# CHARACTERIZATION OF THE PLASMIDIC OR CHROMOSOMAL *CPE* GENE AND METABOLIC ACTIVITIES IN *CLOSTRIDIUM PERFRINGENS* ISOLATES FROM FOOD IN SAN LUIS – ARGENTINA

**Mariana Georgina Corigliano[1], Ana María Stefanini de Guzmán[1], Patricia Virginia Stagnitta[2]**

[1]General Microbiology, Area of Microbiology, Department of Biochemistry and Biological Sciences, Faculty of Chemistry, Biochemistry and Pharmacy, San Luis National University, Argentina

[2]Biological Chemistry, Area of Microbiology, Department of Biochemistry and Biological Sciences, Faculty of Chemistry, Biochemistry and Pharmacy, San Luis National University, Argentina

**SUMMARY**

Food poisoning and non-food poisoning illnesses due to *C. perfringens* (by enterotoxin production) have been associated to chromosomal or plasmidic location of the *cpe* gene, respectively. Clostridial pathogenicity has been correlated to protease and azoreductase production.

The aim of this work was: i) to assess the sanitary-hygienic quality of dehydrated soups (100 samples) consumed in San Luis – Argentina; ii) to verify the presence of *C. perfringens* in these food products using the "Most Probable Number" method (MPN) and plate-counting methods; iii) to characterise enterotoxigenicity in strain isolates by RPLA; iv) to determine the chromosomal or plasmidic location of the *cpe* gene in enterotoxigenic strains previously isolated from food in our lab, using PCR; v) to correlate chromosomal *cpe* and spore heat-resistance; vi) to compare protease activity in *cpe*+ and *cpe*– strains; and vii) to compare azoreductase activity in *cpe*+ and *cpe*– strains. Twenty-six isolates had a count a 3–43 bacteria $g^{-1}$ count using MPN; 7.7% exceeded the Argentine Food Code (CAA) limit. All isolates showed protease activity: enterotoxigenic isolates had higher protease activity than non-enterotoxigenic isolates. All isolates showed azoreductase activity: enterotoxigenic isolates had higher activity and shorter reducing times. Enterotoxigenic isolates showed chromosomal location for the gene responsible for the enterotoxin.

*Key words: clostridium perfringens*, enterotoxin, protease, azoreductase, food

**Address for correspondence:** P. V. Stagnitta, Area Química Biológica. Departamento de Bioquímica y Ciencias Biológicas. Facultad de Química, Bioquímica y Farmacia. Universidad Nacional de San Luis, Chacabuco y Pedernera, 5700-San Luis, Argentina. E-mail: pvstag@unsl.edu.ar

## INTRODUCTION

*Clostridium perfringens* are Gram-positive anaerobic bacteria widely spread in the environment, soil and water, and which can usually be found in the gastrointestinal tract of humans and animals (1–5). It is an important pathogen causing illnesses such as, among others, human enteritis and enterotoxemia in domestic animals (1, 2, 4, 6, 7). *C. perfringens* can be transmitted through food, particularly by cooked meats that are allowed to cool slowly and are eaten some time later (8–10). In many cooked foods, the presence of spores can be unavoidable because spores are heat-resistant and are frequently found in raw foods.

These bacteria produce at least 17 toxins, including the *C. perfringens* enterotoxin (CPE) (3, 4, 11, 12), which is known to cause human food poisoning. *C. perfringens* intoxication can be due to ingestion of food containing an enterotoxigenic strain in a concentration $\geq 10^5$ CFU/g (13, 14). *In vivo*, enterotoxin production is associated to sporulation in the intestine (3, 15, 16), while an adequate culture medium is needed for in vitro production (13, 17). Vegetative cells that reach the intestine and undergo sporulation produce CPE, which in turn is responsible for clinical symptoms. This toxin-infection is characterized by nausea, diarrhea, abdominal pain and gases, 6 to 12 hrs after the intake of contaminated food. Recovery is fast, usually within 12 hrs (3, 18–21).

*C. perfringens* has been classified to five types (A, B, C, D and E) based on production of four main toxins (α, β, ε and ι) (11, 12, 21–23). These toxins can be determined by serological methods or by molecular methods such as PCR (2, 13, 24). Because these are spore-forming bacteria, they are very resistant to the action of external agents, which in turn favours their distribution (4).

