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Insight of Prevalence, Toxin Typing and Antimicrobial Susceptibility of Egyptian *Clostridium perfringens* Isolates Recovered from Broiler, Layer and Breeder Chicken Flocks

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ABSTRACT

Clostridial infection is associated with significant health problems in poultry today, as its enteritis affects intestinal integrity in poultry flocks and may cause considerable losses, which caused by *Clostridium perfringens* that found all over the world, so fighting this infection is a continuing challenge for the poultry sector. Preventive actions using dedicated products are a valuable solution to maintain healthy gut flora. In this study the prevalence of *Clostridium perfringens* was detected in different chicken flocks at a rate of 32%. Also, toxin-typing showed presence of *cpa, cpb, etx, iap* and *cpe* toxins among the isolates which mainly associated with necrotic enteritis cases. *Net-B* toxin is a novel toxin that had been recently identified in virulent avian *C. perfringens* isolates and it presence potentiate the necrotic lesions and destroy the enterocytes. Antimicrobial patterns showed high resistance against most common antibacterial drugs as β-lactams, aminoglycosides, macrolides and tetracyclines. *NetB toxin* harboring isolates, originating from diseased broiler, layer and breeder chickens showed the lowest minimum inhibitory concentration MICs values for the penicillin from β-lactams and tylvalosin from macrolides.

Key words: *Clostridium perfringens*, *Net-B* toxins, Chicken flocks, MICs, Penicillin.

INTRODUCTION

Clostridium belongs to *Clostridiaceae* family which contains more than 203 species. *Clostridium perfringens* (*C. perfringens*) is the most commonly isolated species, which is classified to five types according to major lethal toxins they produce (Koneman et al. 1997). Clostridium diagnosis at bacteriological and molecular base still difficult till now for several reasons, including their specific growth (Collins et al. 1994). Clinically the important *Clostridium* species that associated are with clinical aspects in humans, animals and birds ranged from 40-50 species that produce a variety of toxins which leads to the distinctive clinical features of the diseases they cause (Hatheway 1990). PCR-based methods for *C. perfringens* genotypes are accumulating as more diagnostic and research laboratories adopt for toxin gene detection. There were various genotypes by geographic region, although *C. perfringens* type A remains the most commonly isolated type overall (Garmory et al. 2000), which act as the causative agent of necrotic enteritis, and its clinical form is most often seen in broilers but may also be seen in broiler breeders and layers kept on litter system, while subclinical form appear in the intestine and liver. The disease is characterized by distinct ulcers and necrosis in the mucosa of the anterior small intestine and hepatitis (Fossum et al. 1988; Kaldhusdal and Hofshagen 1992). *C. perfringens*-related hepatitis is characterized by cholangiohepatitis or focal necrosis in the liver and bile ducts, leading to obstruction of the bile ducts (Randall 1991), as the pathogens migrate from duodenum to liver through bile ducts or via liver veins (Sasaki et al. 2000).

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Virulent *C. perfringens* isolates produce 16 protein toxins that are important for the development of different diseases, such as food poisoning, antibiotic associated diarrhea, fatal gas gangrene, enterotoxaemia, and hemorrhagic gastroenteritis (Jihong et al. 2016). *C. perfringens* α-toxigenic strains of are the most common type isolated from chickens suffering from necrotic enteritis (Timbermont et al. 2009), but recently *netB* is a new toxin, in avian *C. perfringens* type A strains (Keyburn et al. 2008). Alpha toxin is a phospholipase that hydrolyzes phospholipids and promotes membrane disorganization, also hydrolysis of lecithin results in stimulation of the arachidonic acid cascade that induces the synthesis of inflammatory mediators which causes blood vessel contraction, platelet aggregation and myocardial dysfunction, leading to acute death, while beta toxin induces hemorrhagic necrosis of the intestinal mucosa (Titball et al. 2000; Awaad et al. 2019). The most common predisposing factors for NE include environmental stress, high stocking density, concurrent infection with IBD and coccidiosis, high protein and fat levels in diet change in mucus production and gut transit time and other dietary factors including lectins, trypsin inhibitors, tannins and mycotoxins (Prescott et al. 2016), so NE has been controlled by reducing exposure to risk factors such as coccidiosis, unsuitable diets and adding antibiotics in the feed and water of poultry (Gabriel et al. 2003). Unfortunately, antibiotic resistance in bacteria may make the commonly used antibiotics less effective (Yegani and Korver 2007), so antibiotic growth promoters (AGPs) have been banned from animal feed worldwide to avoid the spread of antimicrobial resistance and this has contributed to the higher prevalence of economically important diseases such as necrotic enteritis (Van Immerseel et al. 2009). The present study aimed to assess the prevalence of *netB* harboring *C. perfringens* among Egyptian chicken flocks either broiler, or breeder with special attention to its antimicrobial susceptibility and minimum inhibitory concentration.

