Isolation and typing of *Clostridium* spp. 16S rRNA from soil samples obtained in areas with sudden mortality history in Colombia

Diego Ortiz Ortega, Luis Carlos Villamil Jiménez, Rodrigo Martínez S.

Corporación Colombiana de Investigación Agropecuaria, CORPOICA. Km 14 vía Mosquera, Cundinamarca, Colombia.
Universidad de La Salle. Cra. 7 172.85

Accepted 09 April, 2012

A Longitudinal epidemiologic study was developed for the isolation, biochemical characterization and molecular typing the bacterial pathogen *Clostridium* spp found in soil from areas affected by bovine sudden mortality. We included 10 herds, located in three localities. The genetic variability of *Clostridium* genus was analyzed by DNA sequence of a 1500 bp fragment from the 16S rRNA gene. Twenty four *Clostridium* isolates were biochemically classified as *Clostridium sordellii* (41.7%), *Clostridium glycolicum* (12.5%), *Clostridium hastiforme* (12.5%); *Clostridium botulinum* (8.3%), *Clostridium butyricum* (8.3%); *Clostridium chauvoei* (4.3%); *Clostridium limosum* (4.3%), *Clostridium septicum* (4.3%) and *Clostridium tertium* (4.3%). The bacteria that showed pathogenic activity were studied further by 16S rRNA gene sequencing at, which 55.5% was classified as *C. botulinum*, where the native strain isolated from areas with outbreaks was found in a different group from *Clostridium* spp. used by commercial laboratories for vaccine production. Additionally, the native strain identified here differs from others reported in Gen Bank, indicating that the native pathogenic *Clostridium* spp. is genetically different to other *Clostridium* spp. used to prepare immunogens affecting vaccine efficiency. Our results indicate that the use of native strains could improve commercial vaccine preparations, increasing bovine immune response.

**Key words:** *Clostridium*, 16S rRNA, sudden mortality, genetic variability

**INTRODUCTION**

The *Clostridium* genus are an aerobae, Gram-positive bacteria, bacilli, fermentative, catalase negative and negative oxidize. The *Clostridium* is ubiquitous and some species can be associated with defined geographic areas (Songer & Post, 2005). In the soil *Clostridium* diversity has been associated with geographic area, pH, climate, soil type and the presence of other microorganisms. The *Clostridium* has the capacity to adapt to different environmental conditions due to the possibility to sporulate (Gamboa et al., 2005).

*Clostridium* has more than 100 species known, but less than 20 are pathogenic species to the human and domestic animals. The *Clostridium* spp. pathogenic species have been classified in four groups, the first
includes the neuro toxic *Clostridium* (*C. tetani* and *C. botulinum*); the second group are his to toxic (*C. chauvoei, C. septicum, C. novyi, C. per fringens, C. sordellii, C. haemolyticum and C. novyi*); the third are grouped by their enter pathogenic action (*C. per fringens*) and finally, the fourth group integrated by the others *Clostridium* (*C. colinum, C. difficile, C. piliforme and C. spiroforme*) (Quinn et al., 2004).

All the *Clostridium* groups present on the soil are very important due to their pathogenic effect and can be responsible for huge economical losses in animal production because they can produce sporadic disease episodes, acute and sudden mortality in animals on set by the toxin effects delivered by *Clostridium* after they have been ingested by the animals or in infectious processes (Gamboa et al., 2005; Ortiz, 2000).

The aim of this work was to isolate, characterize biochemically and to identify the variation within 16S rRNA in native *Clostridium* obtained from soil samples belonging to ten different localities, with history of sudden mortality.

**MATERIAL Y METHODS**

**Sample soil and bacterial isolation**

The sample size, established by the methodology described by Otte, 1991 and De Blas et al., 1998 was defined as sampling objective for the totality of farms located in the area of influence, where the unit sampling was the farm (Otte, 1991; De Blas et al., 1998). Soil samples were taken from ten different farms located in Mariquita, Nemocón, Puerto López and Ubaté villages.

