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Identification of meat-isolated Enterobacteriaceae by an *In silico*-ARDRA approach

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Abstract: A collection of 20 complete and published sequences were obtained from the NCBI Genbank. Then, an « electronic PCR » was realised for the sequences using the universal primer set S-D-Bact-0008-a-S-20 and S-D-Bact-1495-a-A-20 which target the conserved regions of the 16S ribosomal DNA gene. The *In silico* enzymatic restriction of amplified products by 180 restriction enzymes allowed the selection of *Alu*I, *Msp*I, and *Rsa*I as the most discriminative restriction enzymes. Sixty isolates were collected from 10 different meat samples (chicken, turkey hen, sheep, pig, dromedary, cow, horse, fish, and ostrich). Preliminary morphological identification delimited the range of the concerned genera. Thus, the studied strains were assigned to one of the following genera: *Klebsiella, Enterobacter, Salmonella, Proteus, Escherichia, Yesinia* and *Shigella* spp. Enzymatic restriction of the 16S rDNA gene of the isolated strains using the same enzymes chosen for the theoretical restriction gave 12 ARDRA groups. Comparison of theoretical and experimental ARDRA profiles, using length and number analyses of the restricted fragments, confirm and identify each strain at the genus level, and sometimes up to species level. Epidemiological investigations can optimally be performed by the ARDRA technique, employing restriction enzymes designed electronically through public databases.

Keywords: 16 rDNA, ARDRA, Enterobacteriaceae.

Abbreviations: ARDRA - Amplified Ribosmal DNA Restriction Analysis; rDNA - ribosomal Deoxyribonucleic acid; mM - millimolar; PCR - Polymerase Chain Reaction; tpm - Tour per minute; µl - Microliter; h - Hour; M - Molar; TE - Tris-EDTA; min - Minute; Tris - Trihydroxymethyl aminomethane; EDTA - Ethylenediaminetetraacetic acid.

تحديد هوية الباكتيريا المتواجدة فى اللحوم باستخدام طريقة التقطيع الانزيمي الالكترونية

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مختبر الاحياء الدقيقة، قسم البيولوجيا، كلية العلوم بتونس ، المركب الجامعي 2092 تونس ، الجمهورية التونسية.
قسم جراحة المسالك البولية ، جراحة المسالك البولية لدى الاطفال والذكورة ، جامعة جيسوس ليبج – غيسن ، المانيا.

ملخص: تم تجميع 20 قطعة مادة وراثية كاملة ومنشورة من بنك الجينات NCBI . ثم ، قمنا بعملية تفاعل البلمرة المتسلسل الالكترونية وذلك باستخدام primers التالية S-D-Bact-0008-a- S-20 و S-D-Bact-1495-a- A-20 والتى تستهدف المنطقة المحفوظة للجين rDNA وقد تم اختيار ثلاثة انزيمات من بين 180 لخصائصها التمييزية بين مختلف القطع الوراثية هذه الانزيمات هي Alul, Mspl و Rsal تم عزل ستين بكتيريا من 10 انواع من اللحوم المختلفة والتي شملت الدجاج والدجاج الرومي والاغنام والخنازير والابل والبقر والخيول والاسماك والنعام . التحديد الاولي للاجناس اعتمد على البنية المورفولوجية وتم حصر الاجناس فى واحدة من التالية *Klebsiella, Enterobacter, Salmonella, Proteus, Escherichia, Yesinia and Shigella* spp. التقطيع الانزيمى للجين rDNA لمختلف البكيريا المعزولة و مقارنته بالتقطيع الانزيمي النظري وذلك مع استخدام نفس الانزيمات المذكورة سابقا وبعد القيام بعملية التجميع ARDRA انتجت 12 مجموعة . المقارنة بين تقنية ARDRA العملية والنظرية مكنتنا من تأكيد هوية كل سلالة علي الصعيد جنس، واحيانا تصل الى مستوى الانواع، يمكن استخدام هذه التقنية فى تحديد هوية البكتيريا المتسببة فى الامراض الوبائية.

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Introduction

The Enterobacteriaceae are Gram-negative bacteria, most of which are mobile with polar flagella, they grow easily on the usual media in 24 h at 37°C aerobically and anaerobically, their nutritional requirements are generally reduced, most of them multiply in synthetic medium with a simple carbon source like glucose (Brenner, 1980). On the biochemical species of this family are generally oxidase negative, reduced nitrate to nitrite with the exception of some species of *Erwinia* and *Yersinia*. The name of Enterobacteriaceae was given because these bacteria are usually normal or pathological host, following the microbial species of digestive tract of humans and animals (Williams, 1965).

Many members of this family are a normal part of the microbial community found in the intestines of humans and other animals (Hormaeche and Edwards, 1960), while others are found in water or soil, or are parasites on a variety of different animals and plants. *E. coli*, is one of the most important model organisms, and its genetics and biochemistry have been closely studied.

The accurate identification of Enterobacteriaceae and other glucose-fermenting and nonfermenting gram-negative bacilli has been the subject of many hundreds of publications over the years (O'Hara et al., 1965). The use of commercial kits, either manual or automated, to identify these organisms is common practice. Some species are difficult to identify with phenotypic and biochemical identification schemes commonly used outside reference laboratories. 16S ribosomal DNA (rDNA)-based identification of bacteria potentially offers a useful alternative when phenotypic and biochemical characterization methods fail (Michel et al., 2000).

