu and Hall, 2018). Based on the diversity in its produced toxin, *C. perfringens* is classified into 7 subtypes, including A (α), B (α, β, ε), C (α, β), D (α, ε), E (α, ι), F (α, *Cpe*) and G (α, NetB) (Rood *et al*., 2018). For years, studies have focused only on toxins, their phospholipase C, produces hydrolysis of the cell membrane phospholipids and causes cell death. The function of CPA in the generation of NE was, however, returned when CPA-deleted mutants maintained full virulence *in vivo* as a result the NetB toxin was discovered (Keyburn *et al*., 2006; Paiva and McElroy, 2014). Recently, two novel types of toxins (F and G) were described, Type F produce enterotoxin (*cpe* gene), which is the causative agent of food poisoning disease, while type G produces NetB toxin (*netB* gene) which causes NE in the broiler chickens (Anju *et al*., 2021). NetB is a spore-forming toxin, it is the major virulence agent (Profeta *et al*., 2020). It is in charge of the new strain (type G) synthesis which was previously belonged to *Clostridium perfringens* type A (AKM *et al*., 2021). NetB +ve strains can produce lesions of NE, while -ve strains unable to induce NE lesions (Keyburn *et al*., 2008). The NetB toxin causes pores eukaryotic and prokaryotic cell membrane, permitting ions leading to osmotic cell lysis (Datta *et al*., 2014).

Antibiotic resistance has spread more widely as an outcome of improper and excessive use of antibiotics in chicken farms (Sofos, 2008; Kimera *et al*., 2020; Ma *et al*., 2021) and increasing the incidence of multi drug resistance (MDR) (Manyi-loh *et al*., 2018). Haider *et al*. (2022) reported that all strains of *C. perfringens* were MDR with higher resistance against Neomycin, Trimethoprim,

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Tetracycline and Lincomycin which were used in poultry farms. Agnes *et al*. (2018) discovered that pathogens with multidrug resistance react differently when co-pathogens, commensals, probiotics, and host cells are present. Commensal species, especially those isolated from intra-abdominal and wound infections, affect the expression of antibiotic resistance genes, virulence factors, and eventually pathogenicity of bacteria.

Multiplex PCR can be carried to detect more than toxin genes, so it is used for typing of *C. perfringens* toxin (Fahimeh *et al*., 2018). However, this typing method has some limitations in strain-typing. Thus, new typing methods, including MLST (Nakano *et al*., 2017) and ERIC-PCR (Yuan *et al*., 2010) have been employed to investigate the genetic relationship of bacterial isolates and traceability.

ERIC-PCR fingerprinting is one of the molecular methods; its strategy is to explore specific bacterial genome sequences. The targets are extra genic regions of bacterial genomes that include 126 bp-long core inverted repeats that are highly conserved. Using consensus primers, researchers have effectively subtyped Gram -ve bacteria and examined the variety of Gram +ve bacteria (Ranjbar *et al*., 2009; 2011; Ruiz *et al*., 2011).

Referring to the available literature concerning the use of ERIC PCR for investigation of the genetic diversity and molecular relatedness of any pathogenic anaerobic bacteria only few investigations has been published for *C. perfringens* by Xue-qin *et al*. (2009) who found 12 AFLP genotypes and 8 ERIC-PCR genotypes, Zhong *et al*. (2017) who found that the ERIC-PCR pattern showed 14 genotypes (I-XIV), most of which came from different clones, and the similarity of the 2 clone strains were 99%, and Xiaoting *et al*. (2021) who stated that isolates were divided into 6 genotypes (I-VI) by ERIC-PCR method.

Evaluation of the clonal relatedness between *C. perfringens* strains from poultry is necessary to determine origin and method of infection and contribute to enhance the efficacy of the surveillance and provide evidence to health control measures as previously mentioned by Tenover *et al*. 1997 (2013) and Ranjbar *et al*., 2014). Biofilm formation for *C. perfringens* enhancement of the disease production was due to bacterial biofilm that adhere bacteria to hot cell surfaces, then colonization and infection. But help bacteria to survive unfavorable conditions (Semenyuk *et al*., 2014). Biofilm play role in drug resistance which protect *C. perfringens* from high concentrations of antibiotics (Charlebois *et al*., (2014). Donelli *et al*. (2012) mentioned that *C. perfringens* can forms biofilms from mono, dual and mixed species (Pantaleon *et al*., (2014).

The nano scale materials have antimicrobial agents due to their high surface area to volume ratio and their biological properties (Morones *et al*., 2005; Helenius, 2007).

Now days, there are interest for the use of silver as an antimicrobial agent. Its mechanism depends mainly on Metallic compounds and its ions which have antibacterial properties; but, because ionic silver has toxic effect, so is limited in application. The nanosize of silver used range (1–100 nm) and to have large functional surface area (Luoma 2008). Based on nanoscale of AgNano showed unique biological properties and antibacterial activity against Gram-ve and Gram+ve bacteria (Lara *et al*., 2010).

In poultry production, using of AgNano as an alternative method to antibiotic based growth promoters. Silver has harmful effect on microorganisms (Percival *et al*., 2005), and may also adjusted the digestive microbiota. The application of AgNano could affect the microbial growth, immune response and increase growth of broilers. Silver has antibacterial effect which specially used in industry it has a key role in healthcare systems (Jiang *et al*., (2004).

