ISOLATION OF CLOSTRIDIUM PERFRINGENS FROM SWINE CARCASSES AND FECES

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Abstract Bacterial hazards are the major concern in the production of food of animal origin. Clostridium perfringens is a common cause of food-borne illness. The illness is characterized by profuse diarrhea and acute abdominal pain. At this study 30 carcass swabs from dorsal area and 30 fecal samples from a swine abattoir were analyzed over C. perfringens presence. The frequency of agent in carcasses was 40% and in fecal samples 73.2%. The forty-seven isolated strains were characterized by PCR for the presence of genes codifying the enterotoxin, alpha, beta, epsilon, iota and beta-2 toxins. All strains were positive for presence of alpha and beta 2 toxin gene, and were classified as type A. None were positive to enterotoxin gene through PCR. Single enzyme amplified fragment length polymorphism (SE-AFLP) analyses were used to characterize 47 C. perfringens strains and revealed two common profiles among fecal and carcass strains.

Introduction Clostridium perfringens is the etiologic agent of multiple syndromes in domestic animals, some of the most important conditions that producers and veterinary practitioners have to face (Songer, 1998; Klaasen, 1999). The various toxins produced by the bacteria play key roles in the pathogenesis of the disease and are divided into five biotypes, designated A through E, based on the production of alpha- (α-), beta- (β-), epsilon- (ε-), and iota- (ι-) toxins. The α-toxin is produced by all types, β-toxin is produced by type B and C strains, ε-toxin is produced by type B and D strains, and ι-toxin is produced by type E strains (Garmory et al., 2000). Different biotypes of C. perfringens are associated with different diseases. Type A enterotoxigenic strains are a common cause of food poisoning outbreaks worldwide. Type C, is generally considered to be the primary cause of necrotic enteritis in piglets. In addition to the major toxins, other toxins may play a role in disease (Garmory et al., 2000). A novel toxin produced by C. perfringens, named beta 2-(β2) toxin, has recently been identified and its encoding gene characterized.

The occurrence of C. perfringens in swine carcass at slaughter is not reported in Brazil. The genotyping of these isolates may lead to a better understanding of the potential of this agent to contaminate pork meat and cause outbreaks in humans.

Single enzyme AFLP (SE-AFLP) has previously been used for the differentiation of Clostridium perfringens and others agents as Pasteurella multocida to strain level (McLauchlin et al, 2000; Moreno et al., 2003). The objectives of this trial are to isolate C. perfringens from carcass swabs and swine feces at abattoir, characterize the strains in relation to the presence of enterotoxin, alpha, beta, epsilon, iota and beta-2 toxins genes using polymerase chain reaction (PCR) and analyze the strains through the SE-AFLP technique.

Materials and Methods A total of 30 carcass swabs from dorsal area and 30 fecal samples from one swine abattoir were analyzed. A sample of 5 g of feces and the carcass swabs were submitted to enrichment in 25 ml of liquid thioglycolate medium and cultured overnight under anaerobic condition. After that, the culture was streaked in TSC agar under anaerobic condition. The suspect colonies were identified by the use of Gram stains, lecithinase and lipase reaction in egg-yolk agar and presence of storm clout in Litmus milk.

The colonies of C. perfringens were cultured in 10 ml of thioglycolate broth for 24 hr at 37°C. The DNA extraction was conducted with 200 µl of bacterial culture treated with Lysozyme (140µl of 100mg/ml) and Proteinase K (40µl of 20 mg/ml) for 1 hour at 37°C. The bacterial lysates were submitted to DNA purification with the guanidium thiocyanate method described by Pitcher et al. (1989).

PCR assays were performed using specific primers the toxin genes (alpha, beta, beta2, epsilon, iota and enterotoxin) as described elsewhere (Moreno, 2003). Reference strains C. perfringens type A (ATCC 3624), type C (ATCC 3628), type B (ATCC 3626) and type D (ATCC 3629) were kindly supplied by Instituto Biológico de São Paulo, and were used as positive controls.

