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# USE OF SINGLE-ENZYME AMPLIFIED FRAGMENT LENGTH POLYMORPHISM FOR TYPING *CLOSTRIDIUM PERFRINGENS* ISOLATED FROM DIARRHEIC PIGLETS

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## ABSTRACT

*Clostridium perfringens* is an important pathogen in human and veterinary medicine. In swine, the agent is responsible for necrotic enteritis and enterotoxemia characterized by diarrhea, weight loss, delayed development and, in some cases, death. In the present study amplified fragment length polymorphism analyses (AFLP) was used to characterize 54 *C. perfringens* strains isolated from swine presenting diarrhea. Analysis of the results showed 29 distinct profiles with discriminatory index equal to 0.97. Partial correlation between the origin of the isolates and groups was drawn, and correlation was possible in only 18.5% of the samples. Characterization of the strains in biotypes (A, B, C, D and E), production of beta-2 toxin and enterotoxin were performed by means of the polymerase chain reaction (PCR). Biotypes A, C and D were observed among the strains analyzed. All samples were positive for presence of the gene encoding beta-2 toxin and negative for the gene encoding enterotoxin. AFLP have shown to be a simple, fast, low cost method with high discriminative power and good reproducibility, presenting a great potential in epidemiological studies involving *C. perfringens* strains of animal origin.

**Key words:** AFLP, diarrhea, piglets, *Clostridium perfringens*

## INTRODUCTION

*Clostridium perfringens* is an important cause of enteric disease both in humans and domestic animals (14). The bacteria produce several toxins that play key roles in the pathogenesis of disease. Toxins are classified into five biotypes, designated A – E, based on the differential production of alpha (α), beta (β), epsilon (ε), and iota (I) toxins. *C. perfringens* type C is generally considered the primary cause of necrotic enteritis in piglets from 0 to 2 weeks of age, while type A has been linked to enteric disease in suckling and feeding pigs with mild necrotic enterocolitis (7). A novel toxin produced by *C. perfringens*, named beta 2- (β2-) toxin, has recently been identified and its encoding gene has been characterized. This toxin is cytotoxic for intestinal cells and lethal for mice (2). Preliminary studies suggested that β2-toxin-producing strains are associated with necrotic enteritis in piglets and enterocolitis and typhlocolitis in horses (2,4).

Both phenotypic and genotypic methods have been investigated in the epidemiological typing of *C. perfringens* from human sources, including the use of bacteriocin, bacteriophage, plasmid analysis, pulsed-field gel electrophoresis (PFGE) and single-enzyme amplified fragment length polymorphism (10).

Literature does not present any study involving the typing of samples isolated from enteritis in swine. Genetic analysis of isolates from swine may lead to a better understanding of the transmission of the agent in swine production, as well as aid the choice of the best vaccination program and the recognition of the contamination potential of this agent in carcasses in the slaughterhouse.

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The objectives of this trial were to characterize *C. perfringens* strains isolated from swine for to the presence of alpha, beta, epsilon, iota, enterotoxin and β-2 toxins genes using PCR, as well as to analyze *C. perfringens* genetic variability of isolates through the single-enzyme amplified fragment length polymorphism (SE-AFLP) method.

## MATERIALS AND METHODS

### Bacterial strains

A total of 54 isolates of organisms previously identified as *C. perfringens* were used in this study (11). The strains were obtained from eleven animals presenting neonatal diarrhea in eight herds located in three States of Brazil: Santa Catarina, Paraná, and Minas Gerais. The reference strains of *C. perfringens* biotype A (ATCC 3624), biotype B (ATCC 3626), biotype C (ATCC 3628) and biotype D (ATCC 3629) kindly offered by Instituto Biológico of São Paulo were used as positive controls in toxin genes detection through PCR.

### DNA extraction

*C. perfringens* reference strains and cultures were grown in brain hearth infusion broth (BHI, Difco/BBL- Texas), at 37ºC for 18-24 h. For PCR and SE-AFLP, total cellular DNA was purified with the guanidium thiocyanate method as previously described (13).