Hemeg *et al*. (2017) noted that combining the antibacterial agent with Nano materials overcoming the drug resistance problem and reduce the undesired side effect to some extent. Siddiqi *et al*. (2018) reviewed that nanoparticles can exert their bactericidal effect through many ways such as disruption of bacterial cell membrane, prevention of biofilm formation, damage of proton efflux pump, excitation of the innate host immune responses, induction of oxidative stress by ROS generation and DNA and or protein damage.

The possibilities of using silver nanoparticles in the filed study are promising. However, we need to ensure AgNano interacts with the organism *in vivo* and study its mechanism *in vivo*.

Therefore, this research was done to assess the genetic relationship of *C. perfringens* strains isolated from poultry, its antibacterial resistance and effect of AgNPs through electron microscopy imaging.

MATERIALS AND METHODS

Ethical approval

All experimental design was approved by the Animal Care and Use of laboratory animals committee of Alexandria University. All methods were carried out in accordance with relevant guidelines and regulations (Ethics Committee, Faculty of Veterinary Medicine, Alexandria University, Egypt).

Bacterial strains

A total of 20 *C. perfringens* isolates were isolated from 85 samples from caecum and liver of broiler chickens about two weeks of age suffering from necrotic enteritis were identified according to Smith and Holdeman (1968). Isolation and identification of *C. perfringens* from samples carried according to Quinn *et al*. (2002).

Antibiotic Susceptibility Test

This was done utilizing the Muller Hinton agar (Sparks, MD-based Becton, Dickinson and Company) agar disk diffusion technique in compliance with the standards and recommendations of the Clinical and Laboratory Standard Institute (CLSI, 2014).

Briefly, a sterile cotton swab was used to streak A 0.5 MacFarlane standard on Muller Hinton agar plates. These antibiotic discs were utilized (BBL: Becton, Dickson and Company, NJ): Imipeneme (IPM 10 µg), Ampicillin (Amp. 10 µg), Amoxicillin (Ax30 µg), Penicillin (P 10 µg), Lincomycin (L 2 µg), Vancomycin (VA µg), Colistin (CLM10 µg), Cotrimoxazole (COT 25P), Chloramphenicol (C 30) and Tylosine (TL15). The size of the inhibition zone was used to gauge each strain's susceptibility, which was then interpreted utilizing CLSI (2014) standards.

Molecular typing by PCR and ERIC PCR

Genomic DNA was extracted using kit (Qiagen, Germany) and used for the amplification of 16S rRNA gene of *C. perfringens* isolates. Then, the amplified products were purified using DNA extraction kit (TaKaRa, Japan) subjected to PCR. We examined twenty isolates by PCR for molecular characterization of *C. perfringens* using Cpa (Alpha) primers (Yoo *et al*., 1997); Forward: GTTGATAGCGCAGGACATGTTAAG, and Reverse: CATGTAGTCATCTGTTCCAGCATC; ERIC-PCR typing was preformed (Versalovic *et al*., 1991) using ERIC primers: ERIC-1 (5\-ATGTAAGCTCCTGGGGATTCA-3\) and ERIC-2 (5\-AAG TAA GTG ACT GGG GTG AGC G-3\).

Thermo scientific, Waltham, Massachusetts, USA, developed Gel Image LabTM software to evaluate the electrophoretic bands of the PCR-amplified products, and NTSYSpc 2.10e to create a clustering dendrogram. Each isolate was treated as a separate taxonomic unit (OUT), and strains that were identical to one another ≥ 90% were assumed to have the same origin (Yuan *et al*., 2010).

Quantitative biofilm production assay of C. perfringens

Quantitative biofilm production assay was done according to the method of Stepanovic *et al*. (2000). The bacterial strains were cultivated anaerobically for 24 to 72 hours at 37°C in pre-reduced BHI broth. In each well of a 96-well tissue culture plate, 20 µL of the broth culture (adjusted to 0.5 McF) and 180 µL of fresh BHI enriched with 1% glucose were added. A well with fresh BHI enriched with 1% glucose and no microorganisms was employed as a control. The plate was sealed with a lid and kept anaerobically warm (37°C) for 48 hours. Three times each test was carried out in triplicate. Utilizing a micro plate photometer, the optical density (OD) of each well was determined at 570 nm (Multiscan FC; Thermo Scientific).

Evaluating the antimicrobial effect of Ag-Nps

MIC and MBC determination

Bacterial growth inhibition test

A single colony of bacteria from an MDR strain that was clinically pathogenic was added to BHI broth and cultured anaerobically at 37°C for 24-48 hours in a shaking incubator (144 rpm). To calculate the optical density at OD600, bacterial cultures were diluted in BHI broth to a final concentration of 106 CFU/ ml, which was verified by spectrophotometer. According to Kascat-an-Nebioglu *et al*. (2006) and Hindi *et al*. (2008), Silver Nano particles (Ag-NPs) were diluted using the broth micro dilution technique. In a 96-well microliter plate, diluted bacterial cultures were combined with equal quantities of each dilution, and they were then incubated anaerobically at 37°C. The optical turbidity of the wells before and after incubation was utilized to assess the MIC. Three duplicates of the test were run to confirm its value for the tested bacteria.

Minimum bactericidal concentration (MBC)

Nanoparticle concentrations that inhibited bacterial growth was further diluted to assess the MBC (at which no growth of bacterial was noted on the plates). Aliquots of 50 µl from all wells that had no growth after the Ag-NPs' MIC determination were seeded on blood agar plates that weren't enriched with Ag-NPs and incubated anaerobically for 24 hours at 37°C to identify the MBC.

