Infection, Genetics and Evolution 28 (2014) 210–213

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Letter to the Editor

Genetic and antigenic typing of *Clostridium perfringens isolates from ostriches*

Dear Editor,

Clostridial enteritis is considered as one of the most economically important diseases in ostriches (*Struthio camelus*) and ratites of all ages (Verwoerd, 2000; Zakeri and Kashefi, 2012). The most frequently *Clostridium* bacteria caused enteritis in neonatal ostrich chicks are *Clostridium perfringens* (*C. perfringens*) and *Clostridium difficile* (Shivaprasad, 2003; Verwoerd, 2000). *C. perfringens* is ubiquitous in nature and can be found as a common inhabitant in the intestine of ostriches. This bacterium was classified into five toxinotypes (A, B, C, D and E) based on the presence of four major toxins (α, β, ε and ι) (Slavic et al., 2011).

C. perfringens type A and rarely type C can be associated with necrotic enteritis in poultry (Opengart, 2008). To the best of our knowledge, there isn't information about *C. perfringens* enteritis, types of bacteria and the antimicrobial resistance profiles of *C. perfringens* isolates from ostriches. Hence, it has been proposed that determination the antimicrobial susceptibility of *C. perfringens* isolates from ostriches is important. The objectives of this study were (1) to determine the prevalence of faecal shedding of *C. perfringens* by ostriches, and (2) to determine the toxin genotype and antigenic type and (3) to investigate the antimicrobial susceptibility of recovered isolates from feces of ostriches.

Samples were obtained from 118 ostriches from five farms (Table 1). The fecal samples were collected aseptically in sterile plastic bags and quickly transferred to the laboratory. Fecal contents were processed according to a routine protocol of the bacteriology laboratory from Razi Vaccine and Serum Research Institute (RVSRI). Briefly, 1 g of content was collected into a tube containing 9 ml of sterile phosphate-buffered saline (PBS, pH 7.4), and subsequently they were inoculated onto blood agar plates with 5% defibrinated sheep blood. Inoculated media were incubated overnight at 37 °C under anaerobic condition (10% CO_2, 80% N_2 and 10% H_2) using Anoxomat® (Mart Microbiology B.V Drachten, The Netherlands). Then, colonies were analysed according to the shape, color, type of hemolysis, gram staining smears and biochemical tests as described by MacFaddin (MacFaddin, 2000).

For gives a pure culture, the catalase negative colonies presenting *C. perfringens* characteristics were isolated and sub-cultured in blood agar and incubated at 37 °C for 18–24 h. These cultures were submitted to the following biochemical tests for species identification (MacFaddin, 2000): lecithinase, lipase, gelatinase, motility and skim milk coagulation (stormy reaction). Also for confirmation of *C. perfringens* isolates, all strains were incubated in selective tryptose-sulfite cycloserine (TSC) agar (Merck, 1.11972), as shown black colonies.

Positive and negative controls were used for *C. perfringens* confirmation and detection by multiplex PCR technique. *C. perfringens* type A (ATCC® 13124™) reference strain and *C. perfringens* type B strain CN228 from the bacterial isolates archive of the Razi institute of Iran were utilized as positive controls, as well as *Clostridium septicum* strain CN913 as negative control. Also, distilled H_2O was applied as a negative control to confirm the absence of contamination of material and facilities and removal of experimental errors and to prove the exclusion of non-target DNA.

Each of the isolated *C. perfringens* strains and the reference strains were cultured in tubes with 10 ml thioglycolate broth and were incubated anaerobically overnight at 37 °C. Cultures were centrifuged for 10 min at 7500*g* and collected 10–20 mg of bacterial culture in a 1.5 ml micro centrifuge tube. DNA was extracted using the protocol provided in the DNP™ kit (Cat No: DN8115C, Cinnagen, Iran). Genotyping of isolates were performed by PCR, as described previously (Yoo et al., 1997). Briefly, the PCR assay was performed using a thermal cycler (Bio-Rad, California, USA) with a total reaction volume of 50 μL with the following reagents: 5 μL of 10× PCR buffer (10 mM Tris–HCL, pH 9.0, 50 mM KCl), 2 μL of 50 mM $MgCl_2$, 250 μM of each deoxynucleotide triphosphate, 5U of recombinant Taq DNA polymerase (Cinnagen Corporation, product No: TA7506C), 0.25 μM of each of the primers, 5 μL of template DNA and distilled water. Specific primers (sinaclon Tehran, Iran) were applied for amplification of the genes. Amplicons were obtained with 35 cycles following an initial denaturating step at 95 °C for 3 min. Each cycle involved denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, synthesis at 72 °C for 90 s and the final extension step at 72 ° for 10 min.