To isolate bacteria soil samples were taken from plot areas where cases of sudden mortality have been reported, the protocol for sampling soil was done according to Ferraris, (2005). We selected plots where dead animals by *Clostridium* or plots where dead animals were buried (30 samples by plot). With 1 kg of soil taken from a depth of 0-20 cm was represented the bacterial availability from soil, meaning 0000005 % of average weight of 1 ha (Ferraris, 2005).

**Bacterial culture**

The soil sample of 1g was diluted in 5 ml of saline solution, from this suspension 1,5 ml were taken and each one was heated at 60° C for 10 minutes, then were cultivated on Thioglycolate with cooked meat medium (100µl by tube) pre-reduced by heating 10 min in boiling water and incubated in anaerobic chambers with An aero Gen™ (Oxoid® Laboratories) for 7 days at 37° C. Subsequently were cultured on Blood agar plates (3.0%), and were incubated for 24 hours at 35°C,. once the film of swarming was observed a second culture was carried out in blood agar plates (4.0%), evaluating colony morphology (shape surface and edge) and the hemolytic evidence and their type (alpha, beta).

The colonies were culture don agar with egg yolk to evaluate the lecithinase activity (+) (alpha-toxine, phospholipase C) and lipase (+). Later, the bacteria was cultured on liquid Brain Hearth Infusion agar (BHI) and was incubated for 24 hours at 35° C. The biochemical characterization of the isolates was done using the API®20A methodology for identification of anaerobic bacteria (bio Merieux SA, Marcy l’ Etoile, France). Later the cultures were cryopreserved using 0.5 ml of glycerol and frozen at -70° C.

**16S rRNA sequencing**

The genomic DNA was extracted from cells in the mid-logarithmic growth phase and was purified by the use of FTA® cards and FTA® buffer (What man® International Ltd. United Kingdom) following the methodology described by Dobbs & Madigan, (2002); Rogers & Burgoyne, (2000); Smith & Burgoyne,(2004). The PCR amplification and DNA sequencing of the 16S rRNA genes was performed according to Vaneechoutte et al., (1996). Briefly, The bacterial DNA extracts and control were used to amplified a 1500 bp fragment with 0.5 µM primers 5’ -TGG CTC AGA TTG TAC CTT GTT ACG ACT TCA CCA CA (reverse) and 5’ - TAC CTT GTT ACG ACT TCA CCA CA (forward).

The PCR mixture (25 µl) contained bacterial DNA, PCR buffer (10mMTris/HCl, pH 8.3; 50 m MKCl; 2 mM MgCl2),200 µM of each dNTP and 1.0 U Taq polymerase (Promega). The mixtures were amplified for 35 cycles of 94 °Cfor 1 min, 50 °C for 1 min and 72 °C for 2 min, and final extension at 72 °C for 5 min, in a PCR equipment PTC 100 Termal Cycler (MJ Research). Five micro liters of each amplified product was electro phoresed in a 1.0 % (w/v) agarose gel with molecular size marker (Hyperladder II, Bioline®) in parallel. Electrophoresis in Tris/borate/EDTA buffer was performed at 100 V for 90 minutes. The gel was stained with ethidium bromide (0.5 µg ml–1) for 15 min, rinsed and photographed under UV light illumination.