Ultra structural interaction between Ag-Nps and C. perfringens

This was done at the Faculty of Science, Alexandria University, Egypt. Samples were placed within gelatin capsules before being cut into ultrathin slices using an ultra-microtome. We employed the JEOL JEM-1400 plus for transmission electron microscopy (TEM) imaging (JEOL USA).

Evaluation of the impacts of Ag-Nps on C. perfringens under TEM

In order to microscopically observe the bacterial interaction with Ag-Nps, two bacterial groups were studied by TEM. The first group was the untreated bacterial cells that were used as negative control for the normal bacterial cells. The second group was the treated bacterial cells with AgNPs (20 ug/ml) (MIC).

Detecting the effect of Ag-Nps on C. perfringens and biofilm formation under SEM

Scanning electron microscopy (SEM) was utilized to assess the potential of specific bacterial strains to create biofilm; each well of a 24-well plastic tissue culture plate was filled with 200 lL of a broth culture of the *C. perfringens* strain (adjusted to 0.5 McF) and 1.8 mL of pre-reduced BHI broth containing 1% glucose. The plate was then incubated for 48 hours at 37°C under anaerobic conditions (Donelli *et al*., 2012), with a slight modification to this method by the addition of the MIC of Ag-Nps (20ug/ml) for 10, 15, 20 minutes, 1 hour and for 24 hours. One cover slide without the addition of Ag-Nps was used as a negative control (normal biofilm).

RESULTS

Gross lesions

Included depression, ruffled feathers, and diarrhea. The gross lesions were primarily found in the small intestine (jejunum/ileum), which was ballooned, friable, and contained a foul-smelling, brown fluid. The mucosa was covered with a tan to yellow pseudo membrane (Turkish towel in appearance).

Phenotypic identification of C. perfringens

The mucosal scraping exhibited large, Gram-positive rods straight with parallel sides and smooth ends and sometimes had central or sub terminal oval non protruding endospores.

Histopathological findings

Histopathological examination showed coagulative necrosis of one-third to one-half the thickness of the intestinal mucosa and masses of short, thick bacterial rods in the fibrino necrotic debris.

Prevalence of C. perfringens in broiler chicken

Out of 85 collected intestine samples, 28 showed diarrhea, necrotic enteritis yielded 20 isolates showed blood agar with a double zone of hemolysis (Fig. 1) and lecithinase activity on egg yolk agar medium (Fig. 2).

Fig 1. *Clostridium perfringens* growth on Neomycin Sheep Blood Agar Medium with characteristic double zone of hemolysis.

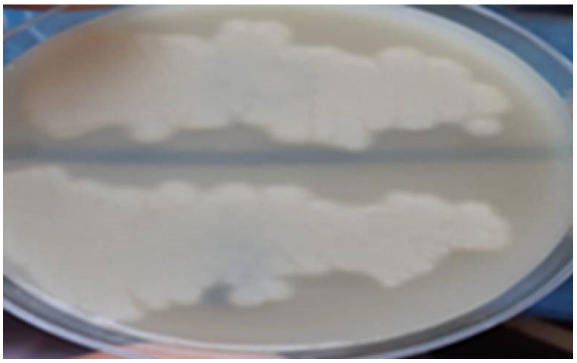

Fig. 2. Opaque zone due to lecithinase action of alpha toxin of *C. perfringens* on egg yolk agar medium.

Interpretation of antibiotic sensitivity test

All strains were MDR highly resistant to Colistin (Polymexine B) (100%), Lincosamides (Lincomycin) (100%), Sulfonamides (Cotrimoxazole) (100%) and Macrolides class (Erythromycin) (100%), Tylosine (macrolides class) (100%).

Biofilm formation of C. perfringens

11 isolates produced moderate biofilms, but only 1 isolate produced weak biofilm.

PCR and ERIC PCR

The twenty studied isolates were defined as *C. perfringens* as all isolates revealed (alpha-toxin) that formed a clear band at 402bp. as showed in Figure 3.

ERIC PCR

The fifteen isolates showed different banding patterns as showed in Figure 4. ERIC-PCR analysis revealed the presence of 4 clusters; cluster I included two strains (9 and 15) recovered from the liver with a similarity ratio about 97% between them and 2 strain (13,7) that recovered from the intestine with a similarity ratio about 92 % between them, cluster II included 2 strains (1,3) that were recovered from the liver with a similarity ratio about 96%, cluster III which contained 4 strains (8,12, 2,5) that recovered from the intestine with a similarity ratio about 99 % and one strain recovered from the liver (11) with a similarity ratio about 92%, cluster IV contained 2 strains (10,14) that recovered from the intestine with a similarity ratio about 99 % and 2 strains were recovered from the liver (4,6) with a similarity ratio about 99% as shown in Figs. 5, 6.

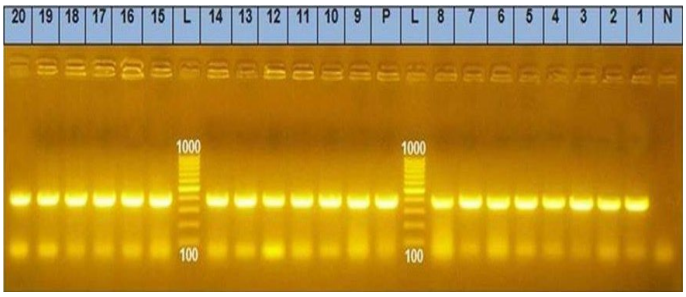

Fig. 3. Agar gel electrophoresis of PCR products of *C. perfringens* isolates from broiler chickens for 20 samples for alpha toxin at 402 bp and agarose gel 1.5% stained by Ethedium bromide.

