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Antibiogram pattern and molecular characterization of *Clostridium perfringens* isolated from Different Species

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ABSTRACT

Clostridium perfringens (*C. perfringens*) is a foodborne pathogen that represent a high risk to animals and poultry due to its high outbreak incidences worldwide causing diseases such as necrotic enteritis in chickens, enterotoxemia in rabbits and necro-hemorrhagic enteritis in calves leading to high economic losses in disease prevention and control. This study aimed to investigate the drug resistance pattern and genetic characterization of *C. perfringens* isolated from different species (chickens, rabbits, and calves) in El-Gharbia governorate, Egypt. The sensitivity test showed that 30 *C. perfringens* isolates (18 from chickens, 9 from rabbits and 3 from calves) were resistant to erythromycin, spectinomycin and sulphamethoxazole–trimethoprim and sensitive to ampicillin-sulbactam, ofloxacin, cefotaxime, meropenem, cefaclor and amoxicillin-clavulanic acid. Polymerase chain reaction (PCR) revealed that all *C. perfringens* isolates were type A carrying *cpa* gene, whereas *net* B and *cpe* genes were detected in only (40%) of the 5 tested isolates and were mainly restricted in isolates from chickens. Antibiotic-resistance genes *erm*B and *sul*1 were detected in (80%) and (100%) of isolates respectively. The variation of the drug resistance pattern between the current studied locality and others within Egypt may reflect the differences in the levels of the previous misuse of antibiotics. This may be taken as an alarm for the necessity of conducting a whole country well planned and controlled study of *C. perfringens* antibiotic resistance and their responsible genes to monitor the antibiotics resistance situation allover Egypt and draw a clear map of the current situation and a plan for reducing and even preventing further multidrug resistance (MDR) development of this serious bacteria.

1. INTRODUCTION

C. perfringens is a Gram-positive anaerobic bacterium that can spread in the environment and occurs within the normal gut microbiota. Despite lacking flagella, *C. perfringens* exhibits gliding movement by type IV pilli and can form spores that spread widely in the environment causing serious disease (McClane, 2001). *C. perfringens* live normally in the balanced gut microbiota, while any disturbance such as stress conditions, starvation, and continual administration of anthelmintics and antibiotics stimulate its attaining pathogenicity causing various diseases such as enteric infection, food poisoning and enterocolitis (Freedman et al., 2015; Heida et al., 2016; Navarro, 2018). *C. perfringens* strains can be classified into five groups from (A to E) based on their ability to produce major lethal toxins : alpha (α), beta (β), epsilon (ε)and iota (ι) that are encoded with *cpa*, *cpb*, *etx* and *itx* genes respectively. In addition to alpha toxin which is the only produced in serotype A, *C. perfringens* produces beta and epsilon toxins (type B), Beta toxin (Type C), epsilon toxin (type D) and iota toxin (type E) (Cooper et al., 2010). Besides, the conventional presence of alpha toxins encoding genes, the recently used in the new toxinotyping scheme *cpe* and *net*-B genes encoding enterotoxin and necrotic enteritis B like protein (Rood et al., 2018; Goossens et al., 2020). This *cpe* gene associated with *cpa* gene were considered typing genes for the new type F and responsible for enterotoxigenic infection as food poisoning and antibiotic-associated diarrhea in human (Dolan et al., 2016) and animal (Lahti et al., 2008), while the newly serotype G produces the toxin encoded by *cpa* gene and the necrotic enteritis toxin encoded by the *net*-B gene (Uzal et al., 2018) which causes avian necrotic enteritis. Another type of chicken necrotic enteritis was demonstrated to be caused by *C. perfringens* β2 (*cpb2*) and toxin *perfringens* large (tpel) toxins which can be produced by all serotypes (Uzal et al., 2010). Toxinotyping scheme is based on plasmid as *cpa* "the basic gene for *C. perfringens* strains" is the only present in a stable region of chromosome (Jewell et al., 2015), while other types of *C. perfringens* toxins encoding genes are encoded on different sizes plasmids except for *cpe* which exists on either chromosome in food poisoning isolates or on large plasmid in non-foodborne disease causing isolates (Freedman et al., 2016). Alpha toxin was known as the major toxin in necrotic enteritis in broiler (Ashraf et al., 2020) before the role of net Band *tpel* toxins in necrotic enteritis was recently identified (Lee et al., 2021). Net B toxin's role in the disease came from its pore forming ability (Rood et al., 2018) in addition, *tpe*l toxin became a member of clostridial cytotoxic family through detection in necrotic enteritis cases caused by *C. perfringens* type A. (Mwangi et al., 2019).