MATERIALS AND METHODS

Isolation and Identification of *C. Perfringens*

During 2020, three hundred intestinal contents of enteritis lesions from broiler (n=160), layer (n=100) and breeder (n=50) chicken's suspected cases from 40 flocks (broilers (n=25), layer (n=30) and breeder (n=10) were inoculated into tryptone proteose peptone glucose, then transfer to fluid thioglycollate (Becton, Dickinson, USA) and plated onto TSC agar (Oxoid, UK) at 37ºC in an anaerobic condition (Martin et al. 2009). Colonies were further sub-cultured on sheep blood agar and checked visually for double-hemolysis zone surrounding the colonies of *C. perfringens* and confirmed biochemically using API 20 A Anaerobes system. The recovered isolates bacterial suspensions were then frozen in BHI broth with 20% glycerol at –80°C (Quinn et al. 2011).

C. perfringens Toxin Typing by using PCR Assay

The boiling technique according to Kanakaraj et al. (1998), was used to extract DNA from the isolates and the primers for toxins typing were *cpa*, *cpb*, *etx*, *iap*, *cpe* according to Yoo et al. (1997), while *netB* toxin primers were used according to Datta et al. (2014) at which selected *netB* toxin positive isolates were sequenced and accessed on Genbank from broiler, and breeder chicken flocks.

Antimicrobial Susceptibility Testing

Agar disc diffusion method were used for tested antimicrobial susceptibility *C. perfringens* strains at 37ºC overnight in anaerobic conditions using the following antimicrobial agents (Oxoid, Hampshire, UK) were tested: penicillin G (10U), penicillin V (30µg), ampicillin (10µg), amoxicillin (20µg), ceftifur (30µg), bacitracin (10µg), lincomycin (30µg), florphenicol (30µg), clindamycin (2µg), erythromycin (15µg), tylosin (30µg), tilmicosin (15µg), spiramycin (100µg), tylvalosin (0.6µg), flumequine (30µg), ciprofloxacin (5µg), enerofloxacin (5µg), difloxacin (10µg), oxytetracycline (30µg), doxycycline (30µg), metronidazole (30µg) rifampicin (5µg), vancomycin (5µg), colistin(10µg), neomycin (30µg), gentamycin (10µg), streptomycin (10µg), spectinomycin (100µg) and trimethoprim-sulfamethoxazole (25µg) (Perelman et al. 1991; BSAC 2011).

Measuring MICs of *Clostridium perfringens net-B* Toxins Isolates

Brucella broth was used for broth micro dilution susceptibility testing. Refreshes of *C. perfringens* strains from frozen condition by subculture twice on TSA supplemented with 5% defibrinated sheep blood followed by inoculation into brucella broth supplemented with Oxyrase to generate anaerobiosis. Inoculum was grown at 37ºC for 24±12h then diluted in brucella broth to a 0.5 McFarland turbidity standard. Sixteen antibiotics were obtained from Sigma (St Louis, MO) as the following: penicillinV and G, ampicillin, amoxicillin, ceftifur, bacitracin, lincomycin, tylosin, tilmicosin, tylvalosin, ciprofloxacin, enrofloxacin, difloxacin, flumequine, vancomycin, metronidazole and rifampicin and Stock solution prepared according to the guidelines of the producer. Stocks were either frozen or freshly prepared and subsequent dilutions of the stock solutions were performed using the procedure listed in NCCLS (CLSI 2018).

Statistical Analysis

Data were analyzed by with SPSS version 7.5 software. All values were expressed as the mean±SD. significant differences between the groups were statistically analyzed by one way analysis of variance (ANOVA). A statistical difference of $P<0.05$ was considered significant.

RESULTS

The prevalence of *C. perfringens* during the different season of 2020 was 37% distributed as the following in broilers (42%), layers (38%) and breeders (44%) with high incidence in autumn (30%) followed in winter (26%), spring (22%) and summer (19%). Toxin typing of the recovered isolates by using PCR assay revealed that; *cpa* toxin in broilers (73%), layers (79%) and breeders (68%). Whereas *cpb* toxins was found in 25% broilers, 66% layers and 45% breeders. The *etx* toxin was detected in 14.5% broilers, 32% layers and 18% breeders. The *iap* toxin was noted in 27% broilers, 37% layers and 27% breeders. The *cpe* and *netB* toxin were found in 45 and 82% broilers, 53 and 89% layers and 45 and 90% breeders, respectively.