Strains type A carry the *cpe* gene in 5–8% of the global population (3, 21–23, 25, 26). C and D strains can also carry *cpe* and produce CPE (4, 23). The *cpe* gene encoding the CPE toxin can be located in the plasmid or in the chromosome of *C. perfringens* (11, 22, 27, 28). It is accepted that *C. perfringens* strains associated to food poisoning carry their *cpe* gene in the chromosome; while *C. perfringens* strains causing illnesses not transmitted through food, such as antibiotic-related diarrhea and sporadic diarrhea, carry the *cpe* gene in a plasmid (18, 26, 29, 30). In opposition to the description above, Tanaka et. al (2003) studied an outbreak in which 90 of 192 individuals showed symptoms of diarrhea and abdominal pain, within an incubation period of 15.5 hrs. Boiled peas were suspected to be the vehicle for *C. perfringens* because they were cooked in large quantities, slowly cooled and inadequately reheated before the meal was

served. It should be noted that the location of the *cpe* gene in this study was concluded to be plasmidic, as determined by Pulse Field Gel Electrophoresis (PFGE).

Spores from strains containing the chromosomal *cpe* gene are more resistant to heat than spores from strains with the gene in a plasmid (31). Heat resistant spores survive the cooking process and continue to cause food poisoning; this may explain why most of the strains generating food poisoning have their *cpe* gene located in the bacterial chromosome (29, 32).

Microbial enzymes are the major cause of food quality decay and putrefaction (33). Protease formation has been related to food poisoning and to pathogenicity in a great number of clostridia, including *C. perfringens* (34). Casein and azocasein are different compounds used as protease substrates. Poilane and Zarnowski have studied extracellular proteolytic enzyme production in *C. difficile* and *Histoplasma capsulatum*, respectively. Also, azocasein was used by Brock to study the proteolytic activity of the microflora in rumen (35–37). In addition to extracellular protease production (33, 38–41), *C. perfringens* synthesizes intracellular proteases (42).

*C. perfringes* is a commensal in the human low gastrointestinal tract (4, 5) that produces azoreductase, which catalyzes the reducing cleavage of colored azo-compounds (43). Azo-colored compounds represent a large group of chemical products widely used in the textile, pharmaceutical, food and cosmetic industries. These compounds are reduced by enzymes called azoreductases. Azoreductases are produced by intestinal bacteria and − to less extent − by enzymes from the microsomal and cytosolic fractions of the liver (44, 45). Some azo-compounds have been correlated to human gall bladder cancer, liver carcinomas, nuclear anomalies in experimental animals and chromosomal aberrations in mammal cells (46). It has been determined that several hydrolytic and reducing enzymes from *C. perfringens* such as azoreductase can be implicated in mutagenic or genotoxic metabolite production in the human intestinal tract, which constitutes an important virulence factor (44, 47, 48).

The aims of the present work were: i) to establish sanitary hygienic quality in dehydrated soups comsumed in San Luis – Argentina; ii) to determine the presence of *C. perfringens* in these products using the Most Probable Number method (MPN) and plaque counting methods; iii) to characterize enterotoxigenicity of isolated strains using the immunological RPLA method; iv) to determine plasmidic or chromosomal location of the *cpe* gene by PCR from enterotoxigenic strains previously isolated from food in our laboratory; v) to correlate the presence of *cpe* in the chromosome and heat-resistance of spores; vi) to compare protease activity between *cpe*+ and *cpe*– strains; and vii) to compare azoreductase activity between *cpe*+ and *cpe*– strains.

## MATERIALS AND METHODS

### Strains

Two reference strains, NTCC 8798 *cpe*+ and ATCC 3624 *cpe*– were kindly supplied by Dr. Ronald G. Labbé from University of Massachusetts, USA. Fifteen enterotoxigenic strains were previously isolated from meat food, seasoning and spices. Twenty-six isolates from dehydrated soups.

### Samples

One hundred dehydrated soup samples from local markets in San Luis City – Argentina were obtained and processed. One gram of each dehydrated soup sample was aseptically weighed and dissolved in 10 ml of sterile 0.1% peptone water. Ten-fold dilutions were made transferring 1 ml from the original suspension to tubes containing 9 ml of sterile 0.1% peptone water.