The uncontrolled use of antibiotics as treatment or for growth promotion in animal production has led to the spreading of antibiotic resistance in the normal enteric flora and ever-increasing incidence of drug resistant *C. perfringens* (Dahiya et al., 2006). This occurs through the adaptation process of these pathogens to repeated usage of antibiotics that resulted in evolution of MDR/toxigenic strains (Ammar et al., 2021). The difficulty of controlling this modulated pathogen had been increased regardless of splendid use of the advancing next-generation therapy options (Mosallam et al., 2021). There are two mechanisms for *C. perfringen*s antimicrobial resistance including the mutation of inherent genes or acquisition of resistance genes (Hall, 2004). *C. perfringens* strains resistance to macrolides acts as a reservoir for *erm*1gene which assists in its conjugal transfer (Sog et al., 2009) while *sul*1assists in sulphonamides resistance (Xiaoting et al., 2021). This study aimed to investigate the antibiogram pattern of *C. perfringens* isolated from different species and to determine its molecular mechanisms of virulence and drug resistance.

2. MATERIAL AND METHODS

2.1. Sampling:

A total of 195 samples were collected from different species in El-Gharbia governorate in 2021. Out of them (135 samples) from intestine and liver of freshly dead chickens (3-8 weeks old) suffering from diarrhea, (30 samples) from liver and intestine of dead rabbits after weaning clinically characterized by diarrhea and sudden death and (30) rectal swabs from newly borne calves suffering from diarrhea associated with fever. The collected samples were transferred to the laboratory for anaerobic bacteriological examination under aseptic conditions.

2.2 Isolation and identification:

Within sterile freshly prepared Cooked Meat broth (CMB) (Oxoid), each sample was introduced into tubes and incubated anaerobically in gas pack anaerobic jars using anaerobic kits (anaerogen, Oxoid ltd, England) at 37°C for 24 hrs. A loop-full from each incubated broth was streaked in 10% defibrinated sheep blood agar plates containing neomycin sulphate 200 µg/ml and incubated for 48 hr at 37˚C under anaerobic conditions (Carter and Cole, 1990).All samples were inoculated on reinforced Clostridial agar (RCA; Oxoid, CM0151) and anaerobically incubated for 24-48hr at 37˚C (Ahmed et al., 2022).The suspected colonies were kept on CMB for further confirmation on Egg Yolk agar (oxoid, SR00885) and Tryptose Sulphate Cycloserine agar (TSC; oxoid CM 0587B).

Then the isolated colonies were identified by the cultural, morphological, and biochemical characteristics: (Lecithinase, Catalase, Indol, Gelatin liquefaction, Litmus milk and Sugar fermentation test) (Macfaddin, 2002).

2.3. Molecular typing:

Typing of *C. perfringens* isolates was carried out by PCR amplification of *cpa, cpb, etx and itx* genes using the specific primers for each gene according to (Yoo et al., 1997) and following the thermal cycling conditions of primary annealing at 94 ˚C for 5 minutes, then amplification for 35 cycles of denaturation at 94˚C for 30 sec followed by annealing at 55˚C for 40 sec and extension at 72˚C for 45 sec ended with a final extension step at 72˚C for 10 minutes. PCR program was done using the primers shown in table (1).

Table 1 Oligonucleotide primers with specific sequence for amplification of *C. perfringens* typing genes.

Genes	Sequence	Amplified product	Reference
cpa gene (Alpha toxin)	GTTGATAGCGCAGGACATGTTAAG CATGTAGTCATCTGTTCCAGCATC	402 bp	YOO *et al*., 1997
cpb gene (Beta toxin)	ACTATACAGACAGATCATTCAACC TTAGGAGCAGTTAGAACTACAGAC	236 bp	
etx gene (Epsilon toxin)	ACTGCAACTACTACTCATACTGTG CTGGTGCCTTAATAGAAAGACTCC	541 bp	
itx gene (Iota toxin)	GCGATGAAAAGCCTACACCACTAC GGTATATCCTCCACGCATATAGTC	317 bp	

2.4. Antimicrobial sensitivity test:

C. perfringens isolates were examined for their antimicrobial susceptibility to ten antimicrobial discs (Oxoid,Basingstoke,UK): ampicillin+sulbactam (AS;20µg), amoxicillin+clavulinicacid (Amc;30µg), meropenem (MEM;10µg), sulphamethoxazole+ trimethoprim (SXT;25µg), erythromycin (E;15µg), ofloxacin (ofx;5µg), cefaclor (CEC; 30µg), colistin (CT;10µg), spectinomycin (SPC;100µg) and cefotaxime (CTX;30µg), using Kirby–Bauer disk diffusion method based on criteria of the Clinical and Laboratory Standards Institute CLSI (CLSI 2020).