### Toxin gene detection

PCR assays were performed using the DNA Thermal Cycler PT 200 (MJ Research) and using specific primers to toxin genes as previously described (11). The reactions were performed in 25 µL volumes containing 5.0 µL of DNA template, 1.5 mM $MgCl_2$, 200 mM of each dNTP, 250 pmol of each oligonucleotide primer and 1.25 U of *Taq* DNA polymerase in 1 X PCR buffer (Invitrogen, São Paulo). The mixture was subjected to an initial denaturing step of 94ºC for 4 min, followed by 35 cycles of 94ºC for 1 min, 55ºC for 1 min, and 72ºC for 1 min.

### Restriction endonuclease digestion, ligation of adapters and PCR

An aliquot containing 10 µg of DNA was digested overnight (16 h) at 37ºC with 24 U of *H*ind III (Invitrogen, São Paulo) 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 (Invitrogen, São Paulo), ligase buffer, and water, in a final volume of 20 µL incubated at room temperature for 3 h. Ligated DNA was heated to 80ºC for 10 min, diluted 1/5 in sterile distilled water, and 5 µL were used for each reaction.

### PCR reaction

PCR reactions were performed in 50 µL final volumes and contained 5 µL of ligated DNA, 2.5 mM $MgCl_2$, 300 ng of primer HIG- 5´GGTATGCGACAGAGCTTG 3´ and 1.25 U of *Taq* DNA polymerase in 1 X PCR buffer. The mixture was submitted to an initial denaturing step of 94ºC for 4 min, followed by 35 cycles of 94ºC for 1 min, 60ºC for 1 min, and 72ºC for 2.5 min.

The amplified products were analyzed on a 2.0% agarose gel, stained with ethidium bromide (0.5 mg/µL), observed by UV transilumination, and registered by an image capturing system (ImageMaster VDS, Amersham Pharmacia Biotech). A 100 bp DNA ladder (Invitrogen, São Paulo) was included twice on each electrophoresis gel. Banding patterns were assessed visually, considering only strong and moderately stained fragments. Analyses of the SE-AFLP banding patterns were performed blindly and under code with respect to the biotype and epidemiological data.

### Discriminatory index

The discriminatory power of the SE-AFLP typing method was calculated as described by Hunter and Gaston (5).

Discriminatory index:

$$\mathbf{DI} = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j(n_j-1)$$

Where $N$ is the number of strains, $s$ is number of types, and $n_j$ is number of strains with type $j$.

### Statistical analysis

Analysis of the banding patterns was done with the Software NTSYS (Numerical Taxonomy and Multivariate Analysis System) with the Jaccard coefficient. In general, strains were considered clonal if they showed 100% similarity. Band patterns were clustered to deduce a dendrogram by the unweighted pair group method with arithmetic averages.

## RESULTS

Analysis of genes *cpa*, *cpb*, *cpb-2*, *cpe*, *ia* and *etx* using PCR showed the presence of three different biotypes. From the 54 samples tested for the presence of genes encoding toxins, 39 (72.2%) were classified as type A, 7 (13%) as type C and 8 (14.8%) as type D. Samples isolated from the same animal were classified as different biotypes, and isolates from each animal were either *C. perfringens* type A and type C, or type A and type D. All samples presented gene *cpa*, which confirmed their identification as *C. perfringens*, and gene *cpb-2* encoding toxin β-2. Besides, all isolates were negative for genes *etx* and *ia*, and type E, type B or enterotoxin-producing strains were not found.

Analysis of the samples tested by SE-AFLP showed the presence of 29 profiles characterized as AP1-AP29. Each profile produced from 4 to 12 DNA fragments (bands) of approximately 380-2,072 pb (Fig. 1). Profiles found were different in relation to the presence or absence of at least one band, and all of them presented one band of approximately 1,050 and 1,450 pb. In order to test the reproducibility, three separate preparations of 10 *C. perfringens* isolates were submitted to SE-AFLP, and no band variation was observed. However, some variations in the intensities of bands were observed with different PCR assays.