Table 1: Disk diffusion antimicrobial susceptibility of Egyptian *C. perfringens* isolates recovered from various chicken flocks

Antimicrobials	Broilers (n=55)			Layers (n=38)			Breeders (n=22)		
	S/R	+ No.	+ %	S/R	+ No.	+ %	S/R	+ No.	+ %
Penicillin G	S	40	73	S	28	74	S	15	68
Penicillin V	S	55	100	S	38	100	S	22	100
Ampicillin	S	30	54.5	S	30	79	R	22	100
Amoxicillin	S	45	82	S	30	79	S	18	82
Ceftifur	S	25	45	S	15	39	S	12	55
Bacitracin	S	25	45	R	20	53	R	15	68
Lincomycin	S	25	45	R	20	53	R	15	68
Clindamycin	R	55	100	R	28	74	R	22	100
Erythromycin	S	15	27	R	38	100	R	22	100
Spiramycin	R	55	100	R	38	100	R	22	100
Tylosin	S	25	45	S	18	47	S	12	55
Tilmicosin	S	20	37	S	15	39	S	10	45
Tylvalosin	S	40	73	S	25	66	S	15	68
Ciprofloxacin	S	15	27	R	38	100	R	22	100
Difloxacin	S	15	27	R	38	100	R	22	100
Flumequine	S	15	27	R	38	100	R	22	100
Oxytetracycline	R	55	100	R	38	100	R	22	100
Doxycycline	R	55	100	R	38	100	R	22	100
Chlortetracycline	R	55	100	R	38	100	R	22	100
Vancomycin	S	55	100	S	38	100	S	22	100
Rifampicin	S	30	54.5	R	30	79	R	18	82
Metronidazole	S	30	54.5	R	20	53	R	18	82
Colistin	R	55	100	R	38	100	R	22	100
Florphenicol	R	55	100	R	38	100	R	22	100
Spectinomycin	R	55	100	R	38	100	R	22	100
Neomycin	R	55	100	R	38	100	R	22	100
Gentamycin	R	55	100	R	38	100	R	22	100
Streptomycin	R	55	100	R	38	100	R	22	100
Trimethoprim-sulfamethoxazole	R	55	100	R	38	100	R	22	100

R: Resistant S: Susceptible.

Three *netB* toxin harboring *C. perfringens* strains were sequenced and accessed on Genbank under code: MW925054 from broiler, MZ382848 from layer and MW925055 from breeder chicken flock respectively. Antimicrobial susceptibility patterns by disk diffusion methods in Table 1 showed that the all recovered isolates were sensitive to penicillin, amoxicillin, tylvalosin and vancomycin, while were resistant for clindamycin, spiramycin, oxytetracycline, doxycycline, chlortetracycline, colistin, florphenicol, spectinomycin, neomycin, gentamycin, streptomycin and trimethoprim-sulfamethoxazole. The other antibiotics showed sensitivity against broiler isolates only as lincomycin, erythromycin, ciprofloxacin, enrofloxacin, difloxacin, flumequine and rifampicin. Also, there were some isolates sensitive to amoxicillin, ceftifur, tylosin, and tilmicosin. The minimum inhibitory concentration values "MICs µg/ml'' of *net-B* toxin isolates recorded in (Table 2) which showed that the lowest values were for penicillin MIC=0.25; MIC_{50}=0.44; MIC_{90}=0.82 against *C. perfringens* recovered from broiler chicken flocks, while values against breeder were 0.32, 0.52 and 1µg/ml respectively, followed by tylvalosin values 0.25, 0.82 and 1.2µg/ml against all isolates, while the other examined antimicrobials showed high MIC values.

DISCUSSION

Necrotic enteritis (NE) caused by toxigenic strains of *C. perfringens* costs the worldwide poultry community an estimated $2 billion annually due to costs of antimicrobial prophylaxis, loss of weight gain and inefficient feed conversion (Van Immerseel et al. 2009). Elimination of routine antibiotic use has been associated with increased incidence of NE (Cooper and Songer 2009) as the prevalence of *C. perfringens* could be attributed to the unsanitary conditions, poor hygienic measures (McClane et al. 2006). In current study the prevalence of *C. perfringens* was 37% in different Egyptian chicken flocks at which 42% in broiler, 38% in layer and 44% in breeder with high incidence in autumn 30% and in winter 26% than spring and summer which may be due to crowdedness and accumulation behavior of chickens in cold weather, also physiological stress act as an important predisposing factor to NE due to disturbance in corticosteroids hormones which corresponded to increased densities of *C. perfringens* in the small intestine and weight gain impairment in chickens, this emphasizes the importance of managing stress to optimize chicken health (Sarah et al. 2020).