The PCR products were gel-purified using the QIA quick PCR purification kit (QIA gen). Both strands of the PCR products were sequenced twice with an ABI 310 automated sequencer according to the manufacturer's instructions (Perkin-Elmer), using the PCR primers described above. The sequences of the
PCR products were compared with known 16S rRNA gene sequence

Gen Bank (http://www.ncbi.nlm.nih.gov) by multiple sequence alignment using the CLUSTAL W program (Thompson et al., 1994). Later the sequences were analyzed using MEGA software ver. 4 (s.kumar@asu.edu, Kimura, 1980; Tamura, Dudley, Nei, & Kumar, 2007), we employed two genetic distance measures, firstly the Kimura’s two parameter model was used (Kimura, 1980), which corrects for multiple hits, taking into account transitional and transversal substitution rates, while assuming that the four nucleotide frequencies are the same and that rates of substitution do not vary among sites (see related Gamma distance). Later Tamura’s 3-parameter model was used, which corrects for multiple hits, taking into account transitional and transversal substitution rates, while assuming that the four nucleotide frequencies are the same and that rates of substitution do not vary among sites (see related Gamma distance). Later Tamura’s 3-parameter model was used, which corrects for multiple hits, taking into account transitional and transversal substitution rates, while assuming that the four nucleotide frequencies are the same and that rates of substitution do not vary among sites (see related Gamma distance).

The stability of relationships was assessed by using a minimum of 1,000 bootstrap trees generated for each data set.

### RESULTS

A total of 24 Clostridium spp were isolated from the soil samples cultures during the 2-year study period. According to the biochemical characterization the 24 isolates were identified as Clostridium sordellii (41,7%), Clostridium glycolicum (12,5%), Clostridium hastiforme (12,5%); Clostridium botulinum (8,3%), Clostridium butyricum (8,3%); and with the same proportion were identified Clostridium chauvoei (4,3%), Clostridium limosum (4,3%), Clostridium septicum (4,3%) and Clostridium tertium (4,3%) (Table 1).

In the Puerto López locality (Meta department) was found the higher number of isolates (37,5%), followed by the Mariquita locality (Tolima department) (29,2%) and similar isolate numbers in the Ubaté locality (Cundinamarca department) (25%), finally the lower number of isolates was found in the Nemocón locality (Cundinamarca department) (8,3%). The isolates were correlated with precipitation and temperature levels (p<0.05). By 2007, the higher precipitation was shown in the Puerto Lopez locality with 2521,95 mm and temperature annual average of 26,6 °C. In Mariquita locality was showed an annual precipitation score of 2481,31 mm, with a temperature annual average of 24,6 °C. More low annual precipitation average (1183,57 mm) was displayed in the Nemocón locality and the annual average temperature was 17,69 °C. Finally in the Ubaté locality was found the lower annual average precipitation with 1041,37 mm and annual temperature of 13,43 °C.

The selected bacteria were used to obtain the sequence of a 1500 bp fragment from 16S rRNA gene (Collins et al., 1994). The 16s rRNA sequences of 34 strains of clostridia were determined by direct sequencing of PCR amplified rRNA gene products. These new sequences were aligned and compared with homologous sequences of 16S rRNA from 21 other clostridial and selected reference strain belonging to low G+C content gram-positive genera available from Gen Bank and were estimated the genetic differences between bacteria. The results were indicated in figure 1.

In the phylogenetic analysis were estimated the genetic similarity values, by means of Kimura measure (Kimura, 1980); and we found the lower distance values between C. sordellii (DOO5556) and C. sordellii (DOO5557) (0,0007) bacteria; C. sordellii (DOO5557) and C. botulinum (DOO5561) (0,0007) bacteria; C. sordellii (DOO5557) and C. sordellii, commercial strain (0,0140). On average, those bacterial strains displayed a sequence identity of 98,3% and were grouped in the first cluster, which were showed in the 98% of repeats after a 1000 resampling procedure by bootstrapping method. This first bacterial cluster also displayed a high similarity with C. difficile obtained from Gen bank bacterial sequence, which has integrate another clearly defined cluster and was showed in the 100% of repeats.

In the second group, lower distance was found between C. botulinum (DOO5560) and C.

### Table 1. Description of isolated Clostridium spp., from soil samples obtained in ten farms located in five different municipalities in Colombia.