It was observed that all samples isolated from the same animal presented different profiles as accessed by SE-AFLP, and that, in some cases, samples that came from different herds showed similar profiles.

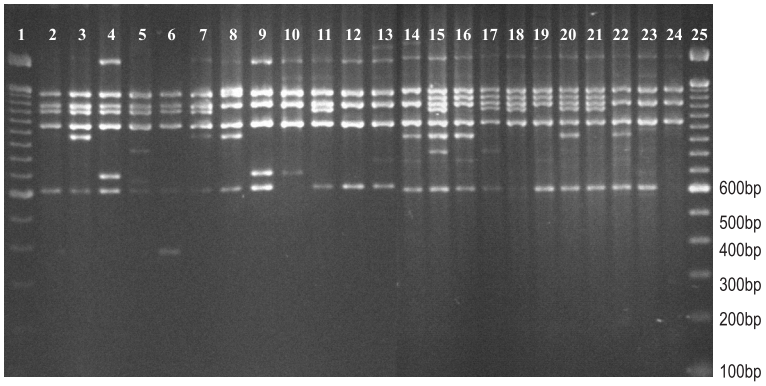



**Figure 1.** SE-AFLP profiles of *Clostridium perfringens* produced by primer HIG. Lanes 1 and 25, 100bp molecular weight standard (Invitrogen, São Paulo). The remaining lanes show AFLP profiles (AP1 to AP23).

Samples belonging to the same biotype, when tested by SE-AFLP presented different patterns. Sample of *C. perfringens* type A presented 21 different profiles in AFLP; type C ones presented four different profiles and type D ones, six different profiles.

AFLP produced a dendogram (Fig. 2) in which two groups may be observed with a similarity rate equal to 27.5%. Group I presented 81.5% (44/54) of the samples analyzed. This group was made up of two subgroups that present at least 42.5% of similarity (Ia and Ib). Subgroup Ia presented 23 samples that belonged to two different biotypes, type A and type C, with similarity rate of approximately 60%. The other subgroup, called Ib, presented 21 samples from three different biotypes, type A, C and D, with similarity rate of 60%.

Group II presented 18.5% (10/54) of the isolates, with at least 83% of similarity. This group showed samples from only one genotype that came from the same herd.

