Egyptian Journal of Animal Health

P-ISSN: 2735-4938 On Line-ISSN: 2735-4946
Journal homepage: https://ejah.journals.ekb.eg/

Evaluation of the efficacy of combination of glutaraldehyde and quaternary ammonium compound disinfectant against different isolated *C. perfringens* strains recovered from broilers

Reham A. Hosny, Azhar G. Shalaby, and Hend K. Sorour

Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Agricultural Research Center, Giza, Egypt

Received in 2/8/2023
Received in revised from 31/8/2023
Accepted in 6/9/2023

Keywords:

C. perfringens
disinfectant
TH4
toxin
concentration
Time
genes

ABSTRACT

Necrotic enteritis caused by *Clostridium perfringens* has been linked to severe economic losses in the poultry industry and represents a serious threat to public health due to spore-forming ability and its role as foodborne pathogen. Although chemical disinfection plays a role in preventing and controlling the spread of bacterial diseases in livestock and poultry housing, little is known about its efficiency against spore-forming *C. perfringens*. This study was designed to investigate the sporicidal activity of a combination of quaternary ammonium and glutaraldehyde disinfectant (TH4) against previously isolated ten *C. perfringens* strains according to European Standard EN 1276, 2019 application method at different concentrations (0.25%, 0.5%, 1%) with different contact times (0, 1, 5, 15, 30, 60 minutes). *C. perfringens* strains were identified, toxinotyped, and screened for the presence of QAC resistance genes (*qacED1 and qac*A/B). Five *C. perfringens* isolates were verified to produce toxinotype A, while the remaining five isolates were confirmed to produce toxinotype G. Screening for *qacED1 and qac*A/B resistance genes revealed their presence in six and four isolates, respectively. TH4 0.25% and 0.5% tested in this study didn't give the required five log10 CFU reduction in the spore count in five *C. perfringens* at all exposure times, while concentration of 1% achieved the required five log reduction in the count of *C. perfringens* spores for relatively long contact times of 60 minutes in five *C. perfringens* type A isolates. These findings recommend the use of concentration of TH 4 1% and increase exposure time to 60 minutes to obtain effective sporicidal activity against *C. perfringens* strains.

INTRODUCTION

Clostridium perfringens (C. perfringens) is a significant pathogen with clinical, food, and veterinary concerns (**Mora et al., 2020). It** causes foodborne infections in humans that are usually linked to the ingestion of contaminated chicken meat, it produces clinical or subclinical necrotic enteritis in broilers (Mora *et al.,*

Corresponding author: Reham A. Hosny; Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Agricultural Research Center, Giza, Egypt, postal code: 264
E-mail address: rehamhosny87@yahoo.com
DOI: 10.21608/ejah.2023.316968

2020). Necrotic enteritis is an opportunistic infection caused by Gram-positive, anaerobic spore-forming *C. perfringens bacterium in chickens* resulting in severe economic losses to the global poultry industry (**He et al. 2022).**

The presence of risk factors can affect the physical properties of the gut, the immunological status of birds, and interfere with the gut microbial homeostasis, resulting in an overgrowth of *C. perfringens* **(He et al. 2022).** *C. perfringens* was classified into seven toxinotypes (A, B, C, D, E, F, and G) based on the production of six toxin types, including four major toxins :alpha (*cpa*), beta (*cpb*), epsilon (*etx*), iota (*iap*), and two minor toxins: enterotoxin (*cpe*), and necrotic beta-like toxin (*netB*) genes **(Fathima et al. 2022; Rood et al. 2018).** *C. perfringens* types A and C are the most common etiological agents of necrotic enteritis in poultry **(Fathima et al. 2022).** The main virulence factors involved in the pathogenesis of necrotic enteritis are alpha and NetB toxins. *C. perfringens types* A and F are also pathogenic for humans and thus have public health implications. Foodborne *C. perfringens* has the ability to produce dormant spores that are resistant to high temperature, high pressure, chemicals, and radiation and can survive in cooked food and germinate during the cooling and storage of prepared foods **(Fathima et al. 2022; Shimizu et al. 2002).**

One of the most significant prophylactic and control approaches for reducing the prevalence of necrotic enteritis in livestock and poultry housing is an efficient biosecurity program. Biosecurity was described as all measures required to keep disease off a farm and to prevent disease transfer from one cycle to the next. Effective cleaning and disinfection are one of the key biosecurity measures for disease control. Chemical disinfection is the most widely used disinfection method for destroying or inactivating germs on inanimate surfaces (**Li et al. 2023).** Chlorine, quaternary ammonium, and glutaraldehyde are the common chemical disinfectants used for broiler houses **(Chidambaranathan and Balasubramanium, 2019; Jiang et al. 2018).** However, abuse of these disinfectants, such as overuse, use of low doses may contribute to the development of disinfectant microbial resistance **(Kamal et al. 2019).** Concerns have recently been raised about the potential role of quaternary ammonium compounds (QACs) in promoting antimicrobial resistance development, particularly cross- or co-resistance to antibacterial drugs **(Davies and Wales, 2019; Kamal et al. 2019).**

QACs resistance genes such as *qacA/B, qacC/D, qacE,* and *qacG* have been identified in multidrug-resistant pathogenic bacterial species (**Ibrahim et al. 2019; Zhang et al. 2015).**

