THE 16S r-DNA RFLP PROFILE OF TOTAL DNA OF INTESTINAL BACTERIA UNDER PATHOLOGICAL CONDITIONS

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ABSTRACT

The study of complex microbial communities by culture-independent techniques has improved the global knowledge of the microbial ecology of complex ecosystems. Differences in the positions of cleavage sites for restriction enzymes in a universal gene, as 16S r-DNA, are a powerful tool to analyse the microbial ecology of complex microbiota, as gut systems are, and a good method to analyse bacteria that are difficult to culture. The aim of the present study was to compare the intestinal bacteria communities present in the gut of healthy or sick rabbits, by restriction fragment length polymorphism (RFLP). Total DNA was extracted from the intestinal contents, collected in ethanol, by the QIAamp DNA Stool Mini Kit (QIAGEN), with some modifications. A fragment of the bacterial 16S r-DNA gene was amplified by PCR with CTACGGGAGGCAGCAGT and CCGTCWATTCMTTTGAGTTT primers and digested with five different restriction enzymes (*Alu* I, *Rsa* I, *Hpa* II, *Sau* 3A I or *Cfo* I). Some intestinal disorders are characterised by a disbiosis, with an increment in the number of the bacterium that cause the characteristic symptomatology of the disease. Divergences in 16S r-DNA sequences between bacterial species can produce specific RFLP profiles because of differences in the cleavage sites of the restriction enzymes. With this method we have observed some RFLP profiles characteristic of different digestive bacterial disorders as Epizootic Rabbit Enteropathy, colibacilosis, clostridiosis or antimicrobial disbacteriosis. The construction of a data base with the RFLP profiles associated with different diseases will be a useful instrument to assist the laboratorial diagnostic in rabbit pathology.

Key words: RFLP, gut bacteria, intestinal disorders, Epizootic Rabbit Enteropathy, colibacilosis, clostridiosis.

INTRODUCTION

Many intestinal disorders are characterized by a microbial disbiosis, with an increment or a reduction of bacterial counts. In other instances the gut disorders are caused by the direct, or the indirect, action of endo- or exo-toxins produced by the causal microorganisms.

In the past, the studies of the changes in the microbial components of the gut were centred in the microbial count, or in the microbial isolation, by classical microbiological methods. But the introduction of new molecular techniques, and specially the amplification of DNA by PCR (polymerase chain reaction), allowed the possibility of cutting off the use of *in vitro* cultures and to broaden the study of the intestinal changes to uncultured microbiota or to microbiota difficult to culture. To conduct some of these molecular studies, specific primers that amplify some particular microorganisms or specific molecular probes that detect a singular microorganism could be used.

Nevertheless, the amplification of a universal gene, as the gene that encodes for the 16S ribosomal-RNA (16S r-DNA) is, could be a useful method to systematically analyse the profile of microorganisms present in the gut of the animals with intestinal disorders. The 16S r-DNA gene is located in the chromosome of all the prokaryotic cells, with the exception of some anaerobic protozoa located in the rumen of polygastric animals, and perhaps in the caecum of rabbits.

The 16S ribosomal-RNA molecule has two additional characteristics, related with its primary structure, that confer its potentiality in the study of complex microbiota: it has some highly conserved areas at the same time that some hyper-variable areas. The first characteristic allows for the use of some universal primers that lead to the amplification of a high percentage of microorganisms. The second characteristic provides the resolution between microorganisms, especially by changes in the cleavage sites of the restriction enzymes.

The combination of DNA amplification of the 16S r-DNA, digestion with restriction nucleases of amplified DNA, and analysis by agarose gel electrophoresis offers an excellent system to study the composition of microbial gut components without the necessity of *in vitro* cultures.

MATERIAL AND METHODS

Sample collection

Approximately one gram of ileal or caecal samples were collected in a sterile plastic tube that contains 3 mL of 98% molecular biology grade ethanol. These tubes were maintained at 4ºC until use.

Ileal and caecal samples from healthy or sick rabbits were collected.