## DISCUSSION

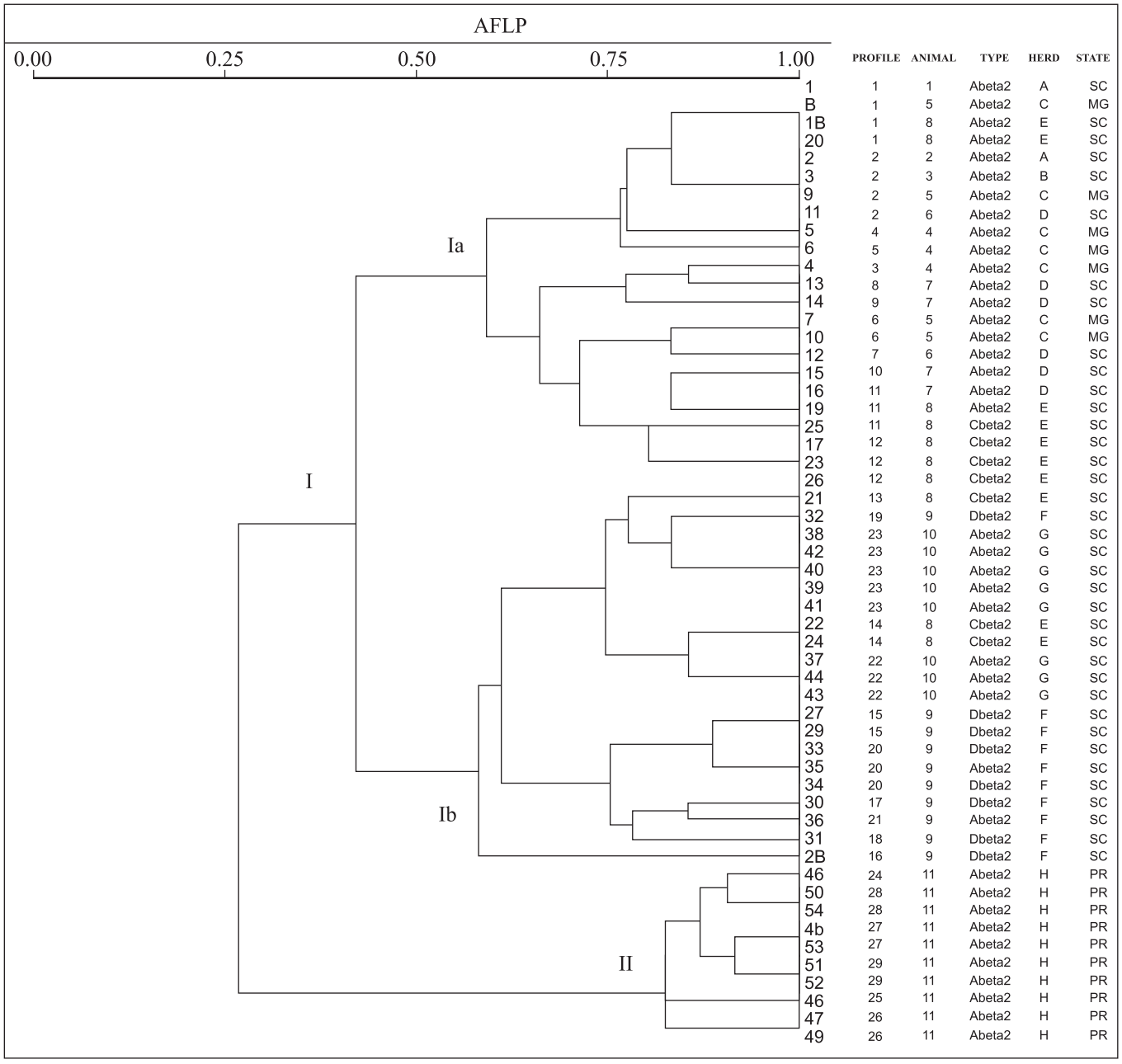
Detection of genes encoding *C. perfringens* toxins by PCR has been used by several authors, for it makes unnecessary to use laboratory animals and does not depend on the *in vitro* sporulation of the organism, what potentially reduces false-negative results (9). Frequency of genes *cpa*, *cpb* and *cpb*-2 in the study was similar to the results obtained by Garmory *et al.* and Klaasen *et al.* (1,7). These authors describe the predominance of isolates in types A and C positive for gene *cpb-2*. No reports were found in the literature on the occurrence of type D samples related to swine diarrhea, as observed in the present study.

Typing is an important epidemiological tool for the recognition of outbreaks, detection of cross-contamination and determination of the source of infection, recognition of virulent strains and monitoring of vaccination programs (12). Different vaccines to be used in the prevention of the infection by *C. perfringens* are available in the Brazilian market nowadays. However, the occurrence of diarrhea caused by this agent has been observed even in herds that have vaccination programs. This fact may be related to the great genetic variability of this agent, as it was observed in the present study.

The analysis of the 54 samples of *C. perfringens* using SE-AFLP showed 29 different subtypes, as it was previously observed (10) in human samples, using the same methodology (16 different subtypes in 35 samples). The results may not be compared with other reports due to the scarcity of studies related to the subtyping of this agent. AFLP produced a dendrogram (Fig. 2), in which two groups may be observed, with similarity rates equal to 27.5% and 70%. This finding is similar to the results reported by Maslanka *et al.* (8), in a study using PFGE. These authors observed that isolates that came from different foodborne outbreaks, although not related, presented similarity rates equal from 40 to 70%.