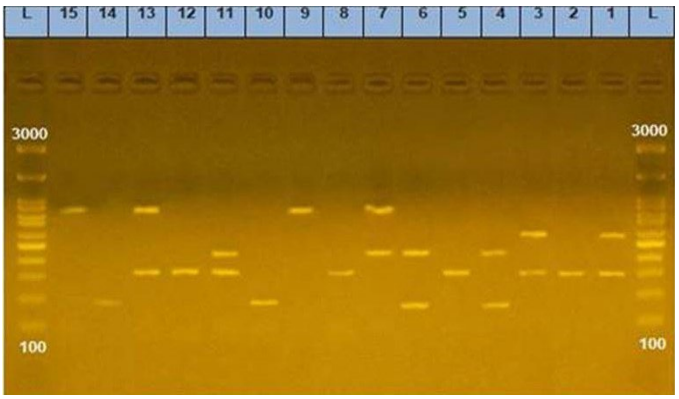

Fig. 4. ERIC PCR showing different bands in each 15 clinical isolates of *C. perfringens*. Lane L =100-3000 bp ladder. Lanes 1,3,4,6,9,15,11: *C. perfringens* isolates from intestine. Lanes 2,5,7,8,10,12,13,14: *C. perfringens* isolates from liver

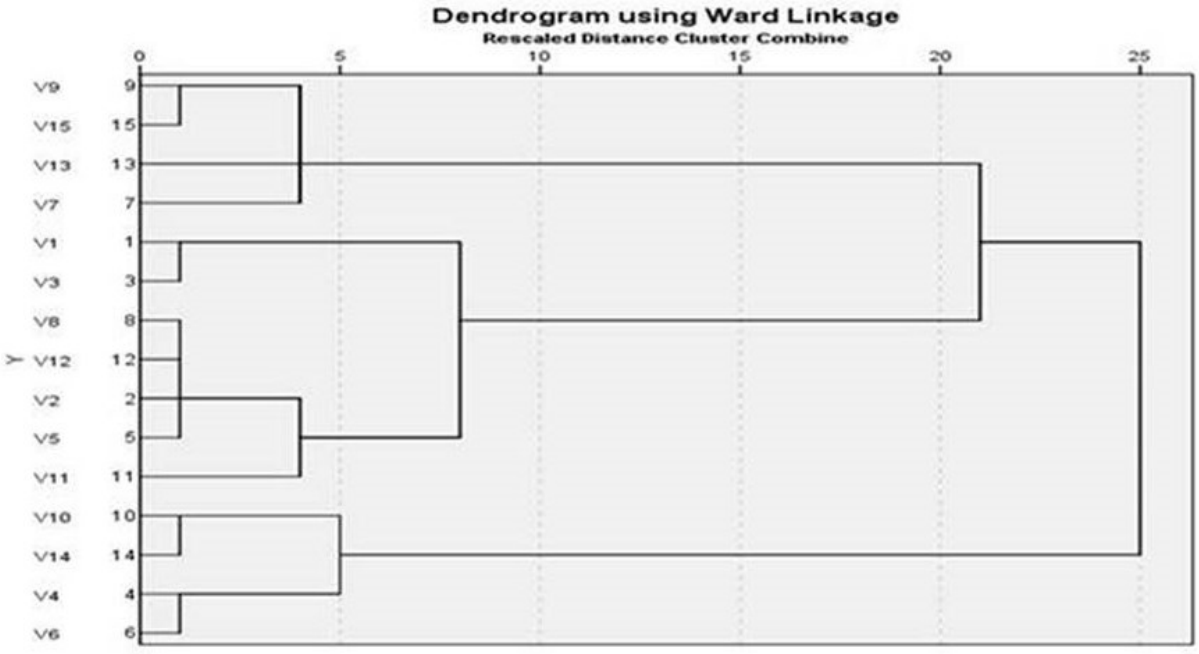

Fig. 5. Dendrogram analysis of fifteen *Clostridium perfringens* strains reveals that *C. perfringens* isolates could be divided into 5 genotypes.

No of intersecting elements	Jaccard / Tanimoto Coefficient														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1		0.5	1	0	0.5	0	0.33	0.5	0	0	0.33	0.5	0.33	0	0
2	1		0.5	0	1	0	0	1	0	0	0.5	1	0.5	0	0
3	2	1		0	0.5	0	0.33	0.5	0	0	0.33	0.5	0.33	0	0
4	0	0	0		0	1	0.33	0	0	0.5	0.33	0	0	0.5	0
5	1	1	1	0		0	0	1	0	0	0.5	1	0.5	0	0
6	0	0	0	2	0		0.33	0	0	0.5	0.33	0	0	0.5	0
7	1	0	1	1	0	1		0	0.5	0	0.33	0	0.33	0	0.5
8	1	1	1	0	1	0	0		0	0	0.5	1	0.5	0	0
9	0	0	0	0	0	0	1	0		0	0	0	0.5	0	1
10	0	0	0	1	0	1	0	0	0		0	0	0	1	0
11	1	1	1	1	1	1	1	1	0	0		0.5	0.33	0	0
12	1	1	1	0	1	0	0	1	0	0	1		0.5	0	0
13	1	1	1	0	1	0	1	1	1	0	1	1		0	0.5
14	0	0	0	1	0	1	0	0	0	1	0	0	0		0
15	0	0	0	0	0	0	1	0	1	0		0	1	0	

Fig. 6. Similarity index of isolates (degree of similarity between isolates) was evaluated using Simpson's diversity index as provided by Hunter and Gaston (1988).

