Genotyping of Clostridium perfringens Isolates from Chicken by Multiplex PCR

R.V. Singh*, B.Bist1, K. Nagrajan2, S.Das3, R.S. Tayde4 and A.Verma5

Department of Veterinary Public Health, College of Veterinary Science and Animal Husbandry, DUVASU, Mathura (UP)

(Received 10.05.2013; accepted 29.09.2014)

ABSTRACT

The aim of the study was to genotype the isolates of Cl. perfringens from chicken meat with multiplex PCR. The study was targeted for 6 genes (cp, cpa, cpb, iA, etx and cpe). Genotyping of 61 isolates of Cl. perfringens (raw meat-60 and fried meat-1) revealed that 55 (90.16%) strains had cpa genes while presence of only one isolate (1.6%) was positive for enterotoxin gene (cpe). None of the isolates showed the cpb, iA and etx genes. All the isolates were positive for cp (Cl. perfringens) gene.

Keywords: Chicken, clostridium perfringens, genotyping, PCR.

Introduction

Clostridium perfringens is a Gram positive, sporogenic, encapsulated anaerobic and ubiquitous bacteria. The organism is regarded as one of the most widespread and pathogenic bacteria in clostridial family (Songer, 1996). It is responsible for causing different diseases like myonecrosis (gas gangrene), diarrhoea and food poisoning in humans. It is also associated with enterotoxaemia and hemorrhagic gastroenteritis in many domestic and wild animals. Cl. perfringens produces 17 extracellular toxins but on the basis of four major lethal toxins (alpha, beta, epsilon and iota), the bacterium is divided into five types A, B, C, D and E (Topley and Wilson, 1998). The major toxins like alpha, beta, epsilon, and iota are responsible for its virulence. Few strains of Cl. perfringens also produce enterotoxin, which is responsible for food poisoning outbreaks in humans and is one of the most common pathogen incriminated in food poisoning. Such foodborne illness mainly occurs by the consumption of meat particularly beef, poultry, etc. (McClane 2001; Erol et al., 2008). Its widespread presence in the environment viz., dust, sewer, soil, water and intestine of domestic, wild animals and humans, etc. make its presence in different foods inevitable (Jay, 2005).

In poultry, it leads to necrotic enteritis cases which create havoc worldwide in poultry industry. The disease is mainly caused by type A and C but sometimes such cases have also been associated with type D strains of Cl. perfringens (Heikinheimo and Korkeala, 2005). The whole poultry industry suffers a setback of about $2 million annually due to necrotic enteritis caused by Cl. perfringens (Thanissery et al., 2010).

The purpose of the present study was to characterize the different strains of Cl. perfringens isolated from chicken meat with multiplex PCR.

Materials and Methods

A total of 61 isolates (raw meat-60 and fried meat-1) of Clostridium perfringens were screened for the presence of various genes namely, cp, cpa (alpha toxin), cpb (beta toxin), etx (epsilon toxin), iA (iota toxin) and cpe (enterotoxin). The primers were procured from Bangalore Genie and details are in Table 1.

The template was prepared by boiling and chilling process. In this process 0.1 ml of inoculum from stock culture was taken in 10 ml alternative thioglycollate broth, followed by heat treatment at 75°C for 20 min and then overnight incubation at 37°C. From this, 1.5 ml aliquot was centrifuged at 8000-10000 rpm for 5 min and supernatant was discarded. The pellet was washed twice with sterile phosphate buffer saline (PBS). The pellet of bacterial cells was re-suspended in 100 µl PBS and was kept in boiling water for 15-20 min, followed by chilling into ice for few sec. The bacterial lysates was centrifuged
Table 1.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5'-3')</th>
<th>Product size(bp) and Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>cp-fcp-r 2006</td>
<td>AACTGGAGGAAGGTGGGGAT AAGAGGTGATCCAACCGCA</td>
<td>370 (Czanderlova et al. 2006)</td>
</tr>
<tr>
<td>cpa-f cpa-r</td>
<td>GCTAATGTTACTGCGCTTTGACCTCTGATACATCGTGTAAG</td>
<td>324 (Meer and Songer 1997)</td>
</tr>
<tr>
<td>cpb-f cpb-r 1997</td>
<td>GCGAATATGCTGAATCATCTAGCAGGAACATTAGTATATCTTC</td>
<td>196 (Meer and Songer 1997)</td>
</tr>
<tr>
<td>etx-f etx-r 1997</td>
<td>GCGGTGATATCCATCTATTCCACTTACTTGTCTACTAACC</td>
<td>655 (Meer and Songer 1997)</td>
</tr>
<tr>
<td>iA-fiA-r</td>
<td>ACTACTCTGAGACAAGACAG CTTTCCTTCTATTACTATACG</td>
<td>446 (Meer and Songer 1997)</td>
</tr>
<tr>
<td>cpe-fcpe-r</td>
<td>GGAGATGGTGAGTAGGAGGACCAGCAGTGTAGATA</td>
<td>233 (Meer and Songer 1997)</td>
</tr>
</tbody>
</table>