To SE-AFLP technique an aliquot containing 10µg of DNA was digested overnight (16 h) at
37°C with 24 U of HindIII (Life Technologies) in the buffer provided with the enzyme and ultra pure water in a final volume of 20 µl. A 5 µl aliquot containing the digested DNA was used in a ligation reaction containing 0.2 µg of each adapter oligonucleotide (ADH1- 5’ ACGGTATGCGACAG 3’ and ADH2- 3’ GAGTGCCATACGCTGTCTCGA 5’), 1U of T4 DNA ligase, ligase buffer, and water, in a final volume of 20 µl incubated at room temperature for 3 h. Ligated DNA was heated to 80°C for 10 min, diluted 1/5 in sterile distilled water, and 5 µl were used for each PCR reaction.

PCR reactions were performed in 50 µl final volumes and contained: 5 µl of ligated DNA, 2.5 mM MgCl₂, 300 ng of primer HIG- 5’GGTATGCGACAGAGCTTG 3’ and 1.25 U of Taq DNA polymerase in 1 X PCR buffer. The mixture was subjected to an initial denaturing step of 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2.5 min.

The amplified products were analyzed on a 2.0% agarose gel, stained with ethidium bromide (0.5 mg/ml), observed by UV transilumination, and registered by an image capturing system. A 100 bp DNA ladder was included twice on each electrophoresis gel. Banding patterns were assessed visually, considering only strong and moderately stained fragments.

**Results** The bacteriological examination of 30 carcasses swabs revealed 12 positive to *C. perfringens* isolation (40%) and twenty-two fecal samples showed positive results (73.2%).

From positive carcasses swabs and fecal samples were selected 47 strains of *C. perfringens* to toxin characterization. All strains were positive for presence of alpha and beta 2 toxin gene, and were classified as type A. None were positive to enterotoxin gene through PCR.

Using SE-AFLP, each culture generated one to five DNA fragments ranging between approximately 400 -1750 bp. Among the 47 cultures tested, 18 SE-AFLP profiles were observed, which varied by one or more bands from all other patterns (Figure 1 and 2). Two identical profiles were present in samples from carcass and fecal strains. Fecal strains presented a high genetic heterogeneity when compared with carcass strains.

**Discussion** Swine slaughter is an open process with many opportunities for the contamination of the pork carcass with potentially pathogenic bacteria; however it does not contain any point were the hazards are completely eliminated. The major contamination points during swine slaughter are pig-related, such as fecal and pharyngeal, and environmental (Borch 1996).

The high occurrence of *C. perfringens* on feces (73.2%) are expected, since the agent is a component of intestinal micro biota, but the frequency of this agent in carcass was higher than expected in this first trial (40%). In Japan, a study with 50 pork meat samples showed that 12% were positive to *C. perfringens*, but none of isolates were positive to enterotoxin presence (Miwa, 1998). At present study enterotoxin positive isolates of *C. perfringens* were not found too.

The AFLP technique showed results comparable with all other molecular typing methods described to analyze *C. perfringens* strains, and appears to have several advantages in terms of being easy to perform, rapid, discriminatory, cheap and highly reproducible. The strains observed in carcass swabs presented less genetic heterogeneity than that isolated from feces. The presence *C. perfringens* with identical profiles in carcass and feces confirm the potential of fecal contamination of carcass during slaughter process.

The absence of enterotoxin positive strains among the isolates studied suggest a low risk of human infections from carcass contamination, but a constant monitoring of this agent must be done, considering the high percentage of it detection at the samples analyzed.

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**References**

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**Figure 1 - Electrophoresis in agarose gel. SE-AFLP profiles of *Clostridium perfringens* produced by primer HIG.**

Lanes 1 and 24, 100bp molecular weight standard. The remaining lanes show AFLP profiles of 20 *C. perfringens* strains isolated from swine carcass swabs.