Evaluating the antimicrobial effect of Ag Nps

Results of MIC and MBC

By measuring the actual decrease in cell viability (cfu/ml) at intervals of 8, 16, and 24 hours, the bactericidal activity was assessed. By 8 hours, growth began to slow down, and at 24 hours, it was completely inhibited. After 24 hours of incubation under anaerobic condition at 37ºC, turbidity was noticed in all wells ranging from 2.5 to 15 ug/ml concentrations of Ag-NPs indicating growth of bacteria, very weak and no turbidity was seen in wells of 20 and 30 ug/ml exhibiting inhibition of bacterial growth. Blood agar plates were inoculated with the suspension from the wells at 20 and 30ug/ml and incubated for 24 hours. In the concentration of 30ug/ml, no bacterial growth was seen. Bacterial growth at different concentrations of Ag- NPs was assessed after (18-24) hours, the MIC and MBC of *C. perfringens* was observed maximum for a concentration of 20ug/ml, and 30ug/ml, indicating it has both bacteriostatic and bactericidal activity. These results thus confirm that the MIC and MBC of Ag-NPs was found to be effective at dilution of 20 ug/ml.

Effect of Ag-Nps on biofilm formed by *C. perfringens* under SEM

Figure 7 shows the formation of biofilm micro colony and EPS (Exo Poly Saccharides) of 48 h old *C. perfringens* biofilm that was grown anaerobically at 37°C on a glass cover slide.

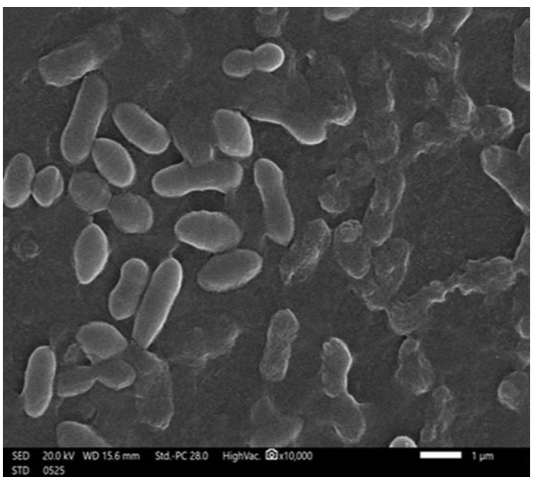

Fig. 7. SEM image showing the formation of biofilm microcolony and EPS (Exo Poly Saccharides) of 48h old *C. perfringens* biofilm grown anaerobically at 37°C on a glass cover slide (control).

As shown in Figure 8, no significant changes in the external appearance of cells were observed after treatment with Ag NPs for 10 minutes. However, not all cells were damaged; there were still many cells in normal size with intact intracellular structures and well-organized intracellular contents.

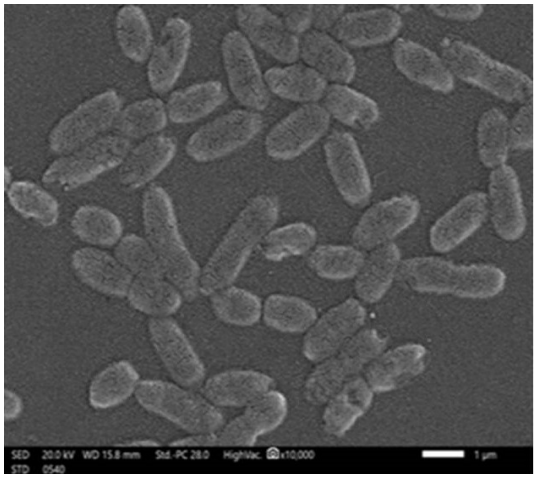

Fig. 8. SEM image of *C. perfringens* biofilm treated with 20ug /ml of Ag NPs for 10 minutes.

Cell membrane deformation was seen in treated cells and the density and amount of biofilm decreased as show in Fig. 9.

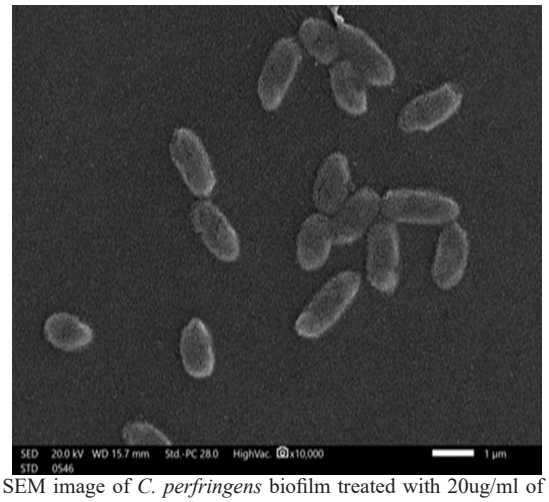

Fig. 9. SEM image of *C. perfringens* biofilm treated with 20ug/ml of Ag NPs for 15 minutes.

