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Short communication

PREVALENCE OF *CLOSTRIDIUM PERFRINGENS* TYPE A ISOLATES IN DIFFERENT TISSUES OF BROILER CHICKENS

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Summary

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Clostridium perfringens (C. perfringens) is an important pathogen in both human and veterinary medicine. Necrotic enteritis is the most severe clinical bacterial enteric disease of poultry induced by *C. perfringens.* Study was done on 100 broiler chickens (400 different tissues of chickens) in Southwest Iran. *C. perfringens* was isolated from different tissues of chickens (meats, liver, gizzard and intestine) using bacterial culture methods. DNA extraction from grown colonies was carried out by boiling method and finally, PCR assay was used for definitive diagnosis of type A *C. perfringens.* The results of present study showed that from 400 different tissues of chickens 169 (42.25%) samples were positive for *C. perfringens* infection that is a comparatively high prevalence of *C. perfringens.* The highest rate of *C. perfringens* infection in tissues was in intestine (55%) and meat (42%). The high prevalence of type A *C. perfringens* in different tissues observed in the present study is very disturbing, as it can cause irreparable damage to the poultry industry and human health.

Key words: broiler chickens, chicken tissue, type A *Clostridium perfringens*

Clostridium perfringens (C. perfringens) is a Gram-positive, spore-forming, rod shaped anaerobic non-motile pathogen that is frequently found in the intestinal tract of humans and animals (Obrien & Melville, 2000). It is commonly present in the environment (in soil, on the skin and sewage) (Shimizu *et al.,* 2002) and is found in undercooked or improperly sterilised canned foods (germination of endospores) and in water (surface water). The natural contamination source are human and animal faeces transmitted into water and food products. Owing to its ability to produce spores under adverse environmental conditions, it is one of the

most widespread potential bacterial pathogens in nature as well as in the gastrointestinal tract of most animal species (Mokhtari-Farsani & Doosti, 2015).

C. perfringens has been identified as an anaerobe responsible for a wide range of diseases in human and animals (Yoo *et al.,* 1997). This bacterium was first implicated as a cause of antibiotic-associated diarrhoea (AAD) in 1984 (Asha & Wilcox, 2002). *C. perfringens* is commonly involved in diseases in most domestic animals and some wildlife, including horses, poultry, birds, rabbits, sheep, goats, cattle, mink, ostriches, dogs and cats (Effat *et al.,* 2007). The overgrowth of *C. perfringens* causes necrotic enteritis (NE) disease in the small intestine of broiler chickens throughout the world. The disease usually occurs in broiler chickens 2 to 6 weeks after hatching (Drew *et al.,* 2004). The virulence of this bacterium largely results of its ability to produce at least 15 different *C. perfringens* toxins (Jabbari *et al.,* 2012). *C. perfringens* strains are classified into five groups (types A, B, C, D and E) on the basis of their production of four major toxins known as alpha (CPA), beta (CPB), epsilon (ETX), and iota (ITX) toxins (Shimizu *et al.,* 2002).

C. perfringens type A food poisoning is one of the most common food borne diseases in the United States. The factor responsible has been shown by the characteristic disease symptoms (diarrhoea and abdominal cramps) of *C. perfringens* food poisoning (Wnek *et al.,* 1985). This type of *C. perfringens* was reported as the cause of necrotic enteritis in poultry especially in broiler chickens (Lindstrom *et al.,* 2011; Rahimi *et al.,* 2011). *C. perfringens* types B and C disease begins in the host intestine (Uzal *et al.,* 2010). Type B isolates cause an often fatal haemorrhagic dysentery in sheep, and possibly in other species, while type C isolates cause enteritis necroticans (also called pigbel) in humans and necrotic enteritis and/or enterotoxaemia in almost all livestock species. Both types B and C animal disease are often accompanied by sudden death or acute neurological signs (Uzal *et al.,* 2010). Clinical signs and histopathologic findings in type C infections are very similar in most livestock animal species. The course of disease can be peracute, acute, or chronic, with signs of the acute and peracute condition including intense abdominal pain, depression, and bloody diarrhoea (Songer, 1998). Type B and D strains are causative agents of fatal enterotoxaemia in domestic animals and occasionally humans (Jabbari *et al.,* 2011).

C. perfringens is usually considered to be an exclusively extracellular pathogen that secretes powerful cytotoxins that lyse cells and break down connective tissue (Obrien & Melville, 2000). After the entry of vegetative cells or spores into the body, the organisms grow rapidly in the host tissue, and produce various toxins and enzymes that cause massive destruction of the host tissues (Rood, 1998).

Due to their short reproductive cycle and their worldwide popularity as a food, poultry represent the most highly selected livestock. Selection of broiler chickens (chickens grown for their meat) has been primarily directed at economic traits which have reduced costs of production (Knowles *et al.,* 2008). Throughout the world the majority of broilers are reared using very similar, modern, intensive systems of production where birds are confined for their lifetime within high density housing (Knowles *et al.,* 2008). The *C. perfringens* type A infections in poultry may present as acute clinical disease or subclinical disease. This organism is asso-

ciated with necrotic enteritis, gangrenous dermatitis, clostridial dermatitis, and gizzard erosions in poultry (Rahimi *et al.,* 2011). The acute form of the disease leads to increased mortality in the broiler flocks (Immerseel *et al.,* 2004).