2.5. Molecular characterization of C. perfringens virulence and antibiotic resistance genes:

Five *C. perfringens* isolates (no. 1 from rabbit, 2, 3, 4 from chicken and 5 from calf) were grown anaerobically in CMB overnight at 37 ◦C. The extraction of the genomic DNA from the isolated strains using QIAamp DNA Mini kit (Catalog no 51304.Qiagen) was used according to manufacturer's instructions. PCR amplification of the genomic DNA was performed using Emerald Amp GT PCR master-mix (Takara) Code No. RR310A. Briefly, PCR program was done using the primers shown in table (2) and running conditions as detailed in table (3). PCR products were separated by electrophoresis in 1.5% agarose gel. Bands were visualized by ethidium bromide staining (Sambrook et al., 1989). The run was stopped after about 30 min and the gel were transferred to UV cabinet. The gel was photographed by a gel documentation system (Alpha Innotech) and the data was analyzed through computer software.

Table 2 Oligonucleotide primers used for amplification of *C. perfringens* genes.

Gene	Primer Sequence5'-3'	Amplified product	Reference
cpe (Virulence gene)	F-ACATCTGCAGATAGCTTAGGAAAT R - CCAGTAGCTGTAATTGTTAAGTGT	247 bp	Kaneko*et al*., 2011
*tpe*L (Virulence gene)	F-ATATAGAGTCAAGCAGTGGAG R - GGAATACCACTTGATATACCTG	466 bp	Baile*yet al*., 2013
*net*B (Virulence gene)	F- CGCTTCACATAAAGGTTGGAAGGC R - TCCAGCACCAGCAGTTTTTCCT	316 bp	
*sul*1 (Antimicrobial resistance gene)	F- CGGCGTGGGCTACCTGAACG R - GCCGATCGCGTGAAGTTCCG	433 bp	Ibekwe*et al*., 2011
*erm*B (Antimicrobial resistance gene)	F- GAA AAG GTA CTC AAC CAA ATA R- AGT AAC GGT ACT TAA ATT GTT TAC	638 bp	Soge*et al*., 2009

Table 3 cycling condition of the different primers during PCR.

Gene	Primary denaturation	Amplification				
		Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
Cpe	94°C	94°C	55°C	72°C	35	72°C
	5 min.	30 sec.	30 sec.	30 sec.		7 min.
*tpe*L	94°C	94°C	55°C	72°C	35	72°C
	5 min.	30 sec.	40 sec.	45 sec.		10 min.
*net*B	94°C	94°C	55°C	72°C	35	72°C
	5 min.	30 sec.	40 sec.	40 sec.		10 min.
*sul*1	94°C	94°C	60°C	72°C	35	72°C
	5 min.	30 sec.	40 sec.	45 sec.		10 min.
*erm*B	94°C	94°C	57°C	72°C	35	72°C
	5 min.	30 sec.	40 sec.	45 sec.		10 min.

3. RESULTS

3.1. Isolation and identification:

Out of 195 samples from chickens, rabbits, and calves, 108 isolates were identified as *C. perfringens* (91 from chickens,14 from rabbits and 3 from calves). The isolated colonies showed dark color, turbidity and offensive odor on cooked meat broth, and double zone of hemolysis on blood agar. Meanwhile on reinforced Clostridia agar, colonies were pin-headed, translucent and shiny. On TSC media, appear as typical black colonies.

Microscopically, Smear of suspected colonies revealed Gram positive anaerobic sporulated bacilli.

Biochemically, *C.perfringens* isolates showed positive results for glucose, sucrose and maltose fermentation, gelatin liquefaction and litmus milk test (stormy fermentation) and negative results with indole, oxidase and catalase tests. A clear opalescence zones were produced by all isolates on egg yolk media.

3.2. Molecular typing:

The suspected five isolates (3 from chickens, 1 from rabbits and 1 from calves) were positive only for *cpa* gene with absence of *(cpb, etx and itx*) genes indicating *C. perfringens* type A.