It was reported that glutaraldehyde, formaldehyde, peroxy acids, hydrogen peroxide, iodine, and chlorine disinfectants have sporicidal activity against spore-forming bacteria **(McSharry et al. 2021).** Previous studies have reported that the efficacy of disinfectants is mainly dependent on their mechanism of action, concentrations, and contact times, assuming that the organic wastes have been eliminated prior to disinfection **(McSharry et al. 2021; Omidbakhsh, 2010).** Although there are currently several different formulations that are commercially available for spore-forming bacteria, little information is available on their relative effectiveness against *C. perfringens*. According to **Nasr et al. (2014),** calcium hypochlorite 1% and Germicidan KOK 4% achieved the required reduction in sporicidal viability in *C. perfringens* strains isolated from litter samples following European Standard EN 1276, 2019, after 30 minutes of contact time under dirty (3% yeast) conditions, whereas Biosentury 904, 2%, and Prophyl 75,1% achieved the required reduction after 10 and 5 minutes of contact time, respectively.

The aim of this study was to investigate the sporicidal activity of a combination of quaternary ammonium and glutaraldehyde disinfectant (TH4) against previously isolated *C. perfringens* strains at different concentrations (0.25%, 0.5%, 1%) and contact times (0, 1, 5, 15, 30, 60 minutes) under clean conditions following European Standard EN 1276, 2019 application method. Furthermore, more investigations were performed on the toxinotyping and detection of *qacED1 and qac*A/B resistance

genes in the isolated *C. perfringens* strains.

MATERIALS AND METHODS

Identification of *C. perfringens* isolates:

Ten *C. perfringens* isolates were previously recovered from commercial broiler farms suffering from necrotic enteritis **(Hosny et al. 2021).** Identification of *C. perfringens* isolates was done according to **Tessari et al. (2014)** through inoculation of *C. perfringens* isolates in enriched cooked meat medium broth (Becton Dickinson and Company, Sparks, Maryland, USA) and incubated anaerobically at 37° C for 24 h. A loopful of the inoculums were then streaked onto 10% sheep blood agar plates with neomycin sulphate (40 µg/ml) and tryptose-sulfite-cycloserine (TSC) agar plates and incubated at 37° C for 24 h under anaerobic conditions using GasPakTM (Oxoid Limited, Thermo Fisher Scientific Inc., UK). The plates were examined for double zones of hemolysis and the growth of black colonies. Confirmation of the suspected *C. perfringens* colonies was done according to Tessari et al. (2014) by lecithinase and litmus milk tests using egg yolk salt agar medium containing 10% egg yolk emulsion (Oxoid Limited, Thermo Fisher Scientific Inc., UK) and litmus milk medium (Oxoid Limited, Thermo Fisher Scientific Inc., UK), respectively.

Spore preparation

The confirmed *C. perfringens* isolates were inoculated into Duncan-Strong sporulation medium overnight under anaerobic condition at 37°C **(Almatrafi et al. 2023).** Spore suspensions from each spore stock for each isolate were prepared at a final concentration of 10^7spores/ml to be used in the disinfectant testing.

Spore germination

The spore suspensions were then heat-activated at 80 °C for 10 minutes before being cooled in a water bath at 21 °C for 5 minutes to promote spore germination **(Almatrafi et al. 2023).**

Toxinotyping of *C. perfringens* isolates using conventional PCR

DNAs of the ten *C. perfringens* isolates were extracted using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) according to the manufacturer's recommendations. The primers set and amplicon sizes used for the detection of five toxin genes (*cpα, cpβ, etx, iA,* and *netB*) were listed in Table 1. A negative control of reference *E. coli* strain (NCIMB 50034) and positive controls of *C. perfringens* ATCC 12917 (for *cpα* gene), ATCC 3626 (for *εtx, cpβ* genes), and ATCC 27324 (for *ιA* gene) were used to assess primer specificity. The positive control for the *netB* gene was provided by ISO 17025 accredited biotechnology unit, Reference laboratory for veterinary quality control on poultry production, Animal Health Research Institute, Egypt **(Merati et al. 2017).** The PCR reactions were optimized using Applied biosystem 2720 thermal cycler as described by (**Hosny et al. 2021).** Multiplex PCR was performed for the detection of *cpα, cpβ, εtx, and iA* gene and each reaction consisted of a 50-µL total volume mixture with 25 µL of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µL of each primer, 6 µL of DNA templates, and 11 µL of sterile distilled water. Uniplex PCR was performed for the detection of *netB* gene and each reaction consisted of 25 µL volume mixture with 12.5 µL of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µL of each primer, 5µL of target DNA templates, and 5.5 µL of sterile distilled water. Multiplex PCR conditions were optimized to 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 seconds, 50 °C for 45 seconds, 72 °C for 45 seconds, whereas uniplex PCR settings were optimized to 94 °C for 30 seconds, 55 °C for 40 seconds, 72 °C for 45 seconds with a final extension cycle for both at 72 °C for 10 minutes. The PCR products were separated on a 1.5% agarose gel (Applichem, Germany, GmbH) in 1x-Tris/Borate/EDTA buffer (TBE) containing 0.2 µg/mL ethidium bromide at gradients of 5V/cm for 20 minutes. Bands were visualized under ultraviolet transilluminator and the data was analyzed through Biometra software.