Recently the NE pathogenesis in poultry has been the subject of considerable investigation, following identification of the novel pore forming toxin *netB* on a 42kb plasmid-encoded pathogenicity locus (NELoc-1) harbored specifically by NE strains (Keyburn et al. 2006; Keyburn et al. 2008) which were all highly conserved in both nucleotide and amino acid sequence (Menestrina et al. 2001) and the recovered *C. perfringens* strains from necrotic enteritis lesions were *netB*-positive, so these results provide a further evidence that *netB* is an essential virulence factor in the pathogenesis of necrotic enteritis (Nauerby et al. 2003). There was a strong correlation between *cpb2* gene and *netB* gene (Crespo et al. 2007; Martin and Smyth 2009; Abildgaard et al. 2010).

Table 2: Minimum inhibitory concentration values (µg/mL) of Egyptian *C. perfringens* isolates harboring *net-B* toxin recovered from broiler, layer and breeder chicken flocks

C. perfringens origin from	Broilers			Layers			Breeders		
MIC values (µg/mL)	MIC	MIC_{50}	MIC_{90}	MIC	MIC_{50}	MIC_{90}	MIC	MIC_{50}	MIC_{90}
Penicillin G	0.5	1.5	1.82	1.5	2.5	3.6	2.3	4.2	4.4
Penicillin V	0.25	0.44	0.82	0.32	0.48	0.92	0.32	0.52	1.00
Ampicillin	0. 56	2.1	2.8	2.2	3.5	5.5	2.3	4.2	8.3
Amoxicillin	0.52	0.62	1.2	0.83	1.5	1.8	0.72	1.2	1.8
Ceftifur	0.58	2.2	4.5	2.2	4.4	8.3	2.2	4.4	8.3
Bacitracin	0.56	2.3	3.6	2.5	4.5	5.4	2.2	4.4	6.3
Lincomycin	0.57	4.2	8.4	2.1	3.6	8.4	2.1	3.6	8.4
Tylosin	1.2	2.3	4.4	1.2	2.3	4.4	1.2	2.6	4.2
Tilmicosin	1.2	2.3	4.4	1.5	2.5	4.8	1.4	2.8	4.5
Tylvalosin	0.25	0.82	1. 2	0.25	0.82	1. 2	0.25	0.82	1. 2
Ciprofloxacin	2.8	4.3	8.4	R	R	R	R	R	R
Difloxacin	2.8	4.3	8.4	R	R	R	R	R	R
Flumequine	2.8	4.3	8.4	R	R	R	R	R	R
Vancomycin	0.25	1.2	1.8	0.25	1.2	1.8	0.25	1.3	1.8
Rifampicin	1.33	4.3	6.2	1.8	3.5	4.5	2.3	4.3	8.4
Metronidazole	0.52	2.5	4.3	1.6	2.8	4.2	1.2	2.4	3.6

R: resistant. Dilution factor: 0.25-512µg/mL.

NE develops when *C. perfringens* establish and multiply in the chicken's intestinal tract due to reduction of intestinal motility with mucosal damage and leakage of serum proteins into the intestinal lumen (Drew et al. 2004). *C. perfringens* has a generation time of 8 to 10min, so can increase very quickly (Stevens and Bryant 2002). In the present study, toxin typing of the recovered isolates revealed that *cpa, cpb, etx, iap* and *cpe* toxin in broiler isolates represent 73, 25, 14.5, 27 and 45%, respectively and in layers were 79, 66, 32, 37 and 53%, respectively while in breeder isolates 68, 45,18, 27 and 45%, respectively. Toxin typing of *C. perfringens* is critical for understanding of the epidemiology criteria of *C. perfringens* infections and may be helpful in the development of effective preventive measures (Nowell et al. 2010) as the release of these toxins is believed to play a major role in determining pathogenesis properties of *C. perfringens* (Ronco et al. 2017). Datta et al. (2014) showed that out of 30 *C. perfringens* isolates from healthy birds, 33.3% were positive for α toxin alone and 6.7% for β-2 toxin alone. In addition, seven (23.3%) isolates were positive for both α and β-2 toxins. In case of the diseased birds, 16 (53.3%) isolates were positive for alpha toxin alone and 2 (6.7%) for β-2 toxin alone. Ten (33.3%) isolates were positive for both α and β-2 toxins. Thus, 19 and 28 isolates from healthy and diseased birds respectively were toxin producing. Agrawal made similar observations et al. (2009) who reported 39.2% isolates of *C. perfringens* to be positive for α toxin alone and 32.10% isolates were positive for both α and β toxins. Fan et al. (2016) reported that the *C. perfringens* type A isolates expressed only the *cpa* gene encoding for alpha toxin. Several studies have indicated that *cpe*-positive strains of *C. perfringens* from poultry occur in low number and can be less than 5% of global *C. perfringens* isolates (Zhang et al.2018). This study showed that the presence of α and β-2 toxin producing strains of *C. perfringens* in healthy as well as enteritis-affected broiler chickens. There was an increase in number of the toxin producing isolates from diseased birds.