Since the understanding of the pathogenicity and physiology of *C. perfringens* is still poor compared with other pathogenic bacteria and by reason of the importance of this bacterium in the poultry industry and human nutrition, the purpose of present research was to study the frequency of *C. perfringens* type A infection by culturing methods and PCR in different tissues of broiler chickens in Chaharmahal Va Bakhtiari province (southwestern Iran).

In the present study, 100 broiler chickens suspicious to be infected with *C. perfringens* (with signs of enteritis) were obtained from ten locations between May to August 2014 from Chaharmahal Va Bakhtiari province located in southwest of Iran and were tested for detection of *cpA* gene of *C. perfringens*. Their body weight ranged from 150 to 500 g and their age – between 2 to 6 weeks. Initially cooked meat broth medium (Merckoplate, Germany) was prepared in sterile tubes by mixture of 500 mg of cooked meat with 10 mL distilled water and was heated at 80°C for 5 min in bain marie. Five g of individual chicken tissues samples (100 from meat, 100 from liver, 100 from gizzard and 100 from intestine) were mixed with 5 mL of PBS (phosphate buffer) in a sterile mortar separately. The above suspension was added to the cooked meat broth medium and heated at 65 °C for 10 min in order to select bacterial spores. Then, tubes were incubated for 72 h at 37 °C in an anaerobic CO_2 incubator (Finetech, Germany). In the next step each sample were cultured by streak plate method using a sterile loop on already prepared blood agar (Merckoplate, Germany) plates supplemented with 5% sheep blood and was incubated for 18 h at 37 °C under anaerobic conditions. The circular, disordered, flat and bright colonies were indicative for the presence of *Clostridium* (Doosti & Mokhtari-Farsani, 2014).

DNA extraction was performed by boiling method. A few colonies were scratched of the blood agar plates, dissolved in 200 µL distilled water in test tubes, and were incubated in boiling water bath for 10–15 min. The last step was a 12,000 rpm centrifugation for 5 min, and DNA samples were kept at –20 °C until used. PCR method was used for definitive diagnosis of *C. perfringens*. Detection of *C. perfringens* was performed by amplification with the following primers: C.P-F: 5'-GTTGATAGCGCAGGACATGTT AAG-3' and C.P-R: 5'-CATGTAGTC ATCTGTTCCAGC ATC-3' [Gene Bank: KF914160.1]. The PCR reactions were performed in a total volume of 25 µL in 0.2 mL tubes containing 60 ng of DNA sample, 1 µM of each primers, 5 µL of 10× PCR buffer AMS, 200 µM dNTPs, 2 mM $MgCl_2$ and 1 unit of Taq DNA polymerase (CinnaGen Co, Iran). The PCR reaction mixtures were placed in an Eppendorf PCR thermal cycler (Eppendorf, Hamburg, Germany). Amplification was obtained with 32 cycles following an initial denaturation step at 95 °C for 5 min. Each cycle comprised denaturation at 94 °C for 40 s, annealing at 58 °C for 40 s, and extension at 72 °C for 60 s. The final extension step was carried out at 72 °C for 5 min.

The PCR-amplified products (*cpA* gene, 602 bp) were analysed by electrophoresis in a 1% agarose-ethidium bromide gel. Aliquots of 10 µL of PCR products were applied to the gel. Constant

voltage of 90 V for 20 min was used for products separation. The PCR products were identified by 100 bp DNA size marker (Fermentas, Germany). After electrophoresis, the gel was examined under ultraviolet illumination and photo was obtained using a UVI doc gel documentation systems (UK).

All data for determination of frequency of *C. perfringens* infection in different tissues of broiler chickens were analysed by the chi-square test using the SPSS 17 (SPSS Inc. Chicago, IL, USA) software. P values <0.05 were considered significant.

From 400 samples, 223 samples (55.75%) were positive for *Clostridium* by bacterial culture method. After culture of *C. perfringens* and DNA extraction, the quality of the extracted DNA was examined through 1% agarose gel and was confirmed. All isolates were examined for the presence of the *cpA* gene by PCR. Agarose gel electrophoresis of positive samples revealed a 602 bp fragment (Fig. 1). The results showed that from 100 broiler chickens 55 were infected with type A *C. perfringens*. Overall, 169 of the 400 (42.25%) tissues were positive for *C. perfringens* by PCR assay. The rate of *C. perfringens* infection in different tissues of chickens was 42/100 for meat, 55/100 for intestine, 34/100 for gizzard and 38/100 for liver.