Table 1. Target genes, primers, and amplified products used for toxinotyping and detection of disinfectant resistance genes of *C. perfringens* isolates

Toxin	Target genes	Primers sequences	Amplified product (bp)	Reference
Alpha	*cpα*	GTTGATAGCGCAGGACATGTTAAG	402	(Hosny et al., 2021)
		CATGTAGTCATCTGTTCCAGCATC		
Beta	*cpβ*	ACTATACAGACAGATCATTCAACC	236	
		TTAGGAGCAGTTAGAACTACAGAC		
Epsilon	*etx*	ACTGCAACTACTACTCATACTGTG	541	
		CTGGTGCCTTAATAGAAAGACTCC		
Iota	*iA*	GCGATGAAAAGCCTACACCACTAC	317	
		GGTATATCCTCCACGCATATAGTC		
netB	*netB*	CGCTTCACATAAAGGTTGGAAGGC	316	
		TCCAGCACCAGCAGTTTTTCCT		
*qac*A/B		GCAGAAAGTGCAGAGTTCG	361	
		CCAGTCCAATCATGCCTG		
qacED1		TAA GCC CTA CAC AAA TTG GGA GAT AT	362	Ibrahim et al. 2019
		GCC TCC GCA GCG ACT TCC ACG		

Molecular detection of disinfectant resistance genes

Uniplex PCR was performed for the detection of *qacED1 and qac*A/B resistance genes in the isolates. The primers sequences used were as follows: *qac*A/B: forward primer 5′-GCAGAAAGTGCAGAGTTCG, reverse primer 5′- CCAGTCCAATCATGCCTG - 3′; *qacED1*: forward primer5′- TAA GCC CTA CAC AAA TTG GGA GAT AT, reverse primer 5′- GCC TCC GCA GCG ACT TCC ACG -3′ (Ibrahim et al 2019). The positive controls for the *qacA/B* and *qacED1* genes used to assess primer specificity were provided by ISO 17025 accredited biotechnology unit, Reference laboratory for veterinary quality control on poultry production, Animal Health Research Institute, Egypt **(Ibrahim et al. 2019).** The PCR amplifications were performed using Applied biosystem 2720 thermal cycler according to **(Ibrahim et al. 2019)** and each reaction consisted of 25 µL volume mixture as previously described. The PCR conditions included an initial denaturation at 94°C for 5 minutes, then 35 cycles of denaturation at 94°C for 30 seconds, primer annealing at 58 and 53°C, respectively for 40 seconds, and extension at 72°C for 40 seconds, and final extension at 72°C for 7 minutes. The expected sizes of amplified bands of *qacED1 and qac*A/B genes were 362 bp and 361 bp, respectively.

Evaluation the sporicidal activity of TH4 disinfectant against *C. perfringens* isolates

Disinfectant

TH4 was obtained from (Theseus company, Laval, France). It is a synergistic combination of glutaraldehyde and quaternary ammoni-

um compound disinfectant that consisted of 18.75 g Didecyl dimethyl ammonium chloride, 19.75 g dioctyl dimethyl ammonium chloride, 37.50 g octyl decyl dimethyl ammonium chloride, 50 g alkyl dimethyl benzyl ammonium chloride, and 62.50 g glutaraldehyde, and 1 l water.

Neutralizer

The neutralizers used for TH4 were combination of tween80 (Sigma Chemical Co., St Louis, MO, USA), 3 g/l lecithin (Sigma Chemical Co., St Louis, MO, USA), and 1 g/l histidine (Sigma Chemical Co., St Louis, MO, USA) **(European Standard EN 1276, 2019).**

Method

A dilution–neutralization method was used to evaluate the sporicidal activity of TH4 disinfectant against the ten isolated *C. perfringens* strains according to preliminary European Standard EN 1276, 2019. Disinfection testing was performed by thoroughly mixing 0.2 mL of the spore suspension of *C. perfringens* (10^7) with 0.8 mL of TH4 disinfectant previously diluted with distilled water at concentrations of 0.25%,0.5%, and 1%. The mixture was then heated in water bath at 20 °c for different contact times (1, 5, 10, 15, 30, and 60 minutes). After that, transfer 0.1 ml of the mixture to 0.8 ml of the neutralizer and 0.1 ml of water and left for 5 minutes.to prevent further inactivation. Samples of the final mixture were then seeded on two plates of reinforced clostridial agar and incubated anaerobically at 37°C for 48 hours. Colonies were counted and expressed as colony-forming units ($CFUmL^{-1}$). According to the European Standard, the sporicidal effect was described as the 5 log_{10} CFU reduction in the viable count from the initial inoculums.

RESULTS

Toxinotyping of *C. perfringens* isolates using conventional PCR

Toxinotyping of the ten *C. perfringens* isolates revealed that all isolates produced positive amplification for the alpha toxin *cpa* gene at 402 bp, while five isolates were positive for the *netB* gene and produced positive amplification at 560 bp (Table 2 and Figs 1 and 2). None of the tested isolates were positive for *cpβ, etx,* and*, iA* genes which indicate the absence of *C. perfringens* toxinotypes B, C, D, E, F in this study (Table 2 and Figs 1 and 2).