3.3. Antimicrobial susceptibility of Clostridium perfringens isolates:

All *30 C. perfringens* isolates (18 from chickens, 9 from rabbits and 3 from calves) showed (100%) resistance to spectinomycin, erythromycin and sulphamethoxazole+ trimethoprim. *C. perfringens* isolates showed high sensitivity to ampicillin + sulbactam (90%), moderate sensitivity to ofloxacin and cefotaxime (73%, 63%) respectively and (50%) for each meropenem and cefaclor. The lowest sensitivity of the isolates was (43%) for amoxicillin+clavulanic and (40%) for colistin. An exception to this pattern, however all isolates (3) from calves were (100%) resistant to cefotaxime and amoxicillin+clavulanic.

3.4. Molecular characterization of C.perfringens antibiotic resistance genes:

Out of five *C. perfringens* isolates that underwent resistance genes investigation, (*erm* B) gene was detected in four isolates (no. 1,2,3,4) while (*sul*1) gene was detected in all five isolates as shown in figures (1, 2).

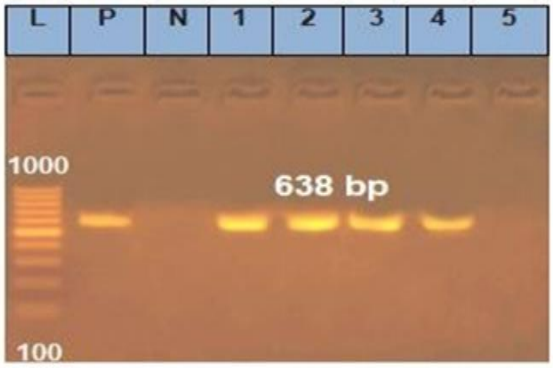

Figure 1 Agarose gel electrophoresis for an amplified product of (*erm*B) macrolide resistance gene of *C. perfringens* isolates. Lane (L): 100bp DNA Ladder, *Lane (P): positive control,* Lane (N): negative control, Lanes (1-4) positive isolates for *erm*B gene which amplified at 638bp and Lane (5): negative isolate for *erm*B gene.

* Positive and negative controls were represented by field sample that were previously confirmed to be positive or negative by PCR for the related genes in the Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Egypt.

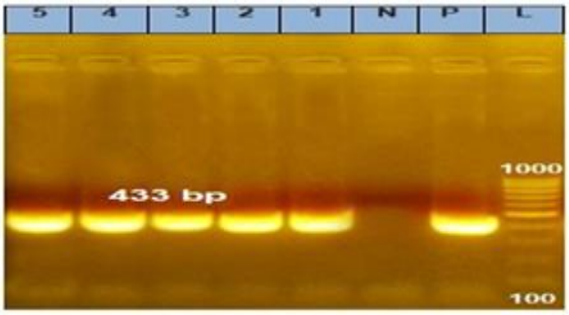

Figure 2 Agarose gel electrophoresis for an amplified product of (sul1) sulphonamide resistance gene of C. perfringens isolates. Lane (L): 100bp DNA Ladder, *Lane (P): positive control, *Lane (N): negative control, Lanes (1-5) positive isolates for sul1gene which amplified at 433bp.

3.5. Molecular characterization of Clostridium perfringens virulence genes:

Genes (*net B, cpe)* were detected in only two isolates (no.2, 3) (from chicken) but *tpe*Lgene was absent in all isolates as shown in figure (3).

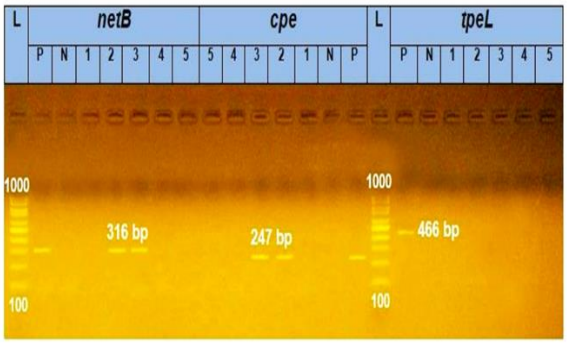

Figure 3 Agarose gel electrophoresis for an amplified product of (netB, cpe,tpeL) toxin genes of C. perfringens isolates. Lane (L): 100bp DNA Ladder,* Lane (P): positive control, *Lane (N): control negative, Lanes (2-3): samples isolated from chicken positive isolates for netB gene which amplified at 316bp and cpe gene at 247 bp.