Fig. 1. PCR of poultry samples. M: Markers; Lane 3-7: Positive for cp (370 bp); Lane 4: Positive for cpe (233 bp); Lane 7: Positive for cpa (324 bp)

Fig. 2. PCR of poultry samples. M: Markers; Lane 1-7: Positive for cp; Lane 2 and 3: Negative for cpa

at 10000 rpm for 2-3 min and 5 µl supernatant was used as DNA template for PCR reaction mixture. The PCR was standardized for different toxic genes of Cl. perfringens as per the method described by Erol et al. (2008) with certain modifications. PCR mixture consisted of 50 µl reaction volume: 5 µl of crude bacterial lysate, 5 µl of 10x Taq DNA polymerase buffer (10 mM tris HCl, pH 8.8, 50 mM KCl, 2 mM MgCl₂, 0.08% nonidet P40), 0.20 mM dNTP, 20 pM of each primer, 3U of Taq DNA polymerase and rest of autoclaved milli Q water. The standardized amplification reaction started with initial denaturation at 94°C for 5 min, followed by 30 cycles of each denaturation (94°C for 1 min), annealing (50°C for 1 min) and extension (72°C for 1 min), with final extension for 5 min at 72°C. The amplification cycles were carried out in gradient thermocycler (Eppendorf, Germany). The amplified PCR product was run along with marker (100 bp ladder) on 1.5% agarose gel containing ethidium bromide and was observed in UV transilluminator.

Results

Out of 60 raw meat isolates, 54 (90%) had alpha gene and only one sample (1.6%) was positive for enterotoxin gene. None of the isolates had shown cpb, iA and etx genes. The lone isolate from fried chicken also harboured alpha gene. All the isolates were positive for cp (Cl. perfringens) gene with PCR. Previous workers have observed that Cl. perfringens type A is a natural inhabitant of gut of chicken and the most cases of necrotic enteritis are associated with type A strain of Cl. perfringens (Chalmers et al., 2008; Gomes et al., 2008; Thanissery et al., 2010).

Few strains of Cl. perfringens didn’t show presence of cpa gene in this study with PCR. Although no such previous studies employing PCR have been reported but few phenotypic studies have revealed similar findings, wherein few strains of Cl.
Clostridium perfringens didn’t show lecithinase activity (Nakamura et al., 1976; Sjelkvale et al., 1979). Brett (1994) has also found that few food-poisoning strains of Cl. perfringens were lecithinase-negative. Likewise, Singh et al. (2005) examined that 3 (10%) chicken meat isolates among 30 were lecithinase negative on egg yolk agar. The lower prevalence of enterotoxigenic Cl. perfringens (1.6%) in the present study may be attributed to the existence of low number of enterotoxigenic strains among Cl. perfringens populations (Miwa et al., 1997; Heikinheimo et al., 2004). Miwa et al. (1998) reported presence of 12% enterotoxigenic strain of C. perfringens in chicken meat samples. Singh et al. (2005) found 7 (9.85%) enterotoxigenic strains among 71 samples of chicken screened. Gurmu et al. (2013) reported that among 50 samples of chicken only one sample (2%) was found to be positive for enterotoxin gene. Previous workers have reported the overall prevalence of 5-6% enterotoxigenic strains among the total population of Cl. perfringens (Engstrom et al., 2003; Jihong et al., 2007) but variation in the occurrence of enterotoxigenic Cl. perfringens have been observed by various workers in different foods, ranging from 1 to 75% (Genigeorgis et al., 1973; Jihong et al., 2007; Lahti et al., 2008; Yang et al., 2010).

References