Higher percentage of α and beta-2 toxin gene producing *C. perfringens* strains in diseased broiler birds indicates its possible role in pathogenesis of enteritis. Alpha toxin hydrolyzes lecithin a major component of the cell membrane and thus destroys the red blood cells, platelets and muscles leading to the myonecrosis. Beta toxin induces hemorrhagic necrosis of the intestinal mucosa. Australian strain of *C. perfringens* type A harbored *NetB* was isolated from necrotic enteritis (NE) affected broiler chicken (Keyburn et al. 2010). In the present study, traceability of *net-B* toxins in the recovered isolates showed that *net-B* toxin distribution in broiler isolates 82%, layer isolates 89% and breeder isolates 90%. Sequencing of three isolates and accessesion numbers on Genbank and under code: MW925054 from broiler, MZ382848 from layer and MW925055 from breeder chicken flock respectively. *Net-B* is a pore forming toxin with structure equal to 3.9 Angstrom (Savva et al. 2013), which can damage the phospholipid membrane bilayer of both human and animal cells, causing an influx of ions (i.e., Na+, Cl, Ca2+that leads to osmotic cell lysis (Keyburn et al. 2010; Sergio et al. 2014). Interestingly, all *netB*-positive isolates were from flocks with the subclinical form of NE, with a moderate increase of mortality rate but an absence of typical pathologic findings of enteritis. In contrast, when severe NE was observed during autopsy of deceased birds, no *netB* gene was detected (Francesca et al. 2019).

Antibiotic as β-lactams, aminoglycosides, macrolides and tetracyclines used in broiler farms for therapeutic purposes of enteric diseases, particularly necrotic enteritis Penicillins as a β-lactams members are known to be particularly active against *C. perfringens* as resistance against penicillin is very rare and β-lactamase has not been demonstrated with three days as minimum duration of treatment, however longer applications may be required (Hughes et al. 2008). In the current study, the antimicrobial sensitivity test showed that all isolates were 100% sensitive for penicillin, amoxicillin, tylvalosin and vancomycin, while were resistant for clindamycin, spiramycin, oxytetracycline, doxycycline, chlortetracycline, colistin, florphenicol, spectinomycin, neomycin, gentamycin, streptomycin and trimethoprim-sulfamethoxazole. Recently, Gad et al. (2011) determined MIC of 16 antibiotics for 100 *C. perfringens* isolates collected between 2008 and the results revealed that there were no

isolates were resistant β-lactam antibiotics, lincospectin, tylosin, doxycycline, tetracycline, trimethoprim/ sulfamethoxazole, lincomycin, and tilmicosin with low frequency of resistance was detected against erythromycin and tiamulin with 5 and 20%, respectively, while the highest incidence of resistance were spectinomycin, neomycin and colistin with 74, 94 and 100%, respectively. In economic point of view controlling of necrotic enteritis cases even clinical or subclinical form in commercial poultry flocks become urgent to overcome its losses in body weight gain and costs of medication (Kaldhusdal and Løvland 2000; Skinner et al. 2010), so strategies to reduce the incidence of clostridial infections become important to avoid economic losses and increase the profitability (McReynolds et al. 2009).

Finally, the present study survey of the minimum inhibitory concentration of *net-B* toxins isolates at which the lowest values were for penicillin MIC_{90}=0.82 against *C. perfringens* recovered from broiler chickens flocks, while values against layers and breeders were 1µg/ml respectively, followed by tylvalosin MIC_{90}=1.2µg/ml against all isolates, while the other examined antimicrobials showed variable values. Resistance of *C. perfringens* the most common antibiotics become recorded (Shojadoust et al. 2010), and also there were MDR isolates as detected in Iran 34.17% (Akhi et al. 2015), this may be due to the wide spread of the antimicrobials in combating infections. Resistance rate of *C. perfringens* to tetracycline 66and 56.2% (Tansuphasiri et al. 2005) and 87.5% to neomycin (Shojadoust et al. 2010), while the percentage of resistance to β-lactams was less than 7% (Silva et al. 2014; Hamza et al. 2017; Chon et al. 2018; Mwangi et al. 2019).

Conclusion

C. perfringens has been shown to develop multiple drug resistance mainly in winter and autumn season, indicating that the treatment for this bacterium is quite challenging, so must depend on MICs values for common used antibiotics which revealed that penicillin is the drug of choice against isolates from different flocks.

Author's Contribution

All authors contributed equally to study the design methodology, interpretation of results, and writing of the manuscript.