Table 2. Molecular detection of toxin and QAC resistance genes

Isolate	*Molecular detection of toxin and QAC resistance genes*	*Toxinotype*
1	*cpa - QacA/B*	*A*
2	*Cpa*	*A*
3	*Cpa*	*A*
4	*cpa – netB- QacED1- QacA/B*	*G*
5	*cpa – netB- QacED1- QacA/B*	*G*
6	*cpa – netB- QacED1*	*G*
7	*cpa - QacED1*	*A*
8	*cpa – QacA/B*	*A*
9	*cpa – netB- QacED1*	*G*
10	*cpa – netB- QacED1*	*G*

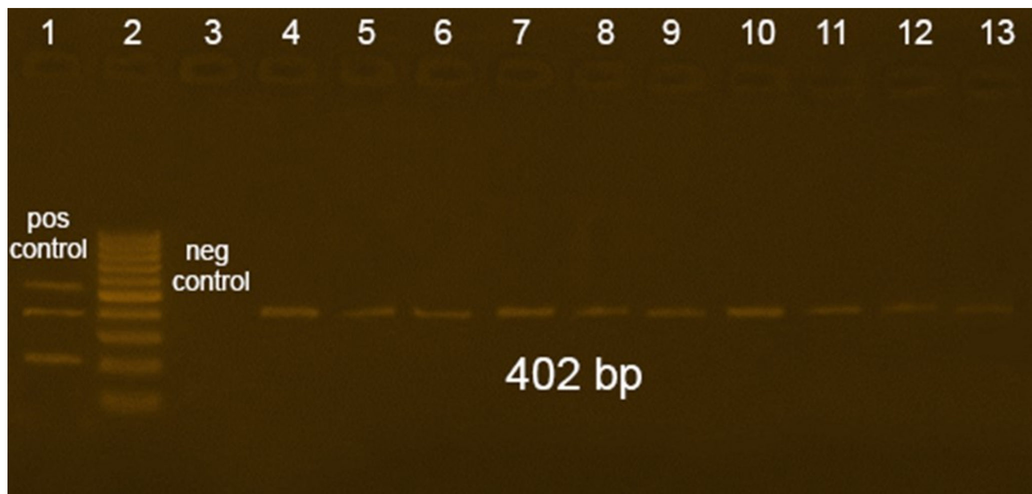

Fig 1. Multiplex PCR for toxinotyping of C. *perfringens* isolates, lane 1: Positive controls of *C. perfringens* ATCC 12917 (for *cpα* gene), ATCC 3626 (for *εtx, cpβ* genes), and ATCC 27324 (for *ιA* gene), lane 2: ladder 100-1000 bp, lane 3: negative control with no amplicon, lanes 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13: positive for *cpa* gene encodes for alpha toxin with production of specific amplicon at 402 bp

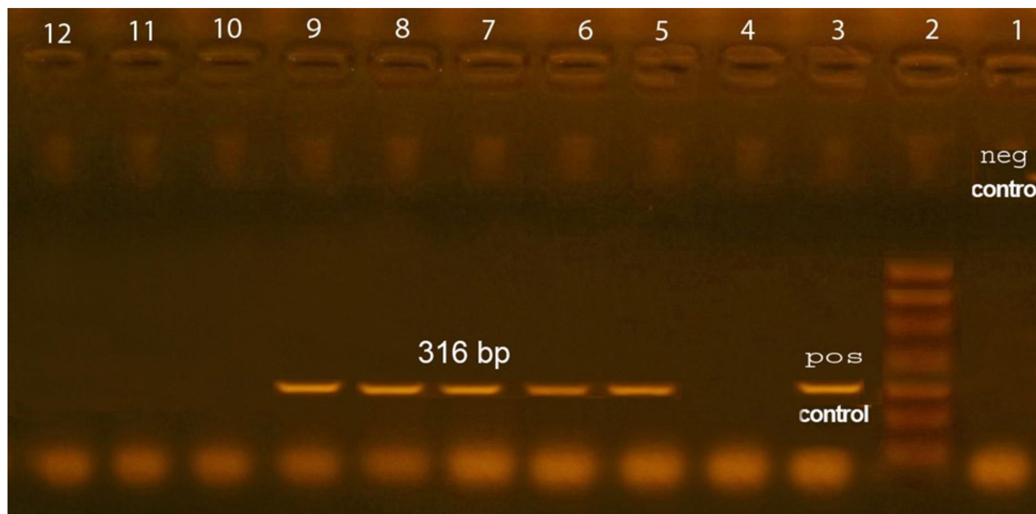

Fig 2. Uniplex PCR for detection of *netB* gene in C. *perfringens* isolates, lane 1: negative control with no amplicon, lane 2: ladder 100-1000 bp, lane 3: positive control (for *netB* gene), lanes 5, 6, 7, 8, and 9:positive for *netB* gene with production of specific amplicon at 316 bp.

Molecular detection of disinfectant resistance genes

Screening for of QAC resistance genes (*qacED1 and qac*A/B) revealed their presence in six and four isolates, respectively (Table 2 and Figs 3 and 4).

Table 2. Molecular detection of toxin and QAC resistance genes

Isolate	Molecular detection of toxin and QAC resistance genes	*Toxinotype*
1	*cpa - QacA/B*	A
2	*Cpa*	A
3	*Cpa*	A
4	*cpa – netB- QacED1- QacA/B*	G
5	*cpa – netB- QacED1- QacA/B*	G
6	*cpa – netB- QacED1*	G
7	*cpa - QacED1*	A
8	*cpa – QacA/B*	A
9	*cpa – netB- QacED1*	G
10	*cpa – netB- QacED1*	G