### Determination of hygienic conditions of samples

**Total aerobic bacteria count:**

Agar medium for plate counts (Britania) was used. 10 ml of sterile medium were plated on Petri dishes and 0.1 ml of each dilution was seeded in duplicate by spreading over the medium surface with spatula. Inoculated media were incubated at 37˚C and colony count performed after 48 hrs.

**Investigation of total coliforms and *E. coli***

Mac Conkey Broth (Merck) was used. One ml of each dilution was seeded in triplicate and incubated at 37˚C. Test readings were taken after 48 hrs. In order to confirm *E. coli* EC broth (Britania) was prepared. 0.1 ml from each positive Mac Conkey tube was seeded and incubated at 45˚C during 24 hrs.

***C. perfringens* count**

Most Probable Number (MPN) in iron-milk medium: The iron-milk medium was prepared according to William et al. (49). One ml of each dilution were seeded in triplicate in tubes containing iron-milk medium and then incubated at 45˚C. Test readings were taken 16–18 hrs after. Tubes showing tumultuous fermentation were considered as positive. Man's Table was used to determine MPN (50). Results were expressed as MPN per food gram ($MPN.g^{-1}$).

**Purification and identification of *C. perfringens***

Differential agar medium for clostridia (Britania) was used. An inoculating loop load from a thioglycolate broth culture was seeded on a Petri dish and incubated at 37˚C during 24 hrs in anaerobic conditions. Black colonies were considered as positive. Suspicious black colonies were identified by Gram's stain in addition to the following biochemical tests: catalase, nitrate reduction, gelatin hydrolysis, starch hydrolysis, lecitinase and hemolysis.

**Strain Maintenance**

Strains were maintained in cooked meat medium and passed into thioglycolate broth or brain-heart infusion broth (BHI) (Britania) according to each assay.

***In vitro* production of enterotoxin**

Strains cultured in BHI broth were obtained after 12–18 hrs at 37˚C and further grown at 37˚C during 10–12 days in Tórtora medium (51) Modified (Tm) by Stagnitta et al. 2002 (13).

**Enterotoxin titration**

Reverse passive latex agglutination (RPLA) was used to determine enterotoxin levels in culture supernatants. The PET-RPLA kit (Oxoid), which has 2 ng/ml sensitivity, was used. Supernatants of CPE-positive and CPE-negative reference strains were used as controls.

**Correlation of the chromosomal location of the *cpe* gene and spore heat-resistance in enterotoxigenic strains.**

Enterotoxigenic strains cultured in BHI broth were obtained after 12–18 hrs at 37˚C. Sporulated cultures were obtained after incubation at 37˚C during 10–12 days in (Tm) medium. Cultures were heated at 80˚C and 100˚C for 10 min, respectively. One ml of each Tm culture was incubated in 10 ml of BHI in anaerobiosis over night (31, 52).

## Protease activity determination

### Kinetics

In order to determine proteolytic activity of the strains in function of substrate concentration, a kinetic assay using the modified Domínguez and Cejudo method was performed to assess optimal azocasein (Sigma) concentration. One *cpe*+ and one *cpe*– strain were randomly chosen and used (53).

### Protease activity

Based on the kinetic study, the assay conditions in tube 3 were the conditions of choice to study protease activity in 15 *cpe*+ strains and 13 *cpe*– strains (53). One Enzymatic Activity Unit was defined as 1 Absorbance Unit increase per reaction time (Unit/hour). Determination of protease specific activity was completed with the quantification of total proteins present in the supernatant using the Lowry method (54).

### Azoreductase activity determination

Azoreductase activity in the supernatant was determined by discoloring the substrate Direct Blue 15 (Sigma) according to Raffi et al. (44, 55). A culture grown in anaerobiosis for 12 hrs at 37˚C was then centrifuged at 15000 x g for 15 min. Two ml of supernatant were taken under anaerobiosis and added with Direct Blue 15 to a final concentration of 100 µl/ml. Tubes were monitored until Direct Blue 15 was completely reduced and discolored. Required times for total reduction was registered. Total protein concentration was determined using the Lowry method by taking a supernatant aliquot before incubating the supernatant with the susbstrate.