Based on the results, it may be observed that different SE-AFLP patterns were found in isolates of the same animal, which suggests that the technique is highly discriminatory and that the agent presents high variability. The analysis of the dendogram shows that group I was made up of highly heterogeneous samples in relation to biotype, state of origin, animal and herd, and it was not possible to draw a direct epidemiological correlation between them. Isolates of different origin and different biotypes presenting identical SE-AFLP profiles were observed, what suggests a clonal relationship between the strains. This finding may be related to the large distribution of genetic material throughout the country, what leads to the dissemination of different genotypes by the animals that carry the agent.

Samples studied in this trial were obtained from cases of diarrhea and from a small number of animals, what may have influenced the small correlation between the isolates gathered in group I. Although other agents that cause diarrhea have been excluded as a cause of infection in the animals studied,




| | PROFILE | ANIMAL | TYPE | HERD | STATE |
|---|---|---|---|---|---|
| 1 | 1 | 1 | Abeta2 | A | SC |
| B | 1 | 5 | Abeta2 | C | MG |
| 1B | 1 | 8 | Abeta2 | E | SC |
| 20 | 1 | 8 | Abeta2 | E | SC |
| 2 | 2 | 2 | Abeta2 | A | SC |
| 3 | 2 | 3 | Abeta2 | B | SC |
| 9 | 2 | 5 | Abeta2 | C | MG |
| 11 | 2 | 6 | Abeta2 | D | SC |
| 5 | 4 | 4 | Abeta2 | C | MG |
| 6 | 5 | 4 | Abeta2 | C | MG |
| 4 | 3 | 4 | Abeta2 | C | MG |
| 13 | 8 | 7 | Abeta2 | D | SC |
| 14 | 9 | 7 | Abeta2 | D | SC |
| 7 | 6 | 5 | Abeta2 | C | MG |
| 10 | 6 | 5 | Abeta2 | C | MG |
| 12 | 7 | 6 | Abeta2 | D | SC |
| 15 | 10 | 7 | Abeta2 | D | SC |
| 16 | 11 | 7 | Abeta2 | D | SC |
| 19 | 11 | 8 | Abeta2 | E | SC |
| 25 | 11 | 8 | Cbeta2 | E | SC |
| 17 | 12 | 8 | Cbeta2 | E | SC |
| 23 | 12 | 8 | Cbeta2 | E | SC |
| 26 | 12 | 8 | Cbeta2 | E | SC |
| 21 | 13 | 8 | Cbeta2 | E | SC |
| 32 | 19 | 9 | Dbeta2 | F | SC |
| 38 | 23 | 10 | Abeta2 | G | SC |
| 42 | 23 | 10 | Abeta2 | G | SC |
| 40 | 23 | 10 | Abeta2 | G | SC |
| 39 | 23 | 10 | Abeta2 | G | SC |
| 41 | 23 | 10 | Abeta2 | G | SC |
| 22 | 14 | 8 | Cbeta2 | E | SC |
| 24 | 14 | 8 | Cbeta2 | E | SC |
| 37 | 22 | 10 | Abeta2 | G | SC |
| 44 | 22 | 10 | Abeta2 | G | SC |
| 43 | 22 | 10 | Abeta2 | G | SC |
| 27 | 15 | 9 | Dbeta2 | F | SC |
| 29 | 15 | 9 | Dbeta2 | F | SC |
| 33 | 20 | 9 | Dbeta2 | F | SC |
| 35 | 20 | 9 | Abeta2 | F | SC |
| 34 | 20 | 9 | Dbeta2 | F | SC |
| 30 | 17 | 9 | Dbeta2 | F | SC |
| 36 | 21 | 9 | Abeta2 | F | SC |
| 31 | 18 | 9 | Dbeta2 | F | SC |
| 2B | 16 | 9 | Dbeta2 | F | SC |
| 46 | 24 | 11 | Abeta2 | H | PR |
| 50 | 28 | 11 | Abeta2 | H | PR |
| 54 | 28 | 11 | Abeta2 | H | PR |
| 4b | 27 | 11 | Abeta2 | H | PR |
| 53 | 27 | 11 | Abeta2 | H | PR |
| 51 | 29 | 11 | Abeta2 | H | PR |
| 52 | 29 | 11 | Abeta2 | H | PR |
| 46 | 25 | 11 | Abeta2 | H | PR |
| 47 | 26 | 11 | Abeta2 | H | PR |
| 49 | 26 | 11 | Abeta2 | H | PR |