4. DISCUSSION

In this study *C. perfringens* isolates recovered from different species showed (100%) resistance to spectinomycin, erythromycin and sulphamethoxazole+ trimethoprim. This result agrees with the previous study reporting the necrotic enteritis-causing *C. perfringens* in Egypt has 100% and 98% resistance to erythromycin and sulphamethoxazole-trimethoprim respectively (Osman and Elhariri, 2013). This is also augmented by the reported high resistance of all *C.perfringens* isolates to erythromycin in another study in Taiwan (Fan et al., 2016). This high resistance figure of *C. perfringens* to erythromycin was also reported in another study from Korea (Li et al., 2020). However a lower level of *C.perfringens* resistance to erythromycin reaching to 26% of the isolates was reported in another study (Anju et al., 2020). The discrepancy in the resistance pattern from one study to another can be attributed to the differences in *C.perfringens* exposure to different levels of antibiotic stress in different localities. These antibiotic resistance genes could be developed in the bacteria due to the overuse of antibiotics or even transmitted horizontally from other foodborne bacterial pathogens via conjugative plasmids (Lerminiaux and Cameron, 2019). On the other hand, in the current results, *C. perfringens* isolates showed high sensitivity to ampicillin + sulbactam (90%) in agreement with previous results showing a high sensitivity of *C. perfringens* to ampicillin (Wei et al., 2020). For the quinolone 2nd generation *C. perfringens* showed moderate 73% sensitivity to ofloxacin which is higher than the reported 45 % sensitivity to enrofloxacin (Wei et al., 2020). However, *C. perfringens* sensitivity to cefotaxime and meropenem was 63% and 50% respectively, which is lower than the previously reported 90% sensitivity to each of them ((Li et al., 2020). The current study-revealed *C. perfringens* 60% resistance to colistin is lower than the previously reported (100%) resistance (Park et al., 2015) which may reflect differences in exposure of the microorganism from one country to another and the lower previous use of colistin in the studied area compared to other areas. This overall higher *C.perfringens* resistance to antibiotics in Egypt than in other countries may reflect the higher antibiotic stress that renders the bacteria more resistant which indicates the disastrous misuse of the antibiotics in Egypt that impose a high economic loss in microbial management and control and the high risk of the probability of developing MDR strain that can evoke endemic outbreak crises. Antibiotic resistance genes development in bacteria occurs due to misuse and undue use of such antibiotics for animal disease control and growth promotion which force the bacteria to seek the way to survive the antibiotic challenge. In the current study the detection of the macrolide resistance gene (*erm*B)is not fully in accordance with the erythromycin resistance in all the studied isolates. This could be explained by presence of other macrolides resistance genes such as *erm* (Q) or *erm* (A). The detection of the macrolide resistance gene (*erm* B) in the 80% of the isolates reflects the hazardous and undue use of macrolides in the studied area that push the bacteria to develop this gene. This is to be considered alarming for the health practitioners as *C. perfringens* as intestinal inhabitant may act as a reservoir for the macrolide's resistance gene (*erm* B) and others and the possibility of conjugal transfer to other foodborne pathogens (Soge et al., 2009). Noteworthy, the detection of erythromycin resistance was reported to be lower in another locality in Egypt to be only 72% (Bendary et al., 2022), while a further lower report of 26% of erythromycin resistance isolates rate among *C. perfringens in* another country (Anju et al., 2020). This variation may reflect the different levels of macrolide previous use that may be associated with the developed resistance to this antibiotic in each locality. Although antimicrobial investigation-revealed resistance of all *C. Perfringens* isolates to sulfonamide is in consistence with the previously reported study of the high sulfonamide resistance rate among *C. perfringens* isolates (Xiaoting et al., 2021), in this study the detection of the sulfonamide antibiotic resistance gene (*sul*1) in all the studied isolates is higher than the lower rate of *sul*1 gene detection rate 6.4% among *C. perfringens* isolate in a recent study conducted in China (Xiaoting et al., 2021). Thus, we can propose (*sul*1) is totally responsible for the sulfonamide resistance in the studied isolates in our study while sulfonamide resistance in the previous study was mediated by other proteins encoded by different genes as *sul*2 or *sul*3. Thus, in our study the detection of the presence of *C. perfringens* isolates having resistance to more than 3 antibiotics family members indicates the MDR type of these isolates. Hence *C. perfringens* has been considered among the bacterial resistance-monitoring bacterial pathogens (Xiaoting et al., 2021), therefore, this MDR resistance encountered in this study is worth to be considered as a monitoring data.