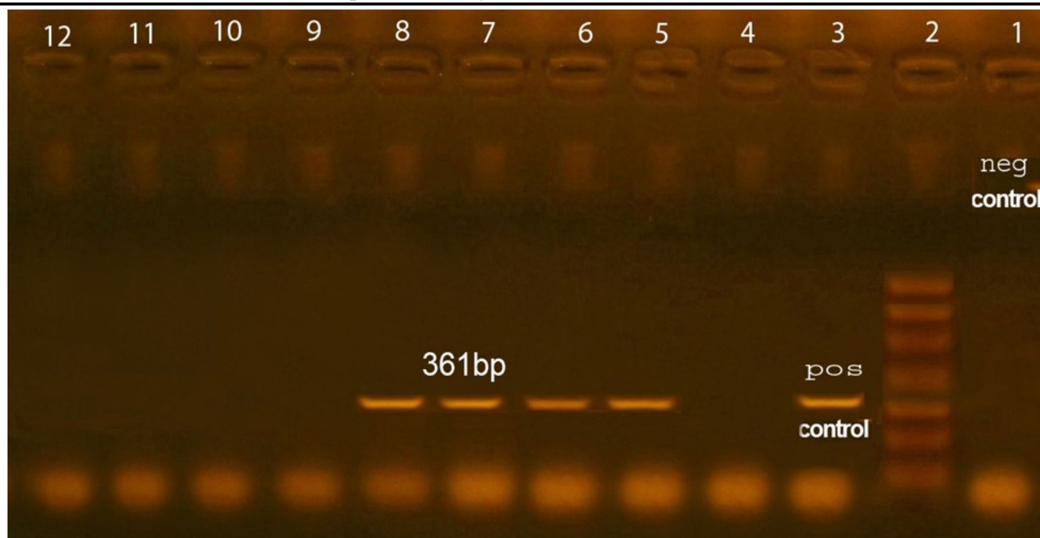

Fig 3. Uniplex PCR for detection of *qac*A/B gene in C. *perfringens* isolates, lane 1: negative control with no amplicon, lane 2: ladder 100-1000 bp, lane 3: positive control (for *qac*A/B gene), lanes 5, 6, 7, and 8 :positive for *qac*A/B gene with production of specific amplicon at 361 bp.

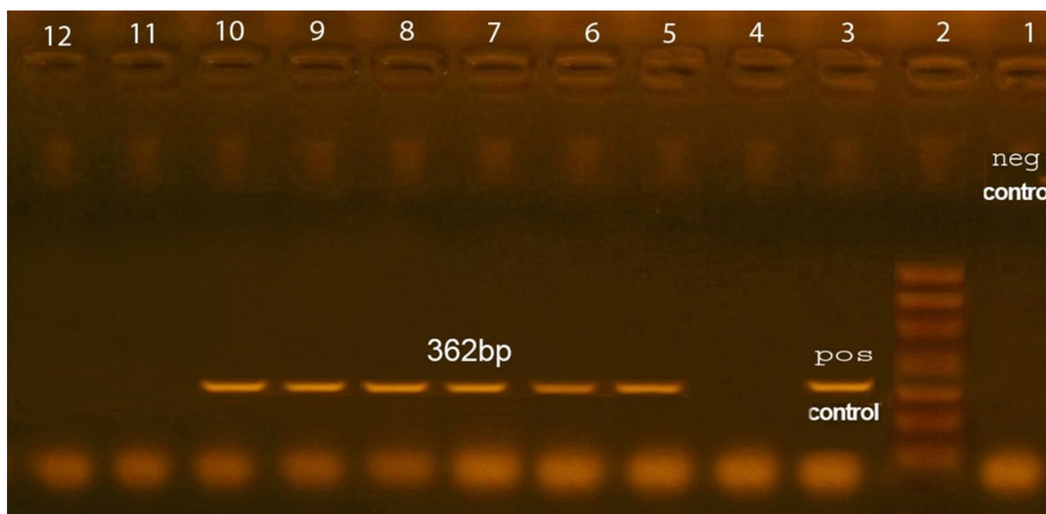

Fig 4. Uniplex PCR for detection of *qacED1*gene in C. *perfringens* isolates, lane 1: negative control with no amplicon, lane 2: ladder 100-1000 bp, lane 3: positive control (for *qacED1*gene), lanes 5, 6, 7, 8, 9, and 10:positive for *qacED1*gene with production of specific amplicon at 362 bp.

Evaluation the sporicidal activity of TH4 disinfectant against *C. perfringens* isolates

The results revealed that the efficacy of TH4 in this study was significantly lower when the exposure time was reduced to 1 minute and increased with an increase in concentration. A Kruskal-Wallis test revealed that there was a statistically significant difference in spores count at different disinfectant concentrations, $\chi2(2) = 25.29, p = 0.000$. A Freidman test revealed that there was a statistically significant difference in spores count depending on the exposed contact time $\chi2(2) = 114.36$, 141.72, and 140.16, respectively $p = 0.000$ for different concentrations of 0.25%, 0.5%. and 1%. A Wilcoxon test revealed significant difference in the *C. perfringens* count in a time-dependent manner $p = 0.000$.

Table 3. Sporicidal activity of different concentrations of TH4 against *C. perfringens* strains at different contact times.