<table>
<thead>
<tr>
<th>Municipality</th>
<th>Clostridium botulinum</th>
<th>Clostridium butyricum</th>
<th>Clostridium chauvoei</th>
<th>Clostridium glycolicum</th>
<th>Clostridium hastiforme</th>
<th>Clostridium limosum</th>
<th>Clostridium septicum</th>
<th>Clostridium sordellii</th>
<th>Clostridium tertium</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mariquita</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Nemocón</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Puerto Lopez</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Ubaté</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td>1</td>
<td>24</td>
</tr>
</tbody>
</table>
Botulinum (DOO5564) (0,000), where the identity between strains were 99.7%, indicating high similarity. Additionally the distance between these bacteria with C. botulinum bacterial sequence obtained from Gen Bank (ABDP0100008) was 0,003, with a sequence identity of 99.9% and 99.6% respectively; in the dendogram this group was displayed in the 90% of the repeats. Moreover the C. botulinum bacteria (DOO5558) showed anothercluster with a lossgenetic distance of (0,010) with C. botilunum bacteria (DOO5563), and were grouped within of this cluster and were observed in the 100% of repeats.

The third cluster was displayed between C. septicumcommercial strainand C. chauvoei (DOO5555) (0,000), where was found lower distance measure indicating near similarity; and also were shown a low distance with sequence of C. septicum obtained from Gen Bank (Accesion number AB558163) (0,037). Within this cluster also was found another group integrated by C. chauvoei commercial strain (0,007), which showed high similarity with a C. chauvoei sequence obtained from Gen Bank (accession number EU106372). Those bacteria displayed a sequence identity of 94.8 % with C. Septicum and 95.4 % with C. chauvoei respectively, integrate a clearly defined group in the 100% of the repeats.

Finally the sequences obtained from C. botulinum commercial strain and sequence of C. botulinum obtained from Gen Bank (Accession Number NC010723) displayed a lower genetic distances with this last group showed a genetic distance of 0.07 and 98% of sequence similarity between two C. botulinum strains and were grouped in this cluster in the 98% of repeats. Similar results were displayed, when was estimated the genetic similarity values by means of the Tamura method (Tamura, 2007).

**DISCUSSION**
The biochemical identification to *Clostridium* allowed us a more precise classification of the bacterial genus isolated however it has showed a low discrimination power, is expensive and time consuming due to great quantity of labor and material needed. In our work the biochemical characterization was combined with the molecular characterization, but was found than the bacteria classified as *C. sordellii*, *C. tertium*, *C. limosum* and *C. glycolicum* were found as *C. botulinum*. In the biochemical characterization, the bacterial strains were β hemolytic and some reports has found than this criteria as lipase or lecithinase could be an indication of pathogenicity (Hatayew 1988, 1990 and Smith 1968). However it is necessary to review the biochemical traits than included subjective evaluations being inaccurate their evaluation, therefore requires the use of tools with greater sensitivity and specificity. Since several years has been demonstrated the use of 16s rDNA to do the more quick diagnostic of the infections due to different *Clostridium* species. In spite of than the sequence of some regions of 16s rDNA gene have displayed homology in several bacterial strains, other regions have showed a considerable difference (Urtler et al., 1991), becoming a good alternative to identify the clinically important *Clostridium* strains.

**Clostridium sordellii**

In our study, the principal isolated bacteria was the *Clostridium sordellii* (41.7%), similar results were found in Costa Rica (Gamboa et al., 2005) where the *C. sordellii* (42 %) and *C. perfringens* (38 %) were the more frequently bacterial strains isolated in soils.