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REFERENCES

Abildgaard L, Sondergaard TE, Engberg RM, Schramm A and Højberg O, 2010. In vitro production of necrotic enteritis toxin B, NetB, by netB-positive and netB-negative Clostridium perfringens originating from healthy and diseased broiler chickens. Veterinary Microbiology 144: 231-235. https://doi.org/10.1016/j.vetmic.2009.12.036

Agrawal A, Narang G, Rakha NK, Mahajan NK and Sharma A, 2009. In vitro lecithinase activity and antibiogram of *Clostridium perfringens* isolated from broiler chickens. Haryana Veterinary 48: 81-84.

Awaad MHH, Elmenawey MA, Afify MA, Zouelfekar SA, Mohamed FF and Demy V, 2019. The impact of high stocking density and *Saccharomyces cerevisiae boulardii* on productive performance, intestinal microbiota and gut integrity of broiler chickens. International Journal of Veterinary Science 8: 362-370.

BSAC, 2011. Methods for antimicrobial susceptibility testing, Version 10.2, May 2011. British Society for Antimicrobial Chemotherapy, Birmingham, United Kingdom.

Chon JW, Seo KH, Bae D, Park JH, Khan S and Sung K, 2018. Prevalence, toxin gene profile, antibiotic resistance, and molecular characterization of *Clostridium perfringens* from diarrheic and non-diarrheic dogs in Korea. Journal of Veterinary Science 19: 368-374. https://doi.org/10.4142/jvs.2018.19.3.368

CLSI, 2018. M11, Methods for Antimicrobial Susceptibility Testing of Anaerobic bacteria. 9th Ed. Clinical Laboratory Standards Institute. Available at https://clsi.org/media/2577/m11-ed9_sample

Collins MD, Lawson PA, Willems A, Cordoba JJ, Fernandez-Garayzabal J and Garcia P, 1994. The phylogeny of the genus Clostridium: proposal of five new genera and eleven new species combinations. International Journal of Systematic and Evolutionary Microbiology 44: 812-826. https://doi.org/10.1099/00207713-44-4-812

Cooper KK and Songer JG, 2009. Necrotic enteritis in chickens: A paradigm of enteric infection by Clostridium perfringens type A. Anaerobes 15: 55-60. https://doi.org/10.1016/j.anaerobe.2009.01.006

Crespo R, Fisher DJ, Shivaprasad HL, FernandezMiyakawa ME and Uzal FA, 2007. Toxinotypes of *Clostridium perfringens* isolated from sick and healthy avian species. Journal of Veterinary Diagnostic Investigation 19: 329-333. https://doi.org/ 10.1177/104063870701900321

Datta S, Rakha NK, Narang G, Arora D and Mahajan NK, 2014. Prevalence of α, β and Net B toxins producing strains of clostridium perfringens in broiler chicken in Haryana. Haryana Veterinary Journal 53: 39-42

Drew MD, Syed NA, Goldade BG, Laarveld B and Van Kessel AG, 2004. Effects of dietary protein source and level on intestinal populations of *Clostridium perfringens* in broiler chickens. Poultry Science 83: 414-420. https://doi.org/10.1093/ps/83.3.414

Fan YC, Wang CL, Wang C, Chen TC, Chou CH and Tsai HJ, 2016. Incidence and antimicrobial susceptibility to *Clostridium perfringens* in Premarket broilers in Taiwan. Avian Diseases 60: 444-449. https://doi.org/10.1637/11315-110915-Reg

Fossum O, Sandstedt K and Engström BE, 1988. Gizzard erosions as a cause of mortality in white leghorn chickens. Avian Pathology 17: 519-525. https://doi.org/10.1080/03079458808436471

Francesca P, Cristina E, Di Francesco DI and Andrea Di ,2019. Prevalence of *netB*-positive *Clostridium perfringens* in Italian poultry flocks by environmental sampling. Journal of Veterinary Diagnostic Investigation 32: 252–258. https://doi.org/10.1177/1040638719885841

Gabriel I, Mallet S and Leconte M, 2003. Differences in the digestive tract characteristics of broiler chickens fed on complete pelleted diet or on whole wheat added to pelleted protein concentrate. British Poultry Science 44: 283-290. https://doi.org/10.1080/0007166031000096470

Gad W, Hauck R, Krüger M and Hafez HM, 2011. Determination of antibiotic sensitivities of Clostridium perfringens isolates from commercial turkeys in Germany in vitro. European Poultry Science 75: 80-83.