Concentration of disinfectant	log_{10} of C. *perfringens* Count					
TH4 (0.25%)	**Contact times**					
	0 minutes	**1 minutes**	**5 minutes**	**15 minutes**	**30 minutes**	**60 minutes**
1	7	6.2	5.8	5.3	4.7	4
2		6.7	6.3	5.8	5.2	5
3		6.6	6.2	5.7	5	4.8
4		7	7	7	7	6.4
5		7	7	7	7	6.2
6		7	7	7	6.7	5.2
7		6.8	6.5	5.7	5.2	4.8
8		6.8	6.5	5.6	5	4.5
9		6.8	6.4	5.8	5.3	5
10		6.7	6.4	5.8	5.3	5
TH4 (0.5%)	**0 minutes**	**1 minutes**	**5 minutes**	**15 minutes**	**30 minutes**	**60 minutes**
1	7	5.8	5.2	4.8	4	3.8
2		6.3	5.7	5	4.8	4
3		6.4	5. 8	5.2	4.8	4.2
4		7	7	7	5.2	4.6
5		7	7	7	5.4	4.7
6		7	7	7	4.3	3.9
7		6.7	5.8	5.2	4.7	3.8
8		6.7	5.7	5.4	4.8	3.7
9		6.7	6	5.7	5	4.6
10		6.2	5.8	5.3	5	4.8
TH4 (1%)	**0 minutes**	**1 minutes**	**5 minutes**	**15 minutes**	**30 minutes**	**60 minutes**
1	7	5.2	4.72	3.86	3,5	2.6
2		5.52	4.85	4.7	3.8	2.7
3		5.97	5.68	4.67	3.8	2.8
4		7	7	7	4.7	4.2
5		7	7	7	4.8	3.8
6		7	7	7	4	3.7
7		5.7	4.7	4.2	3.7	2.8
8		5.8	5.6	4.7	3.5	2.7
9		6.3	5.7	4.8	4	3.8
10		5.87	5.2	4.8	4.3	3.8

DISCUSSION

Toxinotyping of the ten *C. perfringens* isolates revealed that five isolates were verified to be toxinotype A, while the remaining five isolates were confirmed to be toxinotype G. The main reason for the detection of the alpha toxin gene compared to other toxin genes (beta, epsilon, iota, tpeL, netB) may be due to the presence of alpha toxin on chromosomes, but other toxin genes are found on plasmids, which may be lost throughout the culture and passage processes, affecting their positive detection **(Mohiuddin et al. 2023)**. Previous studies have reported that *C. perfringens* type A is the most toxin type isolated from healthy and diseased chickens in India, Korea and China **(Gharaibeh et al. 2010; Malmarugan et al. 2012; Park et al. 2015; Dar et al. 2017; Zhang et al. 2018)** . **Mohiuddin et al. (2023)** reported isolation of *C. perfringens* types A and G in percentages of 95.5% and 4.5%, respectively in China. **Ni et al. (2009)** have displayed isolation of *C. perfringens* type G in percentages of 5.3%. Previous studies have displayed that *C. perfringens* types G and A are the main toxinotypes causing necrotic enteritis **(Rood et al. 2018; Fathima et al. 2022).** Previous studies have established successful animal models with necrotic enteritis lesions using *C. perfringens* strains types A and G **(Cooper and Songer 2010; Keyburn et al. 2010)**. Screening for of QAC resistance genes (*qacED1 and qac*A/B) in *C. perfringens* isolates has been studied for the first time in this study that revealed their detection in percentages of 60% and 40%, respectively. The widespread usage of QACs in animal husbandry has been linked to the development of bacterial resistance **(Jennings et al. 2015)**. The resistance of QACs in Gram-positive bacteria has been related to the presence of genes conferring resistance to these compounds, which are either carried on mobile genetic components such as plasmids and integrons or encoded on the chromosome **(Jaglic and Cervinkova 2012)**. Previous studies have reported the detection of *qacA/B* on plasmids and *qacEdelta* on the 3' conserved sequence of class 1 integrons **(Kücken et al. 2000; Chuanchuen et al. 2007)**. Plasmids and integrons may also carry different antibiotic resistance genes, such as β-lactamase, aminoglycosides, and trimethoprim, implying the co-existence of QACs resistance with antibiotic resistance in *C. perfringens* **(Chapman 2003)**.

The effect of TH4 disinfectant on the count of spores produced by *C. perfringens* isolates was investigated. TH4 is a combination of quaternary ammonium compound (QAC), cationic surfactant, and glutaraldehyde **(Acsa et al. 2021).** QACs are known to react with the cell membrane lipid bilayer, altering cell permeability and inducing bacterial extravasation, whereas glutaraldehyde reacts with amines and thiol groups, which are functional groups in proteins, altering bacterial metabolism, causing bacterial death, and preventing sporulation **(Jiang et al. 2018; Osland et al. 2020; Acsa et al. 2021)**. Glutaraldehyde disinfectant is commonly used for the disinfection of bacterial spores during epidemics due to its strong effect on the spores produced by *C. perfringens* **(Brantner et al. 2014; Jiang et al. 2018)**.

The efficacy of disinfectants against bacterial diseases primarily depends on chemical composition, concentration, temperature, time of exposure, presence of interfering organic and inorganic matter, and type of targeted microorganisms (inoculum or presence of biofilm) as stated by **Acsa et al. (2021).**

In this study, we investigated effect of concentration, time of exposure, and strain type on the efficacy of disinfectant on the count of spores of *C. perfringens* isolates. This study revealed increased efficacy of disinfectants at long contact times and high concentrations and this complies with the results of previous studies **(Jang et al. 2017; Osland et al. 2020)**. Furthermore, the strains used seemed to be one of the most important factors affecting disinfectant efficacy. TH4 1% tested in this study met the reduction requirement in five *C. perfringens* type A isolates at an exposure time of 60 minutes given in the **European Standard EN 1276, 2019**, this level of disinfection was not reached in the other five *C. perfringens* type G isolates, which displayed a mean $\log_{10}$ reduction less than five. These findings exceeded the recommended user concentration of TH4 (0.25%) as previously described by **Acsa**

et al. (2021). This might be attributed to the ability of these strains to form biofilms, which are difficult to eradicate using sanitation measures and are more resistant to disinfectants and antibiotics than their planktonic counterparts **(Møretrø et al. 2009; Abdallah et al. 2014; Osland et al. 2020)**. It was evident that *C. perfringens* type G isolates had *cpa, netB, and QacED1 genes, which could play a role in the TH4 resistance observed in this study, and more research is needed to investigate this association. These findings exceeded* the recommended user concentration of TH4 (0.25%) as previously described by Acsa et al. (2021).