DNA extraction

Two hundred and twenty milligrams of gut contents were processed for total DNA extraction using the QIAamp DNA Stool Mini Kit (Qiagen Inc., Chatsworth, Calif.) system, in accordance with the instructions of the manufacturer with two additional steps of lysozyme and proteinase K, that implement the DNA extraction of some microorganisms. The purified DNA was maintained at -20ºC until use.

PCR amplification and restriction enzyme digestion

Two primers 5'-CTACGGGAGGCAGCAGT-3' y 5'-CCGTCWATTCMTTTGAGTTT-3' designed for regions of the 16S rRNA gene highly conserved among a wide range of microorganisms (LANE, 1991), were synthesized at Sigma-Genosys.
To prepare the PCR reaction, PCR-Master Mix (Applied Biosystems), with 1.25 IU of *Taq* polymerase, 50 ng of DNA template, 0.2 µM the preceding primers, and distilled water in a total volume of 50 µl were used.
PCR mixtures were heated to 94°C for 5 minutes once, followed by 35 cycles of denaturation at 94°C for 1 minute, primer annealing at 45°C for 1 minute, and DNA extension at 72° for 1:15 minutes. The last extension cycle was continued for 5 minutes. The PCR amplification reaction was conducted in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems).
In general, PCR amplification products were processed just after amplification, but they were maintained at -20ºC until use in other case.
The DNA fragments amplified by PCR were digested with *Alu* I, *Rsa* I, *Hpa* II, *Sau* 3A I or *Cfo* I restriction endonucleasas (Sigma-Aldrich) in accordance with manufacturer specifications.

DNA electrophoresis and image analysis

The restriction endonuclease fragments were resolved using a 2% wide range agarose electrophoresis, supplemented with ethidium bromide. For electrophoresis 150 V during 60 minutes were applied. The bands of DNA were visualized in an UV Chemigenious Image System (SynGene) using the GeneSnap software (SynGene). Pictures with 4.63 seconds exposure were stored. The electrophoretic profiles obtained are known as Restriction Fragment Length Polymorphism (RFLP) and are highly characteristic of microbial genera, and in some cases, of microbial species.
Two molecular weight controls were used: Step Ladder, 50 bp (Sigma-Aldrich) and our own control prepared by digestion of amplified fragments of 16S r-DNA of well characterized animal bacteria. The sizes of all the bands obtained were calculated with the aid of the GeneTools software (SynGene).
With the information resulting from the relative size of the RFLP bands, the information stored in the "SSU_Una.gb" file from the Ribosomal Database Project (Maidak *et al.,* 1997), and a specific software, developed in our Institute, we can identify the genera, and in some cases the species, of the suspected microorganisms involved in the aetiology of the disease.

RESULTS AND DISCUSSION

Epizootic Rabbit Enteropathy by RFLP profiles

Our research on Epizootic Rabbit Enteropathy (ERE) points out that this lethal disease is identified by an specific disbiosis characterized by a significant reduction in the number of *Escherichia coli* present in the gut, and an increment in the population of some Gram-

positive and some Gram-negative bacteria. These changes bring out a specific profile, in many cases characterized by a significant increment in the intensity of two RFLP bands.