Garmory HS, Chanter N, French NP, Bueschel D, Songer JG and Titball RW, 2000. Occurrence of *Clostridium perfringens* beta2-toxin amongst animals, determined using genotyping and subtyping PCR assays. Epidemiology

and Infection 124: 61–67. https://doi.org/10.1017/s0950268899003295

Hamza D, Dorgham S and Hakim A, 2017. Toxinotyping and antimicrobial resistance of Clostridium perfringens isolated from processed chicken meat products. Journal of Veterinary Research 61: 53-58. https://doi.org/10.1515/jvetres-2017-0007

Hatheway CL, 1990. Toxigenic clostridia. Clinical Microbiology Review 3: 66-98. https://doi.org/10.1128/cmr.3.1.66

Hughes L, Hermans P and Morgan K, 2008. Risk factors for the use of prescription antibiotics on UK broiler farms. Journal of Antimicrobial Chemotherapy 61: 947–952. https://doi.org/10.1093/jac/dkn017

Jihong L, Daniel P, Mahfuzur RS, and Clane BAM ,2016. Clostridium perfringens Sporulation and Sporulation-Associated Toxin d Toxin Production. Microbiology Spectrum Journal, 4: 1-26. http://www.doi.org/10.1128/microbiolspec.TBS-0022-2015

Kaldhusdal M and Løvland A, 2000. The economical impact of Clostridium perfringens is greater than anticipated. World Poultry 16: 50–51.

Kaldhusdal M and Hofshagen M, 1992. Barley inclusion and avoparcin supplementation in broiler diets. Clinical, pathological and bacteriological findings in a mild form of necrotic enteritis. Poultry Science 71: 1145-1153. https://doi.org/10.3382/ps.0711145

Kanakaraj R, Harris DL, Songer JG and Bosworth B, 1998. Multiplex PCR assay for detection of Clostridium perfringens in feces and intestinal contents of pigs and in swine feed. Veterinary Microbiology 63: 29-38. https://doi.org/10.1016/s0378-1135(98)00229-6

Keyburn AL, Boyce JD, Vaz P, Bannam TL, Ford ME and Parker D, 2008.NetB, a new toxin that is associated with avian necrotic enteritis caused by Clostridium perfringens. PLoS Pathogen 4: e26. https://doi.org/10.1371/journal.ppat.0040026

Keyburn AL, Sheedy SA, Ford ME, Williamson MM and Awad MM, 2006. Alpha-Toxin of Clostridium perfringens is Not an Essential Virulence Factor in Necrotic Enteritis in Chickens. Infection and Immunity 74: 6496–6500. https://doi.org/ 10.1128/IAI.00806-06

Keyburn AL, Bannam TL, Moore RJ and Rood JI, 2010. Net B, a pore-forming toxin from necrotic enteritis strains of Clostridium perfringens. Toxins 2: 1913-1927. https://doi.org/10.3390/toxins2071913

Koneman EW, Allen SD, Janda WM, Schreckenberger PC and Winn WJ,1997. Color Atlas and Textbook of Diagnostic Microbiology. 5th Ed. Philadelphia: Lippincott Williams and Wilkins, pp: 709-784.

Martin TG and Smyth JA, 2009. Prevalence of netB among some clinical isolates of *Clostridium perfringens* from animals in the United States. Veterinary Microbiology 136: 202–205. https://doi.org/10.1016/j.vetmic.2008.10.026

McClane B, Lyerly DM and Wilkins TD, 2006. Chapter 57: Enterotoxic clostridia: Clostridium perfringens type A and Clostridium difficile. In: Gram-Positive Pathogens, 2nd Ed, Wiley Online Library pp: 703-714. https://doi.org/10.1128/9781555816513.ch57

McReynolds J, Waneck C, Byrd J, Genovese K, Duke S and Nisbet D, 2009. Efficacy of multistrain direct-fed microbial and phytogenetic products in reducing necrotic enteritis in commercial broilers. Poultry Science 88: 2075-2080. https://doi.org/10.3382/ps.2009-00106

Menestrina G, Serra MD and Prevost G, 2001. Mode of action of beta-barrel pore-forming toxins of the staphylococcal alpha-hemolysin family. Toxicon 39: 1661–1672. https://doi.org/10.1016/s0041-0101(01)00153-2

Mwangi S, Timmons J, Fitz-Coy S and Parveen S, 2019. Characterization of Clostridium perfringens recovered from broiler chicken affected by necrotic enteritis. Poultry Science 98: 128-135. https://doi.org/10.3382/ps/pey332

Nauerby B, Pedersen K and Madsen M, 2003. Analysis by pulsed-field gel electrophoresis of the genetic diversity among Clostridium perfringens isolates from chickens. Veterinary Microbiology 94: 257–266. https://doi.org/10.1016/s0378-1135(03)00118-4

Nowell N, Poppe C and Parreira VR, 2010. Clostridium perfringens in retail chicken. Anaerobes 16: 314-315. https://doi:10.1016/j.anaerobe.2009.11.004

Perelman B, Mints S, Zjut M, Kuttin E and Machny S, 1991. An unusual Clostridium colinum infection in broiler chicken. Avian Pathology 20: 475-480. https://doi.org/10.1080/03079459108418785

Prescott JF, Parreira VR, Mehdizadeh GI, Lepp D and Gong J, 2016. The pathogenesis of necrotic enteritis in chickens: what we know and what we need to know: A review. Avian Pathology 45: 288-294. https://doi:10.1080/03079457.2016.1139688

Randall CJ, Sasaki J, Goryo M and Okada K, 1991. A Colour Atlas of Diseases and Disorders of the Domestic Fowl and Turkey, 2nd Ed. Wolfe Publishing Ltd pp: 134-135.