Were analyzed the 16s rDNA gene sequences from bacterial *C. sordellii* and *C. botulinum*, isolated from soil samples obtained at three different locations (*C. sordellii*, DOO5556, municipality of Ubaté, Cundinamarca; *C. sordellii*, DOO5557, municipality of Puerto López, Meta; Commercial Laboratory, Armero, Tolima and *C. sordellii* DOO5561, municipality of Ubaté, Cundinamarca) and displayed a sequence identity of 98,3%, indicating the prevalence of those bacterial strains across the regions. *C. sordellii* is a highly pathogenic strain to animals because can produce two principal virulence factors, the hemorrhagic toxins and the lethal toxins (LT), that are related with diarrhea and enterotoxaemia in domestic animals and caseous gangrene. Moreover, the exposition to hemorrhagic toxin can induce a hemorrhagic activity, while than the exposition to LT can cause severe edema (Just et al., 1996). The *C. botulinum* are compromised in food poisoning in bovine by means of exposition to neurotoxins C and D and their pathogenicity are characterized by outbreaks of mortality with clinical manifestations in nervous paralysis with motor paralysis of hind member (Ortiz & Villamil, 2008).

The *C. sordellii* bacterium was isolated from wounds infections in humans, later, the bacteria was involved as cause of death in cattle in Nevada, USA. Recently, fatal infection by *C. sordellii* has been observed in association with obstetric surgeries (Ramírez & Abel-Santos, 2010)

The *C. sordellii* strain isolated by a commercial laboratory in the Armero village, was obtained from an outbreak of mortality in the nineties and was included in a biological vaccine used to prevent disease. This bacterial strain was studied by DNA sequence, and was found a high similarity with the sequence of the *Clostridium sordellii* bacterium isolated in those geographical areas, indicating that the tropical strains have the same origin, those results are noteworthy by the contribution to determination of the ecology of this bacteria, which might be the main responsible of the bovine mortality in areas included in our study.

**Clostridium botulinum, Clostridium limosum and Clostridium glycolicum**

The *C. Botulinum* can be found in soil samples and acuaticse diments (Hauschild, 1989). By means of the neuro toxin C and D this bacteria can be compromised with the poisoning by foods in bovines, and can be concomitant with mortality outbreak swith clinical symptoms of the nervous and motor paralysis of hindlegs (Hatayew, 1988; Titball et al., 2003; Ortiz and Villamil, 2008). Their neuro toxins are their molabile and can be denature deasly.

The results of this study indicated that *C. botulinum* (DOO5560) and *C. botulinum* (DOO5564) (0,00077) have a high sequence identity, which allow us to deduce that have a similar phylogenetic origin, similar situation were found with *C. botulinum* (DOO5558) and *C. botulinum* (DOO5563) (0,000854).Despite demonstrate in our results the toxic effect found in our cultures it is necessary identify the toxicity peproduced by those bacteria sand analyze the sequence for their coding genes as BoNT (Urtler et al., 1991).

In the Mariquita municipality (Tolima department) and the Ubaté municipality (Cundinamarca department) the *Clostridium botulinum* was also isolated, confirming the presence and importance of this organism as the causative agent of food poisoning in cattle, associated with cases of sudden death (neurotoxins C and D), which had been demonstrated in the epidemic outbreak of mortality in the west
savannas in Colombia (Hateway, 1988, 1990; Ortiz, 2000).

In Brazil was conducted a study of isolation and characterization of \textit{C. botulinum} strains, isolated from soil and clinical samples. It was demonstrated that these areas were poor in phosphorus and that farmers replaced the native pasture grasses by exotic crops and they improved the genetic and reproductive management, but not were corrected the soil deficiencies (Bariloche, 2002). The Botulism epidemic occurs extensively in animals raised on pasture low in phosphorus without adequate mineral supplementation, due to the bovine habits of gnawing animal bones (alotriofagia), with higher incidence in the rainy season (Ortiz, 2000). The prevalence of the disease often affects gestants cows and / or lactation cows, rarely attacking calves and other animals even more rarely adults.

From the soil samples were isolated the 39\% of strains of \textit{C. botulinum}; the isolation of \textit{C. botulinum} samples type C and D had provided information to characterization of \textit{C. botulinum} responsible for bovine botulism in Brazil. The use of strains isolated in the country for the production of tetanus toxoid became a factor that improved rates of protection against field challenges (Bariloche, 2002).