**Figure 2.** Dendrogram showing the relationship between *C. perfringens* isolates on the basis of SE-AFLP pattern. Percentages of similarity between patterns were calculated by use of Jaccard coefficient. Dendrogram was constructed by use of UPGMA.

there is a possibility that some of the samples studied may belong to normal piglet microbiota, and that they are not related to diarrhea outbreaks. Therefore, an even greater variability may be expected between the isolates, as well as the absence of direct correlation between them. On the other hand, group II presents samples with more than 75% of similarity, and it was possible to draw a relationship on their biotype and origin.

Nowadays, most of *C. perfringens* genome has been characterized, and it was observed that genes that encode toxins alpha and theta are located on variable regions of the chromosome, near the origin of the replication process, and that genes encoding toxins beta, beta-2, epsilon and iota are located on plasmids that range from 55 to 140 Kb (2,6). Extrachromosomal location of most of the genes encoding

toxins may be related to the large diversity of *Clostridium perfringens* species. It is well known that the presence of mobile genetic elements, such as transposons, insertion sequences, bacteriophages and plasmids may increase the variability of the profiles produced by AFLP and PFGE (3). Loss and acquisition of plasmids and other elements may explain, for example, the presence of different biotypes in the same animal, and samples of different biotypes in the same AFLP profile (2).

All samples tested were typed by SE-AFLP. Maslanka *et al.* (8) reported that PFGE showed 7% of *C. perfringens* isolates which could not be subtyped due to the absence of bands, and that the amount of untypable strains in serological tests may reach 70%. SE-AFLP showed to be an easy, quick, low cost technique, with good reproducibility.

No reports were found in the literature related to the description *C. perfringens* molecular typing in samples isolated from swine. This fact emphasizes the importance of the present study in the evaluation of the SE-AFLP potential in the characterization of *C. perfringens* samples isolated from swine affected by diarrhea.


## RESUMO

### Uso do polimorfismo do comprimento de fragmentos amplificados para tipagem de *Clostridium perfringens* isolados de suínos com diarréia

*Clostridium perfringens* é um importante agente infeccioso em medicina veterinária e humana. Em suínos, o agente é responsável pela enterite necrótica e enterotoxemia, caracterizadas por diarréia, perda de peso, atraso no desenvolvimento e morte. No presente estudo foi utilizado o polimorfismo do comprimento de fragmentos amplificados (AFLP), para caracterizar 54 isolados de *C. perfringens* obtidos de suínos com diarréia. A análise dos resultados do AFLP demonstrou 29 perfis distintos com índice discriminatório igual a 0,97. A correlação entre a origem dos isolados e os agrupamentos obtidos foi parcial, sendo apenas possível a correlação total de 18,5% das amostras estudadas. A caracterização das cepas em biotipos (A, B, C, D e E), produção da toxina beta-2 e enterotoxina foi realizada através da reação da polimerase em cadeia (PCR). Dentre as cepas analisadas foram observados os biotipos A, C e D, sendo que todas as amostras foram positivas para a presença do gene codificador da toxina beta-2 e negativas para o gene codificador da enterotoxina. Neste estudo, o AFLP demonstrou ser uma metodologia simples, rápida, de baixo custo, com alto poder discriminatório e boa reprodutibilidade, apresentando grande potencial para estudos epidemiológicos envolvendo cepas de *C. perfringens* de origem animal.


**Palavras-chave:** AFLP, diarréia, suínos, *Clostridium erfringens*

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