Using the molecular investigation, the detection of alpha toxin encoded by *Cpa* gene in all the studied *C.perfringens* isolates indicates Type A identity of all isolates. This toxin is necessary for forming gas gangrene in case of animal and human severe infection. The pattern of alpha toxin in the current study is in consistence with the previously reported presence of alpha toxin in all isolates (Hassan et al., 2022) while the absence of beta, epsilon and iota toxin encoding genes excludes the presence of other major *C. perfringens* types in the current study. Moreover, detection of the virulence factors encoding genes *net*-B and enterotoxin in (40 %) of the 5 studied isolates indicate the responsibility of these toxins for causing necrotic enteritis since these isolates were recovered from chickens. This is in agreement with our previous reported detection of *net* B in (3/9) isolates from diseased broilers with necrotic enteritis (Ashraf et al., 2020). The presence of this toxin is critical for inducing necrotic enteritis in chickens by producing alpha hemolysin-pore formation (Rood et al., 2016). Notably *net*-B and *cpe* positive *C.perfringens* were detected only in 2 isolates out of 3 isolates recovered from chickens at a rate of (2/3) 66.7% in an agreement with the previous report stated that the incidence rate of *net*-B gene was (59/85) 68% in *C. perfringens* isolates (Mwangi et al., 2019).

5. CONCLUSION

Based on the result of this study, the anti-microbial resistance and distribution of toxin genes among *C. perfringens* recovered from different species demonstrated widespread drug resistance which reflect the uncontrolled and the undue use of the antibiotics that force the *C. perfringens* to develop this various mechanism of MDR. In spite of the several limitations that faced this study, it monitored the case of the foodborne pathogens and their ability to oppose the effort of treating or controlling diseases. The variation of the drug resistance pattern between the current studied and other areas all over the world and even others within Egypt may reflect the differences in the levels of the previous misuse of

antibiotics. This may be taken as an alarm for conducting a whole country's well planned and controlled study of *C.perfringens* antibiotic resistance and their responsible genes to monitor the antibiotics resistance situation allover Egypt.