In Colombia as a result of the outbreak of mortality in the 1990’s, was conducted a cross sectional epidemiological study to examine the problem of bovine mortality in the Colombian Orinoquia. It was deduced that the low content of calcium and phosphorus in tropical soils led to a condition called alotriophagia, which predispose to the animal to die of food poisoning by consuming the toxins produced by \textit{Clostridium botulinum} (Ortiz, 2000).

It has been found (Eklund \textit{et al.}, 1971; Govind \textit{et al.}, 2009) that some non toxic bacterial strain of \textit{Clostridium botulinum} and \textit{Clostridium difficile} is transformed into toxic because of bacteriophages that influence the gene regulation that induce to toxins production, which could happen in the bacteria found, so is necessary develop research studies in this area.

\textit{Clostridium chauvoei}

We also have isolated \textit{Clostridium chauvoei} bacteria, in Nemocón municipality, (Cundinamarca department). This bacteria cause the disease known as blackleg in bovine specie, characterized by fever, depression, lameness and high mortality rates.This bacterial strain showed a high sequence similarity with \textit{Clostridium septicum}, which also was isolated in the Mariquita municipality (Tolima department), which produce similar clinical symptoms to \textit{C. chauvoei}, indicating that these bacteria are playing an important role as causative agents of mortality found (Kuhnert \textit{et al.}, 1996). \textit{Clostridium chauvoei}, displayed a cross reaction with \textit{Clostridium septicum}, and both have a high immunogenic similarity (Kojima \textit{et al.}, 2000).

In Zambia (Africa), Mudenda \textit{et al.}, (2000), have realized a retrospective study of cattle diseases in extensive production and they found that in soils which was isolated \textit{C. septicum} and \textit{C. chauvoei}, there was presence of malignant edema and black leg in cattle. Were taken soil samples from 5 regions and was observed the presence of \textit{Clostridium} using conventional methodologies; isolated \textit{C. septicum}, \textit{C. novyi} and \textit{C. chauvoei}, which were characterized by direct immune florescence (IFA). The isolation of clostridia from soil was of great importance, because it was shown that in places where bacteria isolated were related to documented clinical cases in bovines.Therefore the soil analysis and its correct location was very useful to implement plans for vaccination and prevention, similar situation to that found in this study.

Kuhnert \textit{et al.} (1996), have obtained the sequences of 16S rDNA genes (genes rrs) of \textit{Clostridium chauvoei} and \textit{Clostridium septicum}, and was found a relationship between \textit{Clostridium}. They amplified a 1507 pb fragment located in the rrs gen; the sequence similarity analysis showed a phylogenetic relationship between \textit{C. chauvoei} and \textit{C. septicum} and was found a 99,3\% of similarity between bacterial strains for this gene.

Similar results were found in our paper, where the \textit{C. septicum} and \textit{C. chauvoei} both strains from commercial laboratory, have showed a high sequence identity (98,6\%), indicating a close phylogenetic relationship, but hinders the accurate identification of one them by conventional methods so that the techniques presented in our paper could be a good alternative.

The conventional methods for diagnostic of \textit{Clostridium} pathogens in clinical or food samples were based on bacterial culture (Peterson \textit{et al.}, 1996), cellular culture to evaluate the citotoxicity produced by these bacteria’s (Delmee \textit{et al.}, 2005), or mouse bioassay (Lindstrom \textit{et al.}, 2001). However in these assays are consuming a lot of time, because it takes several days to complete the procedure. As a result, these methods do not provide timely results affecting the performance or life of patients. Several studies have demonstrated the use of multiplex PCR for the \textit{Clostridium} spp detection (Janvilisri \textit{et al.}, 2010). Sasaki \textit{et al.}, (2000), has shown the use of PCR techniques to detect \textit{Clostridium chauvoei} DNA using primers derived from 16S rDNA and their results were displayed in the identification of 37 \textit{Clostridium} strains and 3 strains of other genus, suggesting than this test can be useful to identification of \textit{Clostridium chauvoei}. 