Cell membrane deformation and intracellular contents leakage were seen in treated cells and marked decrease in the amount of biofilm as shown in Fig. 10

As shown in Fig. 11, treated cells showed membrane damage after 1 hour

As shown in Fig. 12, bacterial strains exposed to Ag NPs for

24 h showed the highest antimicrobial effect. Bacterial cells were surrounded by Ag NPs and null bacterial cells were found in bacterial sample treated with Ag NPs, indicating that intracellular contents had leaked from cells due to damage to cell membrane.

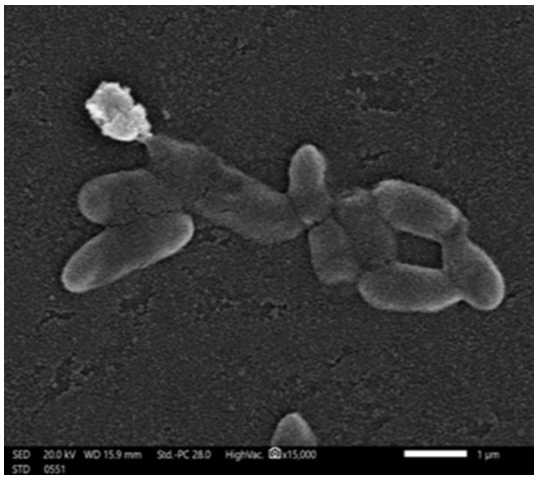

Fig. 10. SEM image of *C. perfringens* biofilm treated with 20ug/ml of Ag NPs for 20 minutes.

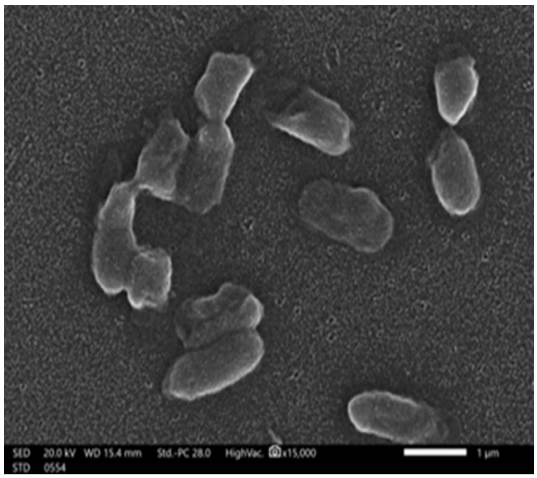

Fig. 11. SEM image of *C. perfringens* biofilm treated with 20ug/ml of Ag NPs for1 hour.

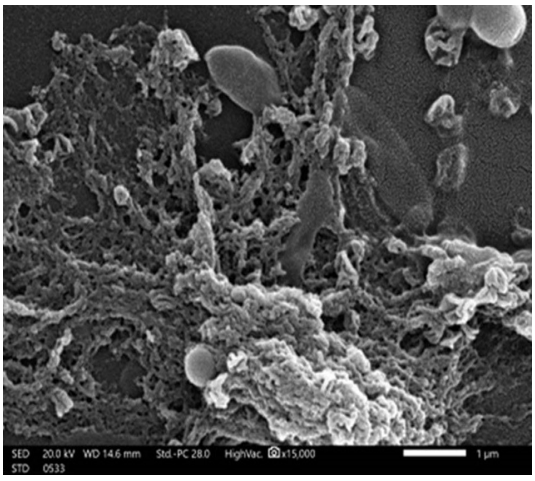

Fig. 12. SEM images of *C. perfringens* biofilm treated with 20ug/ml of Ag NPs for (18-24) hours.

TEM Results

Control sample (untreated)

The bacterial cells were normal in size with intact intracellular structures and well-organized intracellular contents.

The micrographs of untreated cell culture (A and B) without exposure to Ag Nps showed continuous thin, smooth cell walls and other defined cellular structures as shown in Fig 13.

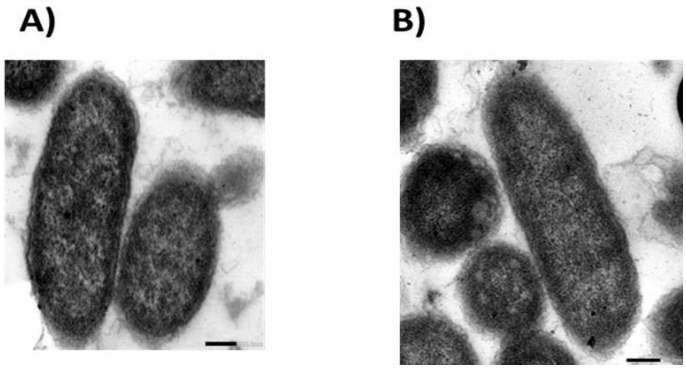

Fig. 13. TEM images of *C. perfringens* (UN treated with Ag Nps).

Treated sample

Many deformed cells and spiral shaped cells were observed in the treated samples. Damaged cells in the treated samples indicated leakage of cell contents. However, deformation of cell membrane and intracellular structure were observed, as shown in Figure 14. Granulation of intracellular material was already observed (A). Cells showed retracting cytoplasm (B). Cytoplasmic membrane degradation and complete cell lysis (C).

As shown in Fig 14, Ag Nps treated *C. perfringens* cells (A, B, and C) showed adulterated morphology, where cell walls had irregularities, less smoothness, less uniformity, and degenerative changes leading to wall ruptures and subsequent cellular lysis in some cases. An unequal cytoplasm distribution caused by the clumping and agglomeration of intracellular material was observed in the treated cell. Furthermore, the cells lacked cytoplasm in certain regions due to the loss of membrane functionality.