Ten microliter of amplicons were evaluated for expected products by electrophoresis on 1.7% agarose gel in 1× TBE buffer [0.089 M Tris-base, 0.089 M boric acid and 0.002 M EDTA (pH 8.0)]. The VC 100 bp DNA ladder (Vivantis, product No: NL1402) and Orange Ruler 50 bp DNA ladder, RTU (Cinnagen, Cat. No: PR901633) were used as molecular markers to indicate the size of the amplicons. DNA safe stain (Cinnagen, product No: PR881603) was utilized for detecting nucleic acid in agarose gels. It is as sensitive as ethidium bromide and can be used exactly the same way in agarose gel electrophoresis. The amplified bands were visualized and photographed under UV illumination.

For antigenic typing, fecal samples were examined for *C. perfringens* alpha toxin by an indirect ELISA commercial kit (ACP12C23; Cypress Diagnostics; Langdorp, Belgium), according to the manufacture's recommendation. Finally, the plates were read at 450 nm by an ELISA reader (Anthos 2020, Wals, Austria).

Antibiotic resistance profiles of *C. perfringens* isolates against 12 selected antibacterial agents were determined by Kirby–Bauer disc diffusion method (Bauer et al., 1966). The isolated strains were cultured in Mueller Hinton agar (Oxoid, Code: CM0337) with the

http://dx.doi.org/10.1016/j.meegid.2014.09.034

Table 1
Multiplex PCR results in different ostrich farms.

Farms			Sample			95% CI
Farm code	Age range (year)	Weight average (kg)	No.	Positive	Percent	
F1	7–13	130	22	8	36.36	15.94–56.05
F2	4–8	105	28	7	25.00	8.96–41.03
F3	4–5	90	25	9	36.00	17.18–54.81
F4	8–9	120	20	11	55.00	33.19–76.80
F5	2–4	65	23	11	47.83	27.41–68.24
Summation			118	46	38.98	30.18–47.78

standard inoculum density and were incubated at 37 °C for 24 h. The following antimicrobial discs (Padtan-Teb, Tehran, Iran) were applied in disc diffusion assay: penicillin (P; 10 μg), oxacillin (OX; 1 μg), amoxicillin (AMX; 25 μg), tetracycline (TE; 30 μg), ciprofloxacin (CP; 5 μg), gentamicin (GM; 10 μg), chloramphenicol (C; 30 μg), erythromycin (E; 15 μg), clindamycin (CC; 2 μg), co-trimoxazole (SXT; 25 μg), cephalothin (CF; 30 μg) and bacitracin (PT; 0.04U). The standard interpretive criteria charts (Padtan-Teb, Tehran, Iran) were used to classify isolates as susceptible or resistant. For quality control, *Bacteroides fragilis* ATCC® 25285, *Staphylococcus aureus* ATCC® 25923 and *Escherichia coli* ATCC® 25922 were utilized as positive controls and uninoculated medium was used as negative control.

Multiplex PCR results showed that 46 isolates (38.98%) were positive from 118 fecal samples (Table 1). Analysis for genotyping showed that all isolates belonged to type A, harbouring the alpha toxin gene (*cpa* gene). No *C. perfringens* type B, type C, type D and type E were detected. In the current study, none of the isolates had enterotoxin gene (*cpe* gene). Alpha toxin was confirmed only in 10 (8.47%) fecal samples by ELISA. Also, antibiogram results were shown relative susceptibility to the most antibiotics among *C. perfringens* isolates. The most prevalent resistances were recorded against to bacitracin and penicillin (89.1% and 63.1%, respectively). While, the most frequent antimicrobial susceptibility of *C. perfringens* isolates were observed to chloramphenicol and cephalothin (100% and 89.1%, respectively) (Table 2). There were different and diverse resistant patterns among the *C. perfringens* isolates.