### Isolation and purification of DNA from *Clostridium perfringens*

Cultures were obtained in 10 ml of BHI broth incubated anaerobically during 12 hrs at 37˚C. A 0.2 ml aliquot was taken and incubated anaerobically in 10 ml of TGY broth (3% trypticase, 2% glucose, 1% yeast extract, 0.1% cysteine) during 12 hrs at 37˚C (22). DNA extraction was performed as described previously (23) and quantification was done by absorption measurements at 280 and 260 nm using a Beckman UV-visible spectrophotometer DU Series 600. Finally, 10 ng/µl DNA dilutions were prepared.

## PCR assay for detection of the chromosome-located *cpe* gene

### Simple *cpe*-IS1470 PCR assay for detection of the chromosome-located *cpe* gene

On the basis of recent studies describing the organization of the chromosomal *cpe* locus, Miyamoto et al. (21) have designed a primer pair that amplifies an ~2.1 kb PCR product from the apparently conserved DNA region between the chromosomal *cpe* gene and the downstream insertion sequences IS1470 in type A isolate strains: 5´-CAGTCCTTAGGTGATGGA-3´(primer CPE-4.5F) and 5´-AACTAAATAGGCCTATAAATACC-3´(primer IS1470F). PCR amplification was carried out by mixing 500–1000 ng of DNA with both primers at 1µM concentration each (Biodynamics), deoxynucleotide triphosphates (dNTPs) at 0.2 µM each (Promega), $MgCl_2$ 2 mM (Promega) and 1.25 U of Taq Polymerase (Invitrogen), in a 50 µl final volume per reaction tube. Cycling was started with 5 min at 94˚C; followed by 33 cycles of: 30 sec at 94˚C, 60 sec at 63˚C and 180 sec at 72˚C; and a final cycle of: 90 sec at 94˚C, 90 sec at 60˚C and 7 min at 72˚C. The Wen et al. technique was used, modifying polymerization temperature to 72˚C due to use of Taq polymerase (22). Amplification was carried out in Perkin Elmer thermal cycler model PCR System 2000. Results were determined by 1.5% agarose gel electrophoresis. Agarose was dissolved in 1X Tris-Borate buffer (TBE). Before gel solidification, 1 µl ethidium bromide was added to reach 1.5 µg/ml final concentration. PCR samples were run in an electrophoresis apparatus (BioRad Power PAC 1000 Midicell EC3500) at 80V during 50 min, in 0.5X TBE buffer. Bands were viewed on a transilluminator (White-Ultraviolet transilluminator UVP Ultraviolet Products Upland Sigma) and photographs were taken with a Polaroid camera (Polaroid Gel Cam EPH5 0.85x Electrophoresis Hood).

### Single *dcm-cpe* PCR assay for detection of the plasmid-located *cpe* gene

On the basis of recent studies describing the organization of the plasmid-borne *cpe* locus, Miyamoto et al. (21) have designed a primer pair that amplifies an ~3.3 kb PCR product from an apparently conserved DNA region in the plasmid and located between the up-stream dcm sequences and the *cpe* gene, in type A isolate strains:
5´-CTCAGAGTTAGGAGCTAGCCCAACCC-3´ (primer MET-1.5F) and
5´-CCTAATATCCAACCATCTCC -3´ (primer CPE-up). PCR amplification was run using both primers at a final concentration of 1 µM each (Promega), in 50 µl final reaction volume containing the other mix reagents and template in equal concentrations as in the chromosomal *cpe* PCR amplification. In addition, the same cycling conditions were applied here as described above (22). Amplification was carried out in a Perkin Elmer thermal cycler model PCR System 2000. Results were determined by 1.5% agarose gel electrophoresis. PCR samples were run at 80V during 50 min. Bands were viewed on a transilluminator and photographs were taken with a Polaroid camera.

### Duplex PCR Protocol for genotyping *C. perfringens* type A strains

Both primer pairs described above were placed in the same reaction mix to create a Duplex-PCR assay capable of distinguishing between the chromosomal or plasmidic location of the *cpe* gene in type A strains (22). PCR amplification was carried out using 0.6 µM of each primer used for "plasmidic-*cpe*" amplification and 0.8 µM of each primer used for "chromosomal-*cpe*" amplification. Final reaction volume was 50 µl, containing the other mix reagents and template in equal concentrations as in the above amplifications. Cycling conditions were repeated as described above. Results were determined by 1.5% agarose gel

electrophoresis. PCR samples were run at 80V during 50 min. Bands were viewed on a transilluminator and photographs were taken with a Polaroid camera.