Ag Nps-treated bacterial cells showed small dense staining aggregate clusters on the bacterial cells when bacteria were viewed using TEM. It seems that *C. perfringens* can be exposed to high concentrations of (accumulate) more silver with keeping integrated cell wall with no lysis. The lysed bacteria showed detachment of the cell wall from cytoplasmic membrane.

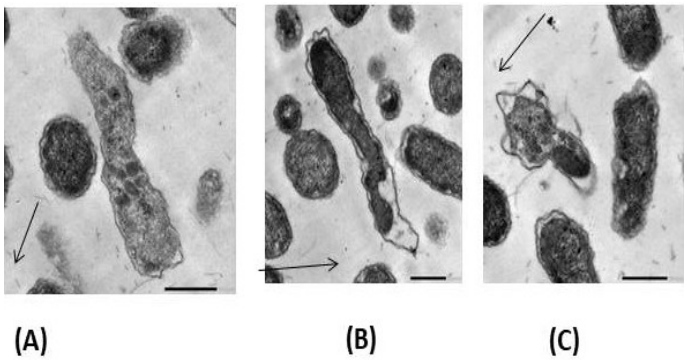

Fig. 14. TEM images of *C. perfringens* treated with 20ug/ml of AgNPs for 24 hours (A, B and C).

DISCUSSION

Taxonomic identification within the *C. perfringens* types have been subjected to considerable debate and differentiation at species level is somewhat perplexed. Based on their capacity to generate four primary toxins, *C. perfringens* is divided into five

toxin types (A to E). These toxins include Alpha (α), beta (β), epsilon (ε) and iota (ι) (Yoo *et al*., 1997; Schlegel *et al*., 2012).

Inspite of the expansion of the classification of *C. perfringens* into seven toxin types (types A to G) (Shrestha *et al*., 2018) *C. perfringens* type F is a global food safety concern, in the USA, Canada, and some developing countries (Charlebois *et al*., 2017; Shrestha *et al*., 2018) and the reports by Keyburn *et al*. (2008); (2010) and Uzal *et al*. (2018) demonstrated that some strains of *C. perfringens* generate a pore-forming toxin called NEB like (NetB), which is crucial in the pathogenesis of NE.. A study by Aboreeda, (2016) showed that type A is the most encountered in this disease. Twenty strains of *C. perfringens* type A were isolated, identified phenotypically and confirmed genotypically from necrotic enteritis chicken reared in different poultry farms at El-Behera governorate, Egypt this agree with Carman *et al*. (2008) and Aboreeda(2016). The Twelve (12) *C. perfringens* strains were producing biofilm (100%), 11 strains were moderate biofilm producers, while one isolate was weak biofilm producer. All 12 strains were MDR which means that biofilm formation can lead to drug resistance. This nearly agreed with Charlebois *et al*. (2017) who illustrated that 96.7% of *C. perfringens* isolated from poultry showed biofilm production. Moreover, clinical strains were moderate and strong biofilm producers, and nearly agreed with Ghareib *et al*. (2018) who recorded that all *C. perfringens* isolated in their research revealed multiple-antibiotic resistance and mostly were biofilm producers.

Generally, a clear determination of strain as well as its type diversity is necessary for conformation of the pathogens and its distinguishment from member of the same species. ERIC PCR in our investigation categorized the 15 *C. perfringens* type A strains into 4 genotypes (I-IV) while, Xue-qin *et al*. (2009) typed 3 *C. perfringens* isolates of type A by ERIC PCR and found 8 genotypes. The subtype of *C. perfringens* appeared to be simple within single farm and mixed with other gene subtypes. The dominant gene subtype was ERIC PCR gene type 1. These results reveal that the diversity of *C. perfringens* in healthy chickens in Sichuan province is low and there is close correlation between epidemiology and regions. In another study by Xiaoting *et al*. (2021) who detected 6 genotypes (I-VI) using ERIC-PCR, confirming that several genotypes occurred in various locations of Xinjiang, sometimes even in the same regions, demonstrating blatant genetic variety.

Our results infer a close relation between organ for isolation and regions of farms this shows obvious genetic diversity. Prior studies have shown that healthy birds have a larger genetic variety of *C. perfringens* than those animals with severe NE (Nauerby *et al*., 2003; Lyhs *et al*., 2013). Most *C. perfringens* strains discovered from NE outbreaks are resistant to gentamicin and streptomycin, two routinely used medicines (Park *et al*., 2015).

In fact, in Egypt, the antibacterials are used from one day old chicken up to slaughter in poultry farms. Every time an antimicrobial agent is used an increase in resistance toward that antimicrobial is developed by bacteria that express genes for resistance to that agent. According to Magiorakos *et al*., (2012), all strains were multi drug resistant (MDR) to Sulphamethoxazole trimethoprim, gentamycin, streptomycin, Colistin and Lincomycin. Few studies have documented the development of *C. perfringens* biofilms (Varga *et al*., 2008; Donelli *et al*., 2012).

Biofilm formation could play a role in the development of *C. perfringens* disease which helps bacteria adhere to host cell surface, colonization, and infection. Moreover, the ability to grow as a biofilm favors survival of bacteria in the environment (Semenyuk *et al*., 2014). There is relationship between drug resistance and biofilm, Kaplan (2011) demonstrated that low doses of antibiotics lead to biofilm formation. Ghareib *et al*. (2018) recorded that all *C. perfringens* isolated in their research revealed multiple-antibiotic resistance and mostly were biofilm producers.