Although *C. perfringens* is a member of normal gut flora, it is also regarded as one of the most important causes of intestinal disease in farm animals and wild

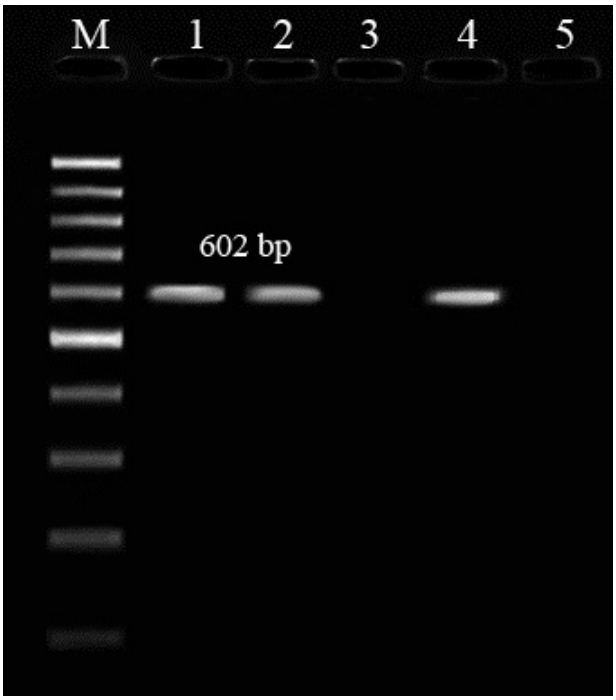

Fig. 1. Agarose gel electrophoresis of PCR amplification products for detection of *C. perfringens* in broiler chickens samples. Lane M: Fermentas 100-bp DNA molecular marker, lanes 1, 2 and 4 are positive samples, lane 3: negative sample, lane 5: negative control.

animals and, to a lesser extent, in humans (Allaart *et al.,* 2011). *C. perfringens* plays a significant role in food-borne human disease and is among the most common food-borne illnesses in industrialised countries (Brynestad & Granum, 2002; Lindstrom *et al.,* 2011). Since *C. perfringens* food poisoning is not a reportable disease, its frequency probably is seriously underestimated. Nonetheless, in recent years a high incidence of *C. perfringens* food poisoning in different country like Japan, Norway, the UK and the USA was reported (Immerseel *et al.,* 2004). If a sufficient number of *C. perfringens* cells are consumed with contaminated food, these cells are capable to pass from the stomach to the intestinal tract where spores are produced, released and cause clinical *C. perfringens* food poisoning (Smedley *et al.,* 2004; Sawires & Songer, 2006).

The broiler chicken industry is a main source of animal protein and fat (Mersmann, 2002), and *C. perfringens* is considered as a serious threat to this industry. In the present study a relatively high prevalence of *C. perfringens* infections (42.25%) in different tissues of broiler chickens was observed. Some studies were performed about *C. perfringens* infection in broiler chickens. In study of Miwa *et al.* (1997) the reported prevalence of *C. perfringens* in chicken intestinal contents was 40% that is somewhat similar to the present study results. Studies conducted on incidence of *C. perfringens* in meat and the intestinal tract of poultry between 1996 to 2001 established that approximately 75% to 95% of the animals were positive, with a remarkable high percentages of positive meat samples (up to 84%) (Immerseel *et al.,* 2004). Perhaps the reason for very high incidence in this study and for the difference with the results of our study is the general detection of all C. *perfringens* types, as we used specific primers only for diagnosis of type A *C. perfringens*. In the investigation of Craven *et al.* (2001) on environmental samples collected from poultry farms, the prevalence of *C. perfringens* was between 43% to 53% indicating that *C. perfringens* is a common intestinal inhabitant. Obviously, in our study and other studies throughout the world the incidence of *C. perfringens* in poultry industry was high. Also, the present results and those from other studies showed that *C. perfringens* can easily spread from the intestine to other tissues of broiler chickens, and that the global prevalence of *C. perfringens* in chicken meat of 40% to 84% is a threat for human health and the poultry industry.

Despite of the known protective effects in other animal species, vaccination of poultry against *C. perfringens* is a seriously under-researched matter. It is documented in numerous studies that coccidiostatic drugs and coccidial vaccines are able to prevent *C. perfringens*-associated necrotic enteritis (Immerseel *et al*., 2004). As to the results of studies indicating successful use of vaccines in other farm animals, vaccination could be a helpful tool in preventing *C. perfringens* infection and necrotic enteritis in poultry. In addition to vaccination, the use of probiotics has been found effective against various pathogens especially *C. perfringens* in poultry (Asadi-Farsani *et al*., 2015).

Our study and other studies shows a high prevalence of *C. perfringens* in broiler chickens. The present study established a relatively high prevalence of this bacterium in meat (42%) (which is related to human nutrition) compared to other tissues according to their importance in human nutrition. In conclusion, to prevent

the increasing growth of *C. perfringens* and further damage to the poultry industry and most importantly transmission of this pathogen from poultry to humans, we suggest regular vaccination, use of probiotics in poultry diets, use of appropriate antibiotics and reduction of imprudent use of antibiotics in order to avoid microbial resistance, and further studies for recognition of the epidemiology of this bacterium.

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