## RESULTS

### *C. perfringens* count

*Most probable number (MPN) in iron-milk medium.*

Twenty six *C. perfringens* strains were isolated out of the total of 100 dehydrated soup samples analyzed (26%). These 26 isolates rendered a 3–43 bacteria $g^{-1}$ count using the MPN method. Two samples (7.7%) surpassed the 10 $g^{-1}$ limit established by the Argentine Food Code (CAA), showing 15 and 43 MPN $g^{-1}$ (Art. 442). Four samples (15.4%) almost reached the limit showing 9.2 MPN $g^{-1}$.

### Determination of the hygienic conditions of samples

*Total aerobic bacteria count*

Total mesophilic aerobes rendered counts between $10^2$ and $10^4$ bacteria $g^{-1}$. These results were found to fall within the CAA limits ($10^5$ $g^{-1}$) for this food.

*Total coliforms and E. coli investigation*

Twelve positive samples were found of the 63 examined (19%) for the total coliforms reaction. The CAA does not limit these microorganisms for foods that are cooked. *E. coli* was not detected.

### *In vitro* production of enterotoxin

*Enterotoxin assessment*

None of the assayed strains isolated from dehydrated soups produced enterotoxin, and thus were defined as non-enterotoxigenic strains.

### Correlation of the chromosomal location of the *cpe* gene and spore heat-resistance in enterotoxigenic strains.

All studied enterotoxigenic strains (100%) were heat-resistant at 80˚C and 100˚C. Non-enterotoxigenic strains used as negative controls did grow after heating at 80˚C, but did not grow after heating at 100˚C.

## Protease activity determination

### Kinetic results

*Protease activity kinetics in enterotoxigenic and non-enterotoxigenic strains*

Kinetic results for the non-enterotoxigenic strain were plotted to show that the highest protease activity was reached at 0.5% of substrate (Fig. 1). Thus, this condition (0.5% substrate) was chosen to study protease activity in the 13 non-enterotoxigenic strains. The kinetic assay for the enterotoxigenic strain showed the same activity pattern as for the non-enterotoxigenic strain when rising substrate concentration: 0.5% showed highest protease activity (Fig. 2). This condition was also chosen to study protease activity in the 15 enterotoxigenic strains.

*Protease activity determination in enterotoxigenic and non-enterotoxigenic strains*

All assayed strains (100%) showed protease activity. Enterotoxigenic strains showed higher protease activity than non-enterotoxigenic strains.