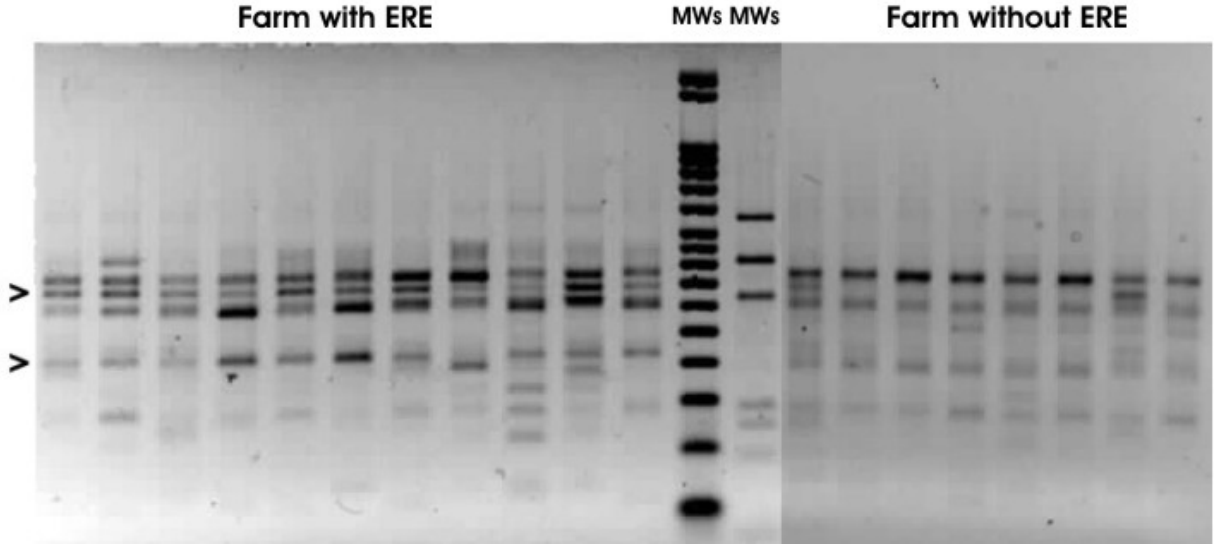


Figure 1: RFLP profiles obtained from caecal samples of ERE infected or non-ERE infected rabbits from different farms. With a > mark are located the bands that are significantly related with the Epizootic Rabbit Enteropathy. (MWs= DNA molecular weight controls: Step Ladder-50 bp from Sigma, and CReSA's DNA molecular weight control).

At figure 1 it can be seen a band of approximately 350 bp, and a band of 210 bp, which are present in almost all the samples of animals from farms clinically affected by ERE, and are absent in almost all the samples of animals from farms clinically non-affected by ERE.

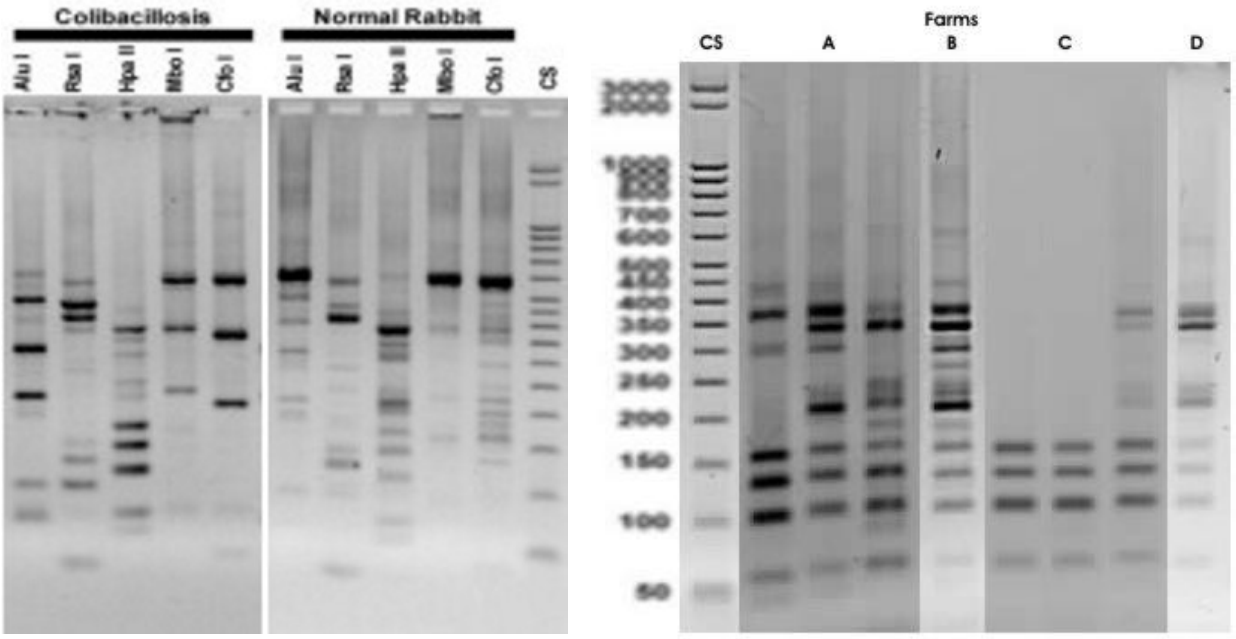
Initially we believed that some of the microorganisms related with these electrophoretic bands were involved in the aetiology of the disease, but the results obtained after inoculation of the bacteria, to healthy or sick animals, point out this microorganism as a reaction to the aetiological agent of ERE and as a possible probiotic that could be used for the control of the disease. More studies on this possibility are conducted at present time.