Limitations of the study

Our study has some limitations including selection of random isolated ten *C. perfringens* strains to be tested, this was attributed to the limitation of our resources and the findings of this study exceeded the recommended user concentration of TH4 which isn't economically to apply in the field. Furthermore, other factors must be considered in future researches including presence of interfering organic matter which might reduce the efficacy of the TH4 disinfectant.

CONCLUSIONS

The findings from this study recommended to use a high concentration of TH4 and increase exposure time to 60 minutes in order to obtain effective sporicidal activity against *C. perfringens* isolates. Furthermore, these results displayed the presence of QACs resistance which can assist policymakers in developing guidelines for the use of disinfectants.

REFERENCES

Abdallah M, Benoliel C, Drider D, Dhulster P and Chihib NE. 2014. Biofilm formation and persistence on abiotic surfaces in the context of food and medical environments. Arch Microbiol 196: 453-472.

Acsa I, Lilly Caroline B., Philip Njeru N and Lucy Wanjiru N. 2021. Preliminary study on disinfectant susceptibility/resistance profiles of bacteria isolated from slaughtered village free-range chickens in Nairobi, Kenya. Int J Microbiol 2021: 1-7.

Almatrafi R, Banawas S and Sarker MR. 2023. Divalent Cation Signaling in *Clostridium perfringens* Spore Germination. Microorganisms 11: 591.

Brantner CA, Hannah RM, Burans JP and Pope RK. 2014. Inactivation and ultrastructure analysis of Bacillus spp. and *Clostridium perfringens* spores. Microsc Microanal 20: 238-244.

Chapman JS. 2003. Disinfectant resistance mechanisms, cross-resistance, and co-resistance. International biodeterioration biodegradation 51: 271-276.

Chidambaranathan AS. and Balasubramanium M. 2019. Comprehensive review and comparison of the disinfection techniques currently available in the literature. J Prosthodont 28: e849-e856.

Chuanchuen R, Khemtong S and Padungtod P. 2007. Occurrence of *qacE/qacED1* genes and their correlation with class 1 integrons in *Salmonella enterica* isolates from poultry and swine. Southeast Asian J Trop Med Public Health 38: 855-862.

Cooper KK and Songer JG. 2010. Virulence of Clostridium perfringens in an experimental model of poultry necrotic enteritis. Vet Microbiol 142: 323-328.

Dar PS, Wani SA, Wani AH, Hussain I, Maqbool R, Ganaie MY, Kashoo ZA and Qureshi S. 2017. Isolation, identification and molecular characterization of *Clostridium perfringens* from poultry in Kashmir valley, India. J Entomol Zool Studies 5: 409-414.

Davies R and Wales A. 2019. Antimicrobial resistance on farms: a review including biosecurity and the potential role of disinfectants in resistance selection. Comprehensive reviews in food science and food safety 18: 753-774.

European Committee for standardization 2019. European standard EN 1276: Chemical disinfectants and antiseptics. Quantitative suspension test for the evaluation of bacteri-

cidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic, and institutional areas. Test method and requirements (phase 2, step 1). British Standards Institute: London, UK.

Fathima S, Hakeem WGA, Shanmugasundaram R. and Selvaraj RK. 2022. Necrotic enteritis in broiler chickens: A review on the pathogen, pathogenesis, and prevention. Microorganisms 10: 1958.

Gharaibeh S, Al Rifai R and Al-Majali A. 2010. Molecular typing and antimicrobial susceptibility of *Clostridium perfringens* from broiler chickens. Anaerobe 16: 586-589.

He W, Goes EC, Wakaruk J, Barreda DR and Korver DR. 2022. A poultry subclinical necrotic enteritis disease model based on natural *Clostridium perfringens* uptake. Frontiers in Physiology 13: 788592.

Hosny RA, Gaber AF and Sorour HK. 2021. Bacteriophage mediated control of necrotic enteritis caused by *C. perfringens* in broiler chickens. Vet Res Commun 45: 409-421.

Ibrahim WA, Marouf SA, Erfan AM, Nasef SA and El Jakee JK. 2019. The occurrence of disinfectant and antibiotic-resistant genes in *Escherichia coli* isolated from chickens in Egypt. Vet world 12: 141.

Jaglic Z and Cervinkova D. 2012. Genetic basis of resistance to quaternary ammonium compounds--the qac genes and their role: a review. Veterinarni Medicina 57.

Jang Y, Lee K, Yun S, Lee M, Song J, Chang B and Choe N. 2017. Efficacy evaluation of commercial disinfectants by using *Salmonella enterica* serovar Typhimurium as a test organism. Journal of veterinary science 18: 209-216.

Jennings MC., Minbiole KP and Wuest WM. 2015. Quaternary ammonium compounds: an antimicrobial mainstay and platform for innovation to address bacterial resistance. ACS Infect Dis 1: 288-303.