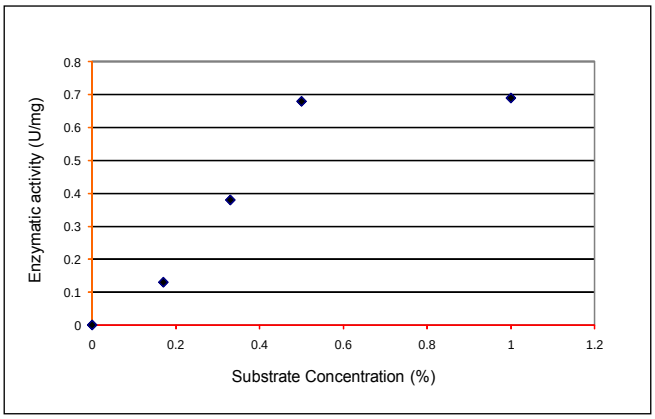


***Fig. 1.*** *Protease activity kinetics of a non-enterotoxigenic strain (NESL 13).*

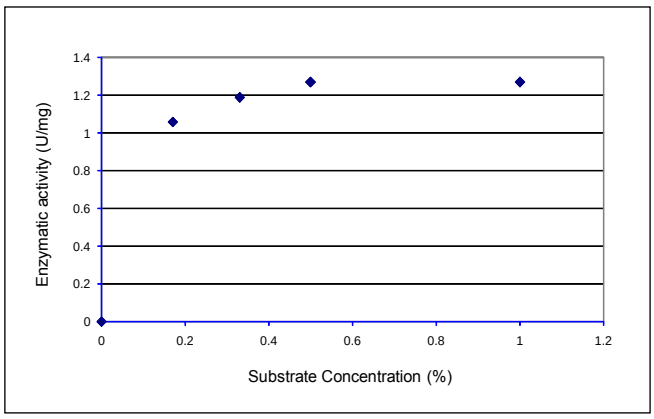


***Fig. 2.*** *Protease activity kinetics of an enterotoxigenic strain (ESL 3).*

The 13 *cpe*– strains showed a mean Specific Activity = 0.22 U/mg of protein, while the 15 *cpe*+ strains showed a mean Specific Activity = 0.3 U/mg of protein (Table 1, Fig. 3). Statistical results from the t-test, which assesses the probability associated with Student's t-test, showed a significant difference <0.05.

*Azoreductase activity determination*

All assayed strains (100%) showed Azoreductase activity. Enterotoxigenic strains showed higher activity along with shorter times needed for reduction of the azo-compound: enterotoxigenic strains needed 6–24 hrs while non-enterotoxigenic strains needed 6–72 hrs.

PCR results for *cpe* gene detection on the chromosome or on the plasmid

The location for the *cpe* gene was determined by setting two standard PCRs and using primers targeted to the gene's possible location on the chromosome or on the plasmid. Enterotoxigenic strains previously isolated from meat foods, spices and seasonings were used. Both amplification products for each strain were seeded side-by-side in order to confirm the presence of the *cpe* gene on the chromosome or on the plasmid (Fig. 4). All 15 enterotoxigenic strains in study showed chromosomal location for the enterotoxin gene, as the amplification product was ~2100 bp (Fig. 5).

***Table 1:*** *Specific Activity results of cpe– and cpe+ strains of C. perfringens*

| ***cpe–*** **strains** | **Specific Activity** | ***cpe+*** **Strains** | **Specific Activity** |
|---|---|---|---|
| Strain | U/mg of prot. | Strain | U/mg of prot. |
| NESL 13 | 0.149 | ESL 1 | 0.33 |
| NESL 16 | 0.612 | ESL 2 | 0.27 |
| NESL 22 | 0.249 | ESL 3 | 0.45 |
| NESL 24 | 0.158 | ESL 4 | 0.23 |
| NESL 43 | 0.168 | ESL 5 | 0.27 |
| NESL 50 | 0.183 | ESL 6 | 0.27 |
| NESL 53 | 0.173 | ESL 7 | 0.23 |
| NESL 55 | 0.176 | ESL 8 | 0.41 |
| NESL 60 | 0.176 | ESL 9 | 0.32 |
| NESL 77 | 0.189 | ESL 10 | 0.23 |
| NESL 85 | 0.200 | ESL 11 | 0.29 |
| NESL 86 | 0.188 | ESL 12 | 0.23 |
| NESL 88 | 0.221 | ESL 13 | 0.35 |
| mean | 0.218 | ESL 14 | 0.23 |
| | | ESL 15 | 0.36 |
| | | mean | 0.298 |

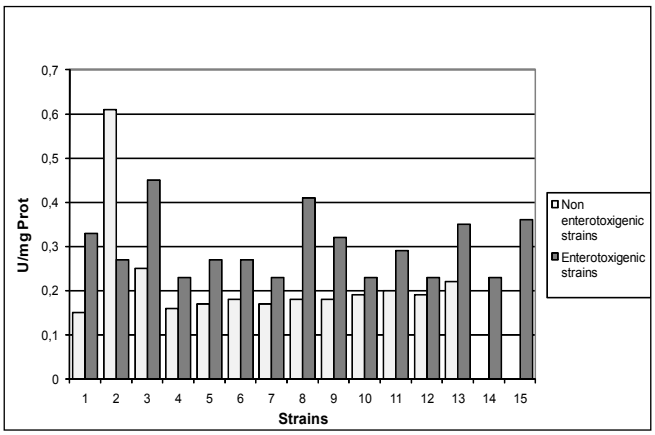


***Fig. 3.*** *Specific activity, protease determination. X axis: strain group, Y axis: U/mg of proteins. Light grey: non-enterotoxigenic strains. Dark grey: enterotoxigenic strains.*

## DISCUSSION

The fact of having isolated *C. perfringens* from dehydrated soup samples did not represent an alarming issue since they were non-enterotoxigenic. Nevertheless, the literature indicates that soups come third in place as the most common source of *C. perfringens* after meat foods, spices and seasonings. In addition, the presence of this microorganism in food has been reviewed in the Argentine Food Code (56), which has set the upper limit at 10 spores gram$^{-1}$ (Art. 442) for dehydrated soups. Two samples have surpassed this limit (43 and 15 *C. perfringens* g$^{-1}$), and 4 other were almost reaching the limit (9.2 *C. perfringens* g$^{-1}$).