In the present study, genotyping of *C. perfringens* isolates by multiplex PCR, revealed that all isolates were only positive for alpha toxin gene, which means that all isolates are type A. This is the most frequently types of bacteria that isolated from the environment and digestive tract of animals and man (Petit et al., 1999). This bacterium is considered as one of the most important pathogens causing necrotic enteritis in ostriches (Zakeri and Kashefi, 2012).

In the current study none of isolates have *cpe* gene. This gene codes the enterotoxin. Enterotoxin, a not secreted toxin, is the major virulence determinant in food poisoning *Clostridia* that only produced during sporulation of *C. perfringens* type A (Briggs et al., 2011). The current results were shown the isolated *C. perfringens* are not important for food poisoning.

One of the available tools for necrotic enteritis antigenic monitoring is ELISA. Here, the alpha toxin of *C. perfringens* type A was confirmed only in 10 (8.47%) fecal samples from 118 fecal samples. Use of this technique for antigenic typing of isolated *C. perfringens* strains from different animal sources had been reported, previously (El Idrissi and Ward, 1992; HADİMLİ et al., 2012; Hale and Stiles, 1999; Lovland et al., 2003; Nagahama et al., 1991; Naylor et al., 1997). Previously, antigenic typing of the toxigenic *C. perfringens* isolates from Iranian ostriches by ELISA were performed (Babe et al., 2012) and they showed that the type A was the most prevalent antigenic type in the intestinal flora of ostriches. This is consistent with our results. As you can see, the ELISA test has confirmed low percent (8.47%) of *C. perfringens*, whereas genotyping has approved a higher level (38.98%) of *C. perfringens* in fecal samples. One of causes of this forecast is the susceptibility of bacterium toxins. Also, other material in feces are affected the sensitivity of ELISA test. These results were shown that the genotyping is better than antigenic typing to determine the prevalence of *C. perfringens* in ostrich fecal samples.

To our knowledge, no studies regarding the antimicrobial susceptibility of *C. perfringens* isolates from ostriches have been published. Investigation the antimicrobial susceptibility of isolates was one of the purposes in the current study, that doing for the first time. Multiple resistance patterns to antimicrobial drugs have been found previously in *C. perfringens* strains isolated from different sources (Catalán et al., 2010; Dutta and Devriese, 1981; Tansuphasiri et al., 2005). In this work, there were different and diverse resistant patterns among the isolates.

The most prevalent resistances were recorded against to bacitracin and penicillin, respectively (Table 2). Bacitracin used as a feed additive in poultry industry (Singer and Hofacre, 2006). High resistance values in *C. perfringens* isolated from poultry have been reported (Chalmers et al., 2008; Silva et al., 2009), but there isn't any published data about resistance of *C. perfringens* to this antibiotic in ostriches. In this study, 89.1% of strains were resistant to bacitracin. The mechanisms of this resistance are not yet clear. Probably, wide use of bacitracin in Iranian poultry industry is associated with this degree of resistancy. Penicillins were shown to be very effective against *C. perfringens* isolates (Johansson et al., 2004). This drug is the choice treatment for *C. perfringens* infections and showed the best activity against isolated *C. perfringens* from broiler chickens (Gharaibeh et al., 2010). In current study, resistancy of isolates to penicillin (63.1%) was shown, too. Resistancy to penicillin is noticeable.

Tetracycline-resistance is the most common antibiotic resistance trait found in *C. perfringens* strains (Park et al., 2010) and it is high 10–76% (Hecht, 2006) and it was described in poultry (Gholamiandehkordi et al., 2009; Johansson et al., 2004). In this study, isolates were shown 80.4% susceptibility to tetracycline. Current results are almost same as the previous results in broiler chickens (LLANCO et al., 2012). In present study, also a high degree of susceptibility in *C. perfringens* isolates from ostriches to chloramphenicol, cephalothin, and gentamicin (Table 2) had been shown for the first time that those are noticeable.

In conclusion, use of the genetic and antigenic typing techniques for detection and typing of *C. perfringens* isolates were shown that the multiplex PCR presented here can be utilized as a useful, reliable and accurate tool, with high specificity and sensitivity. This is the first study that demonstrates the antimicrobial resistances of *C. perfringens* isolates in ostriches. Further molecular studies about other toxins of *C. perfringens* ostrich isolates (such as beta2, *NetB*, *TpeL* and perfringolysin O) and molecular factors related to the antimicrobial resistance of these isolates are recommended.