Jiang L, Li M, Tang J, Zhao X, Zhang J, Zhu H, Yu X, Li Y, Feng T and Zhang X. 2018. Effect of different disinfectants on bacterial aerosol diversity in poultry houses. Front Microbiol 9: 2113.

Kamal MA, Khalaf MA, Ahmed ZAM and El Jakee J. 2019. Evaluation of the efficacy of commonly used disinfectants against isolated chlorine-resistant strains from drinking water used in Egyptian cattle farms. Vet world 12: 2025.

Keyburn AL, Yan XX, Bannam TL, Van Immerseel F, Rood JI and Moore RJ. 2010. Association between avian necrotic enteritis and *Clostridium perfringens* strains expressing NetB toxin. Vet Res 41.

Kücken D, Feucht HH and Kaulfers PM. 2000. Association of qacE and qacE Δ1 with multiple resistance to antibiotics and antiseptics in clinical isolates of Gram-negative bacteria. FEMS Microbiol Lett 183: 95-98.

Li Y, Song Y, Huang Z, Mei L, Jiang M, Wang D and Wei Q. 2023. Screening of *Staphylococcus aureus* for disinfection evaluation and transcriptome analysis of high tolerance to chlorine-containing disinfectants. Microorganisms 11: 475.

Malmarugan S, Boobalan A and Dorairajan N. 2012. Necrotic Enteritis in broiler and layer farms in Tamil Nadu, India. Int J Agro Vet Med Sci 6: 241-249.

McSharry S, Koolman L, Whyte P and Bolton D. 2021. Investigation of the effectiveness of disinfectants used in meat-processing facilities to control *Clostridium sporogenes* and *Clostridioides difficile* spores. Foods 10: 1436.

Merati R, Temim S and AAAFM. 2017. Identification and characterization of *Clostridium perfringens* isolated from necrotic enteritis in broiler chickens in Tiaret, Western Algeria. Kafkas Üniversitesi Veteriner Fakültesi Dergisi 23.

Mohiuddin M, Song Z, Liao S, Qi N, Li J, Lv M, Lin X, Cai H, Hu J and Liu S. 2023. Animal Model Studies, Antibiotic Resistance and Toxin Gene Profile of NE Reproducing *Clostridium perfringens* Type A and Type G Strains Isolated from Commercial Poultry Farms in China. Microorganisms 11: 622.

Mora ZV, Macías-Rodríguez ME, Arratia-Quijada J, Gonzalez-Torres YS, Nuño K and Villarruel-López A 2020. *Clostridium perfringens* as foodborne pathogen in broiler production: pathophysiology and potential strategies for controlling necrotic enteritis. Animals 10: 1718.

Møretrø T, Vestby L, Nesse L, Storheim S, Kotlarz K and Langsrud S. 2009. Evaluation of efficacy of disinfectants against *Salmonella* from the feed industry. J Appl Microbiol 106: 1005-1012.

Nasr SA, Ali MM, Hamoda A, Hatem E, El Agrab HM, Moubarak ST, Samaha HA and Zahran O. 2014. Sporicidal Activity of some Disinfectants Against *Clostridium Perfringens* Isolated from Broiler Poultry Litter. Alex J Vet Sci 42.

Ni X, Song Z, Zeng D, Zheng X and Gong JhJ. 2009. Isolation, identification and genotyping of *Clostridium perfringens* from chickens in Sichuan province. Chinese J Zoonoses 25: 737-786.

Omidbakhsh N. 2010. Evaluation of sporicidal activities of selected environmental surface disinfectants: carrier tests with the spores of *Clostridium difficile* and its surrogates. American journal of infection control 38: 718-722.

Osland AM, Vestby LK. and Nesse LL. 2020. The effect of disinfectants on quinolone resistant *E. coli* (QREC) in biofilm. Microorganisms 8:1831.

Park JY, Kim S, Oh JY, Kim HR, Jang I, Lee HS and Kwon YK. 2015. Characterization of *Clostridium perfringens* isolates obtained from 2010 to 2012 from chickens with necrotic enteritis in Korea. Poultry science 94:1158-1164.

Rood JI, Adams V, Lacey J, Lyras D, McClane BA, Melville SB, Moore RJ, Popoff MR, Sarker MR and Songer JG. 2018. Expansion of the *Clostridium perfringens* toxin-based typing scheme. Anaerobe 53: 5-10.

Shimizu T, Ohtani K, Hirakawa H, Ohshima K, Yamashita A, Shiba T, Ogasawara N, Hattori M, Kuhara S and Hayashi H. 2002. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. Proc Nati Acad Sci 99:996-1001.

Tessari ENC, Cardoso ALSP, Kanashiro AMI, Stoppa GFZ, Luciano RL and de Castro AGM. 2014. Analysis of the presence of *Clostridium perfringens* in feed and raw material used in poultry production. Food Nutri Sci 2014.

Zhang C, Cui F, Zeng GM, Jiang M, Yang ZZ, Yu ZG, Zhu MY and Shen LQ. 2015. Quaternary ammonium compounds (QACs): A review on occurrence, fate and toxicity in the environment. Sci Total Environ 518: 352-362.

Zhang T, Zhang WAID., Zhang R, Lu Q, Luo Q and Shao H. 2018. Prevalence and characterization of *Clostridium perfringens* in broiler chickens and retail chicken meat in central China. Anaerobe 54: 100-103.