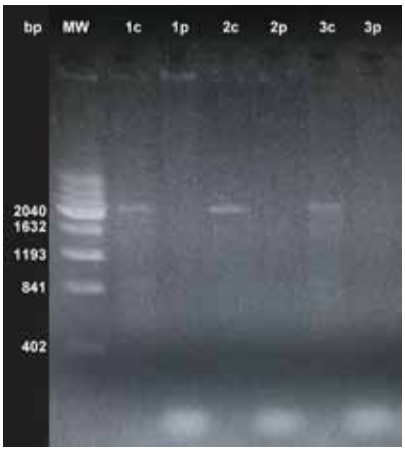


***Fig. 4.*** *MW: molecular weight markers. 1c, 2c and 3c: amplification products from strains 1, 2 and 3 using chromosomal-location primers. 1p, 2p and 3p: amplification products from strains 1, 2 and 3 using plasmidic-location primers.*

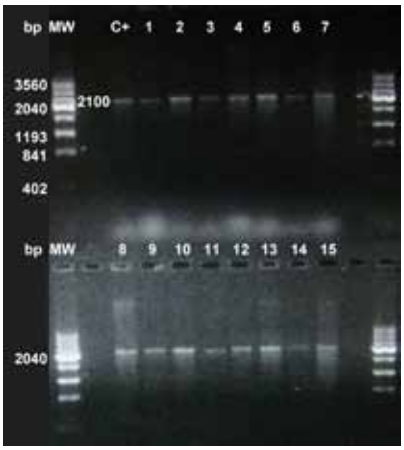


***Fig. 5.*** *MW: molecular weight markers. C+: positive control. Enterotoxigenic strains 1–15, PCR amplification using chromosomal-location primers.*

It is important to perceive the risk associated with heating and reheating the spore contaminated food later. In this scenario, germinated spores can find an adequate environment for multiplication and can therefore reach suitable concentrations for food poisoning, even when the initial load was lower than the limit set by the CAA (8–10).

The assays assessing the sanitary-hygienic quality of dehydrated soups revealed that total mesophilic aerobes were within the limits set by the CAA ($10^5$ g$^{-1}$) for this food. However, coliforms were found in 12 samples, one sample surpassing the maximum allowed limit ($10^3$ g$^{-1}$). *E. coli* was not isolated. In general terms, it can be stated that the sanitary-hygienic quality of dehydrated soups is good and, although the incidence of *C. perfringens* was low, there is a potential risk of food poisoning in case of inappropriate food preparation and handling.

The presence and expression of the *cpe* gene is fundamental in producing gastrointestinal symptoms (13). The critical point in enterotoxin (CPE) determination is enterotoxin expression. As is known, this toxin is produced only upon sporulation and, if the environment is not good enough for this, a possible false negative

can take place (15, 16). However, in addition to the positive and negative controls from the RPLA kit, we use two enterotoxigenic strains as positive controls for the culture medium. The medium of choice was the Modified Tórtora (Tm) medium.

*C. perfringens* may be present in vegetables, spices and seasonings that were used as additives to the different soup varieties described in the literature. Wild strains are understood to be common enterotoxin-producers (3).

Election of an adequate technique to determine enzymatic activity is crucial. Although not much literature was found on *C. perfringens* protease activity, the option of choice was azocasein because it is widely used as a substrate in this type of assays for a large number of genera and species (35–37). Due to results obtained from preliminary assays that compared the modified Domínguez and Cejudo technique to the modified Brock technique, the former one was chosen. This technique does not use NaOH and has the advantage of requiring shorter incubation times, which results in overall reduction in time and costs.