6. REFERENCES

1. Ahmed, H.A.; El Bayomi, R.M.; Hamed, R.I.; Mohsen, R.A.; El-Gohary, F.A.; Hefny, A.A.; Elkhawaga, E. and Tolba, H.M.N., 2022. Genetic Relatedness, Antibiotic Resistance, and Effect of Silver Nanoparticle on Biofilm Formation by *Clostridium perfringens* Isolated from Chickens, Pigeons, Camels, and Human Consumers. Vet. Sci. 9, 109.
2. Ammar, A.M.; Abd El-Hamid, M.I.; El-Malt, R.S.; Azab, D.S.; Albogami, S.; Al-Sanea, M.M.; Soliman, W.E.; Ghoneim, M.M. and Bendary, M.M., 2021. Molecular detection of fluoroquinolone resistance among +multidrug-, extensively drug-, and pan-drug-resistant Campylobacter species in Egypt. Antibiotic. 10, 1342.
3. Anju, K., Karthik, K., Divya V., Mala, Priyadharshini M.L., Sharma, R.K. and Manoharan, S., 2020.Toxinotyping and molecular characterization of antimicrobial resistance in *Clostridium perfringens* isolated from different sources of livestock and poultry. Anaerobe. 67,102298.
4. Ashraf A. Abd El-Tawab, Amira M. Rizk, Mohammed M. El-Bardisy and Shreen Mayouf., 2020. Prevalence of *Clostridium perfringens* infection and virulence genes detection in broiler chickens Benha Veterinary Medical Journal 38, 128-132
5. Bailey, M.A.; Macklin, K.S. and Krehling, J.T., 2013. Use of a Multiplex PCR for the Detection of Toxin-Encoding Genes *net*B and *tpe*L in Strains of *Clostridium perfringens*. *ISRN veterinary science* vol. 865702.
6. Bendary, M.M., Abd El-Hamid, M.I., El-Tarabili, R.M., Hefny, A.A., Algendy, R.M., Elzohairy, N.A., Ghoneim, M.M., Al-Sanea, M.M., Nahari, M.H. and Moustafa, W.H., 2022. *Clostridium perfringens* Associated with Foodborne Infections of Animal Origins: Insights into Prevalence, Antimicrobial Resistance, Toxin Genes Profiles, and Toxinotypes. Biology. 1;11(4):551.
7. CLSI 2020: Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing, 30th ed.; CLSI supplement M100; Clinical and Laboratory Standards Institute.
8. Carter, G.R. and Cole, J.R., 1990. Textbook of Diagnostic procedure in veterinary bacteriology and mycology, 5 th., Ed., Academic press., Harcourt. Boace, publishers, New York, Boston, Toronto
9. Cooper, K.K. and Songer, J.G., 2010. Virulence of *Clostridium perfringens* in an experimental model of poultry necrotic enteritis. Vet. Microbiol.142, 323–328.
10. Dahiya, J.; Wilkie, D.; Van Kessel, A. and Drew, M., 2006. Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. Anim. Feed. Sci. Technol. 129, 60–88.
11. Dolan, G. P., Foster, K., Lawler, J., Amar, C., Swift, C., Aird, H., and Gorton, R., 2016. An epidemiological review of gastrointestinal outbreaks associated with *Clostridium perfringens*, Northeast of England, 2012-2014. Epidemiology and patho., 144(7), 1386–1393.
12. Fan, Y.C., Wang, C.L, Wang, C., Chen, T.C., Chou, C.H. and Tsai, H.J., 2016. Incidence and antimicrobial Susceptibility to *Clostridium perfringens* in Premarket Broilers in Taiwan. Avian Dis. 60(2), 444-9
13. Freedman, J.C., Shrestha, A. and McClane, B.A., 2016. *Clostridium perfringens* Enterotoxin: Action, Genetics, and Translational Applications. Toxins. 8, 73.
14. Freedman, J.C.; Theoret, J.R.; Wisniewski, J.A.; Uzal, F.A.; Rood, J.I. and McClane, B.A., 2015. *Clostridium perfringens* type A-E toxin plasmids. Res. Microbiol. 166, 264-279.
15. Goossens, E.E., Dierick, R., Ducatelle and F. Van Immerseel., 2020. Spotlight on avian pathology: untangling contradictory disease descriptions of necrotic enteritis and necrohaemorrhagic enteritis in broilers. Avian Pathol. 49(5), 423–427.
16. Hall B.G., 2004. Predicting the evolution of antibiotic resistance genes. Nat. Rev. Microbiol. 2,430–435.
17. Hassani, S.; Pakbin, B.; Brück,W.M.; Mahmoudi, R.and Mousavi, S., 2022. Prevalence, Antibiotic Resistance, Toxin-Typing and Genotyping of *Clostridium perfringens* in Raw Beef Meats Obtained from Qazvin City, Iran. Antibiotics. 11, 340.
18. Heida, F.H., van Zoonen, A.G.J.F. and Hulscher, J.B.F., 2016. A Necrotizing Enterocolitis-Associated Gut Microbiota Is Present in the Meconium: Results of a Prospective Study. Clin Infect Dis. 62(7),863-870.
19. Lahti, P., Heikinheimo, A., Johansson, T. and Korkeala, H.J., 2008. *Clostridium perfringens* type A strains carrying a plasmid-borne enterotoxin gene (genotype IS1151-cpe or IS1470-like-cpe) as a common cause of food poisoning. Clin. Microbiol. 46(1), 371-3
20. Ibekwe, A.M.; Murinda, S.E. and Graves, A.K., 2011. Genetic Diversity and Antimicrobial Resistance of *Escherichia coli* from Human and Animal Sources Uncovers Multiple Resistances from Human. 6 (6), e20819.
21. Jewell, S.A., Titball, R.W., Huyet, J., Naylor, C.E., Basak, A.K., Gologan, P., Winlove, C.P. and Petrov, P.G., 2015. *Clostridium perfringens* α-toxin interaction with red cells and model membranes. Soft Matter. 11, 7748–7761.
22. Kaneko, I.; Miyamoto, K.; Mimura, K.; Yumine, N.; Utsunomiya, H.; Akimoto, S. and McClane, B.A., 2011. Detection of Enterotoxigenic *Clostridium perfringens* in Meat Samples by Using Molecular Methods. Applied And Environmental Microbiology, 7526–7532.
23. Lee, K.W., Lilleho, H.S., Kim, W., Park, I., Li, C., Lu, M. and Hofacre, CL., 2021. Research Note: First report on the detection of necrotic enteritis (NE) B-like toxin in biological samples from NE-afflicted chickens using capture enzyme-linked immunosorbent assay.Poult Sci. 100(7), 101190.
24. Lerminiaux, N.A. and Cameron, A.D.S., 2019. Horizontal transfer of antibiotic resistance genes in clinical environments. J.Microbiol. 65(1),34-44
25. Li, Xiu., Liu, Y., Wu, W., Chen, S., Zhong, Z. and Wang, H., 2020. Prevalence and multilocus sequence typing of *Clostridium perfringens* isolated from 4