Rabbit colibacillosis by RFLP profiles

The preliminary cross-analysis of RFLP profiles with the information located in the SSU_Una.gb file from the Ribosomal Database Project, has lead us to the conclusion that the 16S r-DNA genes of *Escherichia coli* of rabbits are clearly different to other 16S r-DNA genes of *E. coli* deposited in the Database. Because that, we introduced in our Database new RFLP fragments corresponding to the strains of *E. coli* isolated from rabbits.

With these new RFLP profiles, characteristics of rabbit's *E. coli*, we can observe that gut samples obtained from colibacillosis were clearly different to samples collected from normal rabbits or from rabbits infected by other microorganisms.

The Figure 2 shows the RFLP profiles obtained from the samples collected from healthy rabbits and from rabbits with colibacillosis.



A B

Figure 2: (A) Example of RFLP profile of a rabbit with intestinal colibacillosis and a RFLP profile of a healthy animal with the five restriction enzymes used. (B) Some RFLP profiles from field samples, obtained with *Hpa* II restriction endonucleasa, clinically compatible with intestinal colibacillosis. (CS= DNA control standard: Step Ladder-50 bp from Sigma).

In the samples collected from colibacillosis we can observe some electrophoretic bands that have greater intensity than the corresponding bands in the sample collected from normal rabbits. The bands obtained from the strain isolated from the colibacillosis sample superpose their bands with the bands with increased intensity in colibacillosis. Furthermore, it is possible to observe that some bands were more intense in normal rabbits than in rabbits with colibacillosis, indicating a reduction of some normal components by clinical colibacillosis (disbiosis).

Rabbit clostridiosis by RFLP profiles

In spite of we have more RFLP profiles, the lack of space for this communication force us to select a final RFLP profile related with clostridiosis. This RFLP profile is not associated with a clear disbiosis, with an increment of bands related with the aetiological microorganism and a reduction of bands related with normal components of intestinal microbiota. In this case, and because the clinical signs are related with production of exotoxins, a significant increment in the number of bacteria is not essential.

In the Figure 3 we can observe the RFLP profile obtained from a clostridiosis sample and the RFLP profile obtained from the *Clostridium sordelli* isolated.

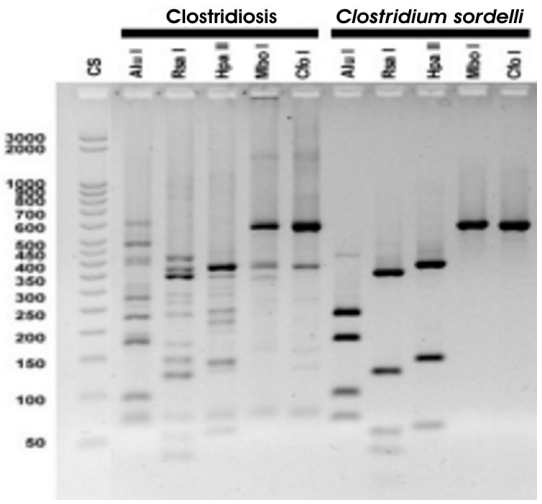


Figure 3: RFLP from rabbit clostridiosis samples and C.sordelli strain. (CS= DNA control standard: Step Ladder-50 bp from Sigma).

CONCLUSIONS

RFLP profiles could be a valuable system to analyse the microbial components involved in gut disorders of rabbits. This method is independent from the culture conditions and, because that, useful to analyse microorganisms with difficult *in vitro* growth. The reproducibility of RFLP profile is very high and is possible to construct a database of RFLP profiles which could be useful at diagnostic laboratories.

ACKNOWLEDGEMENTS

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