...
in culture mediums and animals clinically affected by blackleg. Subsequently determined the partial sequence of 16S rDNA (1465 pb) of Clostridium novyi type A, B and C and the Clostridium haemolyticum which were clustered with Clostridium botulinum type C and D. The sequence of 16S rDNA of Clostridium novyi type B and Clostridium haemolyticum were completely identical only were different by 1 bp (similarity level of 99.9%) from Clostridium novyi type C. Was found an homology of 98.7% with Clostridium novyi type A and shown a greater similarity in the sequence of 16S rADN from C. botulinum C and D. These results suggest that the types of C. novyi B and C and C. haemolyticum can be an independent specie generated from same phylogenetic origin (Sasaki et al., 2001).

**Other Clostridium**

Other types of Clostridium strain as Clostridium hastiforme, Clostridium butyricum, Clostridium tertium, were isolated in this work, many of whom are believed than have not pathogenic effect on Cattle. The distribution of the wide variety of Clostridium on soil is an indicator of high biodiversity present on the colombian soils, which can be associate with grazing animals so it is necessary to increase efforts to know the natural history of these bacteria and its pathogenesis. In our work, C. tertium produce no hemolysis but it have displayed citotoxicity in cell cultures, which help to provide evidence about of its toxic effect it could have acquired by plasmids or transposons. The pathogenic islands (PAIs) are bacterial DNA segments that carry one or more virulence genes, which can be acquired in bulk from an external source (Mainil et al., 2003). The pathogenic genome usually represents a mosaic between these new acquired islands and a relatively old DNA. The PAIs are identified as by their difference in the percentage of G+C content relative to the average chromosome and other features that suggest its acquisition through the mobile genetic elements.

Genes to a wide range of virulence determinants are associated with PAIs, including those that code for certain mechanisms that enable to resist the host defenses, colonization factors and acquisition of nutrient and toxins (Fernández et al., 2004). Other studies have indicated that Clostridium per fringes has a high degree of genetic interchange, a situation that allow the transfer of virulence factors and gives them the ability to produce different toxins as a result of the loss or gain of the specific genes. There are not large differences between different types of Clostridium were it not for the transportation of certain virulence genes.

The evidence indicates that some virulence factors encoded on plasmids can be transferred horizontally. The horizontal genetic movement is also facilitated by transposing (Deguchi et al., 2009; Morris et al., 2009), these evidence could allow explain why some bacteria can be toxics, situation that was showed with these three bacteria in Colombia.

**CONCLUSION**

The Clostridium spp pathogens isolated from soil of farms affected by bovine mortality, showed genetic differences with reference bacteria used by commercial laboratories for the production of bacterins and toxoids. In this work the biochemical characterization allowed us identified bacteria’s as C. sordelli, C. tertium, C. limosum and C. glycolicum and classified them as C. botulinum by ADN analysis, but the principally isolated bacteria in soil samples was C. sordelli, this bacteria is highly pathogenic in animals, producing a highly mortality in the Colombian herds. These results are consistent with observations in the countryside, where animals die despite being immunized. Local characterization studies are needed to know the natural history of bacteria associated with soils and develop strategies for prevention and control including such bacteria in the immunogen.

**ACKNOWLEDGMENTS**

This study was supported by the Research Division of Bogotá (DIB) and the fellowship program to Postgraduate outstanding students from Academic vice rector of National University of Colombia and The Colombian Agricultural Research Corporation (CORPOICA). Additionally, we want express the gratefully with the Universidad de la Salle by the valuable cooperation.

**REFERENCES**


http://www.elsitioagricola.com/