duck farms in Shandong province, China. Poult. Sci. 99, 5105–5117.
26. Macfaddin, J.F., 2000. Biochemical test for identification of medical bacteria 3rd edn., LippinCott Willians & wilkins, Philadelphia
27. McClane, B.A., 2001. *Clostridium perfringens*. In Food Microbiology: Fundamentals and Frontiers, 2nd ed; Beuchat, L.R., Doyle, M.P., Montville, T.J., Eds.; ASM Press: Washington, DC, USA, 2001; pp. 351–372.
28. Mosallam, F.M.; Helmy, E.A.; Bendary, M.M. and El-Batal, I.A., 2021. Potency of a novel synthesized Ag-eugenolnano emulsion for treating some bacterial and fungal pathogens. J. Mater. Res. 36, 1524–1537.
29. Mwangi, S.; Timmons, J.; Fitz-Coy, S. and Parveen, S., 2019. Characterization of *Clostridium perfringens* recovered from broiler chicken affected by necrotic enteritis. Poult Sci. 98(1),128-135.
30. Navarro, A., 2018. Mechanisms of Action and Cell Death Associated Mauricio with *Clostridium perfringens* Toxins. Toxins vol. 10(5), 212-222.
31. Osman, K.M. and Elhariri, M., 2013. Antibiotic resistance of *Clostridium perfringens* isolates from broiler chickens in Egypt. Rev Sci Tech.32(3), 841-50.
32. Park, J.Y., Kim, S., Oh, J.Y., Kim, H.R., Jang, I., Lee, H.S. and Kwon, Y.K., 2015. Characterization of *Clostridium perfringens* isolates obtained from 2010 to 2012 from chickens with necrotic enteritis in Korea. Poultry Science. 94(6), 1158 - 64.
33. Rood, J. I., A.L. Keyburn and R.J. Moore., 2016. NetB and necrotic enteritis: the hole movable story, Avian Pathol. 45 (3), 295–301.
34. Rood, J.I.; Adams, V.; Lacey, J.; Lyras, D.; McClane, B.A.; Melville, S.B.; Moore, R.J.; Popoff, M.R.; Sarker, M.R. and Songer, J.G., 2018. Expansion of the *Clostridium perfringens* toxin-based typing scheme. Anaerobe. 53, 5–10.
35. Sambrook, J., Fritsch, E. R .and Maniatis, T., 1989. Molecular cloning. A laboratory manual. Vol 1, Cold spring Harbor Laboratory press, New York.
36. Soge, O.O.; Tivoli, L.D.; Meschke, J.S. and Roberts, M.C., 2009. A conjugative macrolide resistance gene, *mef*(A), in environmental *Clostridium perfringens* carrying multiple macrolides and⁄ or tetracycline resistance genes. Journal of Applied Microbiology. 106, 34–40.
37. Uzal, F.A., Vidal, J.E., McClane, B.A. and Gurjar, A.A., 2010. *Clostridium perfringens* Toxins Involved in Mammalian Veterinary Diseases. Open Toxinol. J. 2 ,24–42.
38. Uzal, F. A., Navarro, M. A., Li, J., Freedman, J. C., Shrestha, A. and McClane, B. A., 2018. Comparative pathogenesis of enteric clostridial infections in humans and animals. Anaerobe. 53, 11–20.
39. Wei, B., Cha, S.Y., Zhang, J.F., Shang, K., Park, H.C., Kang, J., Lee, K.J., Kang, M. and Jang H.K., 2020. Antimicrobial Susceptibility and Association with Toxin Determinants in *Clostridium perfringens* Isolates from Chickens. Microorganisms. 8(11),1825.
40. Xiaoting, W., Chengcheng, N., Chunhui, J., Yan, L., Jing, L., Qingling, M., Jun, Q., Lixia, W., Kuojun, C., Jinsheng, Z., Zaichao, Z., Weiwei, Y., Yelong, P. and Xuepeng, 2021. Antimicrobial resistance profiling and molecular typing of ruminant-borne isolates of *Clostridium perfringens* from Xinjiang, China. J Glob Antimicrob Resist. 27, 41-45.
41. Yoo, H.S.; Lee, S.U.; Park, K.Y. and Park, Y.H., 1997. Molecular Typing and Epidemiological Survey of Prevalence of *Clostridium perfringens* Types by Multiplex PCR. Journal of Clinical Microbiology. 35 (1), 228-232.