According to the kinetic study using a *cpe*+ and a *cpe*– strain, and different substrate concentrations, tube 3 – presenting 0.5% substrate concentration and corresponding to the highest enzymatic activity – was chosen.

All strains were shown to have protease activity. Results from protease activity and specific activity reveal that there is a quantitative difference between *cpe*– and *cpe*+ strains that can be correlated to higher pathogenicity in enterotoxigenic strains.

Concerning *C. perfringens* azoreductase activity, the dye Direct Blue 15 was chosen. Although it is known that this compound and its cleavage products are extremely toxic, it renders a useful substrate to study and determine the presence and/or activity of enzymes bearing azoreductase activity (57). Currently, several bio-discoloring systems are in development. These systems use microorganisms in order to lower toxicity and contamination – not only ecological, but also visual – generated from Direct Blue 15 usage (58, 59). In our study, all assayed strains showed azoreductase activity, though enterotoxigenic strains showed higher activity and shorter times for Direct Blue 15 discoloring. The presence of azoreductase in the *C. perfringens* strains indicated high virulence and thus correlated the high pathogenicity of the enterotoxigenic strains to the higher azoreductase activity in the strains (44).

The PCR assay that determines plasmidic or chromosomal location of the *cpe* gene gives an idea of the possible reservoirs for potential outbreaks caused by *C. perfringens* in association to food (22).

Both standard PCR assays using primers that locate the *cpe* gene in the plasmid or in the chromosome have the advantage of being capable of identifying *C. perfringens cpe*+ from poorly sporulating cultures that can be otherwise taken as false negatives by serological methods.

The duplex PCR assay has additional advantages such as lowering reagent costs and spent time by combining both primer pairs described above in one single reaction. Also, this assay demonstrated that these strains, isolated from the studied food, carry the *cpe* gene in the chromosome. Although the food in study was not related to any outbreak, the fact of the *cpe* gene having chromosomal location raises worries concerning the heat resistance feature present in these types of strains and the risk of potentially causing food poisoning. It is to note that in our area there is no epidemiological data on food poisoning by *C. perfringens* because this illness develops without complications and practically no routine clinical tests are performed.

Upon learning about the *cpe* gene location in a *cpe*+ strain and of the relationship "chromosomal *cpe* gene / heat-resistant strain" and "plasmidic *cpe* gene / heat-sensitive strain", stress should be laid upon the importance of low temperature storage conditions for food as well as correct heating and/or cooking in prevention of food poisoning caused by *C. perfringes* type A.

Isolation of *C. perfringens* carrying a plasmid-borne *cpe* gene from feces from individuals affected by food poisoning is an atypical case; nevertheless, it supports the need for cooking food properly, preserving hygiene at all times and to not discard heat-sensitive strains of *C. perfringens* as possible causal agents of food poisoning.

Development of a multiplex PCR assay using primers that distinguish insertion sequences (IS) located downstream from the plasmid-borne *cpe* gene is a very important advancement because it brings out the possibility of studying the prevalence of different ISs and, thus, represents another tool for unveiling the mechanisms of horizontal transmission between *C. perfringens* strains. The hypothesis of plasmid transmission by conjugation mechanisms is supported (26). The ability to transfer the plasmid from *cpe*+ strains to *cpe*– strains, and thus transform the latter into enterotoxigenic strains, is a point to be taken into account as the presence of enterotoxigenic *C. perfringens* may convert *cpe*– strains from normal flora into *cpe*+, consequently causing a gastrointestinal illness.

## CONCLUSIONS

1. The incidence of *C. perfringens* in the dehydrated soup samples was found to fall within the limit values stated in the literature. Enterotoxigenic strains of *C. perfringens* were not detected in these samples.

2. In general, the tested soups had good hygienic conditions, as *E. coli* was not detected.

3. The studied foods represent a risk for health, if the particular conditions required for microorganism proliferation are met.

4. The fact of finding azoreductase and protease activity represents the presence of other important virulence factors of *C. perfringens*, that must be taken into account when handling food contaminated with *C. perfringens.*

5. There is a relation between virulence factors and enterotoxigenicity of the strains, which implies a high risk.

6. The presence of the *cpe* gene in chromosomal location indicates a higher risk of suffering food poisoning illness when consuming food contaminated with *C. perfringens*.

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