In the current primary study of Ag NPs antimicrobial activity showing a greater sensitivity of *C. perfringens* and bacterial inhibition is directly related with Ag Nps concentration and exposure time. Colloidal nano silver is a suspension that inactivates the enzymes responsible for respiration, reproduction, and metabolism of the treated microorganisms. Oligodynamic impact of silver is one of its key features that has a great affinity for protein residues in cell membranes and sulfhydryl groups in the ionic state (Domínguez *et al*., 2020). This result in the inactivation of the enzymes necessary for the treated bacteria to breathe, reproduces, and metabolize. Each bacterium will react differently depending on its metabolic properties. Gram-positive bacteria have more negatively charged peptidoglycan layers in their cell walls than Gram-negative bacteria do. Teichoic acids in Gram-positive microorganisms' cell walls, which are substantially thicker than those of Gram-negative bacteria, inhibit the uptake of AgNPs (Feng *et al*., 2000; Rai *et al*., 2012; Dakal *et al*., 2016; Pazos-Ortiz *et al*., 2017; Domínguez *et al*., 2020). Domínguez *et al*. (2020) pointed out that colloidal silver caused the generation of reactive oxygen species in Gram-negative bacteria at lower and higher concentrations, but only to a considerably smaller level in Gram-positive bacteria. This may account for colloidal silver's slower bactericidal effect against Gram-positive pathogens. Kim *et al*. (2007) attributed that Gram-negative and Gram-positive bacteria vary in their cell wall thickness, composition, and structure, which may help to explain why Ag NPs dramatically reduced E. coli activity whereas S. Aureus had a lower sensitivity. Lipopolysaccharides' negative charge promotes the adherence of silver and increases bacteria's sensitivity to it (Dakal *et al*., 2016). Sondi and Salopek-Sondi (2004); Morones *et al*. (2005) and Choi *et al*. (2008) revealed that in the presence of oxygen and water, Ag NPs produced Ag+ ions. It's possible that the Ag+ ions that are released from the Ag NPs alter the actions of membrane-bound enzymes in bacterial cells and make ROS production easier (McDonnell and Russell, 1999; Pal *et al*., 2007). Notified, the inhibition of respiratory chain enzymes, a change in membrane permeability, and eventually cell lysis and death may arise from the electrostatic forces between Ag+ ions and the negatively charged cell membrane or wall (Ratte 1999; Sambhy *et al*., 2006).

It is currently unclear how exactly NPs' antibacterial properties work against various strains of bacteria. Finally, we demonstrated these pits using the SEM picture and verified the leaking of the internal cell contents. Metal depletion may result in the production of irregularly shaped pits on the outer membrane of cells, which may affect the membrane permeability and induce leakage of internal cell contents. Since they don't use electron microscopy, our observation using an electron microscopy image may serve as a nomination or at the very least serve as proof of the antimicrobial mechanism of Ag Nps, which involves the formation of pits with irregular shapes on the outer membrane that cause leakage of the internal contents of bacteria and the aggregation of silver inside damaged cells.

In the current research the MBC of Ag Nps against *C. perfringens* was 30 µg/ mL. Alsadwi (2018) reported that MBCs for all compounds (> 32 µg/mL) were >85 µM for SCCs and >192 µM for AgAc against *C. perfringens* type A. It is well known that Gram positive bacteria have higher MBC values than gram negative as the structure of cell wall is different. Sütterlin *et al*. (2012) revealed that Gram-positive bacteria had an MBC of silver ion that was more than 32 times greater. On the other hand, in anaerobic conditions, silver ions may lose a key antibacterial function and produce ROS. Because it may bind the thiol group when present within the cell and so impede the action of various enzymes, the Oligodynamic impact of silver ions is well recognized. Silver ions are known to inhibit thiol-containing enzymes like NADH dehydrogenase II in the aerobic respiratory chain, which is recognized as a major source of ROS formation (Messner and Imlay, 1999). Silver ions are known to inhibit thiol-containing enzymes (Lansdown, 2002) such as NADH dehydrogenase II. Gram-positive, obligate anaerobe *C. perfringens* employs nitrate (NO3) as the last electron acceptor during anaerobic respiration (Hasan and Hall, 1975). Additionally, the genome of *C. perfringens* has anaerobic fermentation enzymes that result in gas generation but lacks enzymes for the tricarboxylic acid cycle or respiratory chain (CO_2 and H_2) (Shimizu *et al*., 2002). As a result, *C. perfringens* cannot produce ROS because it does not decrease O_2 to produce reac-

tive products like hydrogen peroxide (H2O2), which is needed for the Fenton reaction to produce hydroxyl free radicals (Pesakhov *et al*., 2007). Furthermore, there is no published research comparing SCCs to Gram positive restricted anaerobic bacteria. Previous research on Staphylococcus aureus and E. coli utilizing silver nitrate in anaerobic and aerobic conditions. Despite the fact that silver ion's action in anaerobic circumstances differs from that under aerobic ones (Park *et al*., 2009).

CONCLUSION

Ag NPs has strong antibacterial activity against MDR *C. perfringens* strains. So, this study suggests the use of silver nanoparticles as antibacterial agent instead of traditional antibiotics to avoid the development of drug resistance. Silver nanoparticles are safe, cost-effective antibacterial agents. To the best of the authors' knowledge, this study is the first that focused on the potential alternative antimicrobial role of silver nanoparticles for control of multiple drug-resistant *C. perfringens* in Egypt.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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