Ronco T, Stegger M, Ng KL, Lilje B, Lyhs U, Andersen PS and Pedersen K, 2017. Genome analysis of Clostridium perfringens isolates from healthy and necrotic enteritis infected chickens and Turkeys. BMC Research Notes 10: 270. https://doi: 10.1186/s13104-017-2594-9

Sarah JM, Sarah M, Alexander M, Richard RE, Wesley F, Wade Abbott D and Douglas IG, 2020. Host responses to Clostridium perfringens challenge in a chicken model of chronic stress. Gut Pathogens 10: 12-24. https://doi.org/10.1186/s13099-020-00362-9

Sasaki J, Goryo M and Okada K, 2000. Cholangiohepatitis in chickens induced by bile duct ligations and inoculation of Clostridium perfringens. Avian Pathology 29: 405-410. https://doi.org/10.1080/030794500750047144

Savva CG, Fernandes da Costa, Bokori-Brown M, Naylor CE, Cole AR, Moss DS, Titball RWand Basak AK, 2013. Molecular architecture and functional analysis of NetB, a pore-forming toxin from Clostridium perfringens. Journal of Biological Chemistry 288: 35123522. https://doi:10.1074/jbc.M112.430223

Sergio P, Fernandes da Costa, Savva CG, Bokori-Brown M, Claire E, Naylor, Moss DS, Basak AK and Titball RW, 2014. Identification of a key residue for oligomerisation and pore-formation of Clostridium perfringens NetB. Toxins 6: 1049-1061. https://doi.org/10.3390/toxins6031049

Shojadoust B, Peygambari S and Nik PH, 2010. Isolation, identification, and antimicrobial susceptibility of *Clostridium perfringens* isolates from acute necrotic enteritis of broiler chickens. International Journal of Veterinary Science 4: 147-151. https://doi:10.22059/IJVM.2010.21344

Silva ROS, Ferreira Junior FC, Marques MVR, Oliveira Junior CA, Martins NRdS and Lobato FCF, 2014. Genotyping and antimicrobial susceptibility of *Clostridium perfringens* isolated from Tinamidae, Cracidae and Ramphastidae species in Brazil. Ciência Rural 44: 486-491. https://doi.org/10.1590/S0103-84782014000300016

Skinner JT, Bauer S, Young V, Pauling G and Wilson J, 2010. An economic analysis of the impact of subclinical (mild) necrotic enteritis in broiler chickens. Avian Diseases 54: 1237-1240. https://doi.org/10.1637/9399-052110-Reg.1

Stevens DL and Bryant AE, 2002. The role of clostridial toxins in the pathogenesis of gas gangrene. Clinical Infectious Diseases 35: S93-S100. https://doi.org/10.1086/341928

Tansuphasiri U, Matra W and Sangsuk L, 2005. Antimicrobial resistance among Clostridium perfringens isolated from various sources in Thailand. Southeast Asian Tropical Medicine and Public Health 36: 954-961

Timbermont L, Lanckriet A, Gholamiandehkordi AR, Pasmans F, Martel A and Haesebrouck F, 2009. Origin of Clostridium perfringens isolates determines the ability to induce necrotic enteritis in broilers. Comparative Immunology, Microbiology and Infectious Diseases 32: 503-512. https://doi.org/10.1016/j.cimid.2008.07.001

Titball RW, Naylor CE, Miller J, Moss DS and Basak AK, 2000. Opening of the active site of Clostridium perfringens alpha-toxin may be triggered by membrane binding. International Journal of Medical Microbiology 290: 357-361. https://doi.org/10.1016/s1438-4221(00)80040-5

Van Immerseel F, Rood JI, Moore RJ and Titball RW, 2009. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends in Microbiology 17: 32–36. https://doi.org/10.1016/j.tim.2008.09.005

Yegani M and Korver D, 2007. Application of egg yolk antibodies as replacement for antibiotics in poultry. World's Poultry Science Journal 66: 27-38. https://doi.org/10.1017/S0043933910000048

Yoo HS, Lee SU, Park KY and Park YH, 1997. Molecular typing and epidemiological survey of prevalence of Clostridium perfringens types by multiplex PCR. Journal of Clinical Microbiology 35: 228–232. https://doi.org/10.1128/jcm.35.1.228-232.1997

Zhang T, Zhang W, Ai D, 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. https://doi.org/10.1016/j.anaerobe.2018.08.007