The aim of this study was to identify environmental isolated strains (meat) by an *In Silico*-ARDRA (amplified rDNA restriction analysis) approach.

Material and methods

Sample collection and preparation

Food samples (n = 15), including ground beef, packed pork sausage, retail packages of beef steak, pork chops, hamburger, fish and products from turkey and chicken origin, were randomly collected from retail stores of five supermarket chains in Tunis and from naturally-raised animals (at a farm in Sidi-Thabet, Tunis, Tunisia) (Table 2) (Messaoudi et al., 2009). Sampling visits were made one day per week for 6 months (September 2005 to February 2006). Eight prepackaged raw meat products (two of each meat type) were randomly selected and transported on ice to the laboratory. Each sample was aseptically removed and placed in a plastic bag that contained 200 to 500 ml of buffered peptone (Difco Laboratories, Detroit, Mich.), depending on the sample size. The bag was shaken manually for 3 min and left on ice for 20 min.

The collected meat samples were analyzed as follows: 11 g of each sample was suspended in sterile peptone and then selenite broth at 37°C for 24 h. According to the standard dilution method, 1 ml from each concentration was plated onto Hektoen and MacConkey agar plates and incubated at 37°C for 24 h (Nataro et Kaper, 1998) (Le Minor, 1986). Colonies suspected to resemble *Enterobacter* and *Klebsiella* spp. (on the basis of colony size and morphology) were selected for identification and further studies.

Morphological characterization of isolates

Culture on Hektoen agar media

Hektoen is a selective and differential agar primarily used to recover Salmonella and Shigella from patient

specimens. Hektoen contains indicators of lactose fermentation and H_2S production; as well as inhibitors to prevent the growth of Gram-positive bacteria. The pattern of lactose fermentation and H_2S production aids in the organism identification. Table (1) summarizes the different types of colonies observed on Hektoen medium (Nataro et Kaper, 1998).

Table 1. Colonies observed on the Hektoen agar media.

Colonies with Salmon color	Colonies with Salmon color and black center	Colonies blue-green with black center	Colonies blue-green or green
Escherichia	*Citrobacter*	*Salmonella*	*Shigella* ou
Levinea	*Proteus vulgaris*		
Citrobacter			*Salmonella*
Klebsiella			
Enterobacter			
Serratia			
Yersinia			

Table 2. Origin of strain.

Meat type	Origin	Number of isolates
Lamb's meat	Reared animals	**1**
Meat of ostrich	Carrefour*	**1**
Meat of turkey	Mliha*	**1**
Sausage of Frankfort	Mazreaa*	**3**
Chicken	Mliha*	**3**
Chicken	Mazreaa*	**4**
Scallop of turkey	Mazreaa*	**5**
Chicken	Pinda*	**2**
Meat of turkey	Carrefour*	**4**
Meat of pigs	Carrefour*	**3**
Meat of dromedary	Carrefour*	**3**
Sheep	Reared animals	**2**
Fish	Sea bream	**0**

(*) Meat supermarket

Culture on Mac-Conkey agar media

MacConkey (also McConkey) agar is a culture medium designed to grow Gram-negative bacteria and stain them for lactose fermentation. By utilizing the lactose available in the medium, lactose fermenting bacteria such as *E. coli*, *Enterobacter* and *Klebsiella spp*. will produce acid, which lowers the pH of the agar below 6.8 and results in the appearance of red/pink colonies. Non-lactose fermenting bacteria such as *Salmonella*, *Proteus* and *Shigella spp*. cannot metabolize lactose, and will use peptone instead. This forms ammonia, which raises the pH of the agar, and leads to the formation of white/colorless colonies (Le Minor, 1986).

Chromosomal DNA extraction

Chromosomal DNA extraction was carried out as described by Chen and Kuo (1993) and Birnboim and Doly (1979). After an overnight culture at 37°C on MacConkey agar medium, colonies were suspended in 1.5 ml of Luria broth

medium (Difco Laboratories, Detroit, Mich) and incubated at 37°C for 24 h. The broth was then centrifuged (12,000 tpm during 3 min at 4°C) and the supernatant suspended into 200 µl of extraction buffer (Tris acetate [pH 7.8]; 40 mM, sodium acetate; 20 mM, EDTA [pH 8]; 1 mM and SDS 1%). The cell detritus were removed by adding 66 µl NaCl 5 M and centrifugation at 12,000 tpm for 10 min at 4°C. The supernatant was removed and was then treated with an equal volume of phenol-chloroform (1:1). The aqueous phase was discarded by 2.5 volume of chilled absolute ethyl alcohol and two washings in ethyl alcohol 70% (v/v). DNA was purified by TE ((Tris-HCl [pH 7.4]; 10 mM, EDTA [pH 8]; 1 mM) and stored at - 20°C.

Amplification of 16S rDNA gene

Amplification of 16S rDNA gene was carried out with universal primers S-D-Bact-0008-a-S-20 and S-D-Bact-1495-a-S-20 (Wheeler et al., 1996) (Figure 2). Each reaction tube contained 0.5 µM of each primer, 50 mM Tris-HCl (pH 9.0), 50 mM KCl, 7 mM MgCl2, 2 µl of purified DNA, 100 mM deoxynucleoside triphosphate, and 0.5 U of *Taq* polymerase (Applied Biosystems, Milano, Italy) and was adjusted to a total volume of 50 µl. Samples were amplified in a Biomed model 60 thermal cycler (Bio-Rad, Hercules, CA) programmed for a first cycle of denaturation (10 min at 94°C).

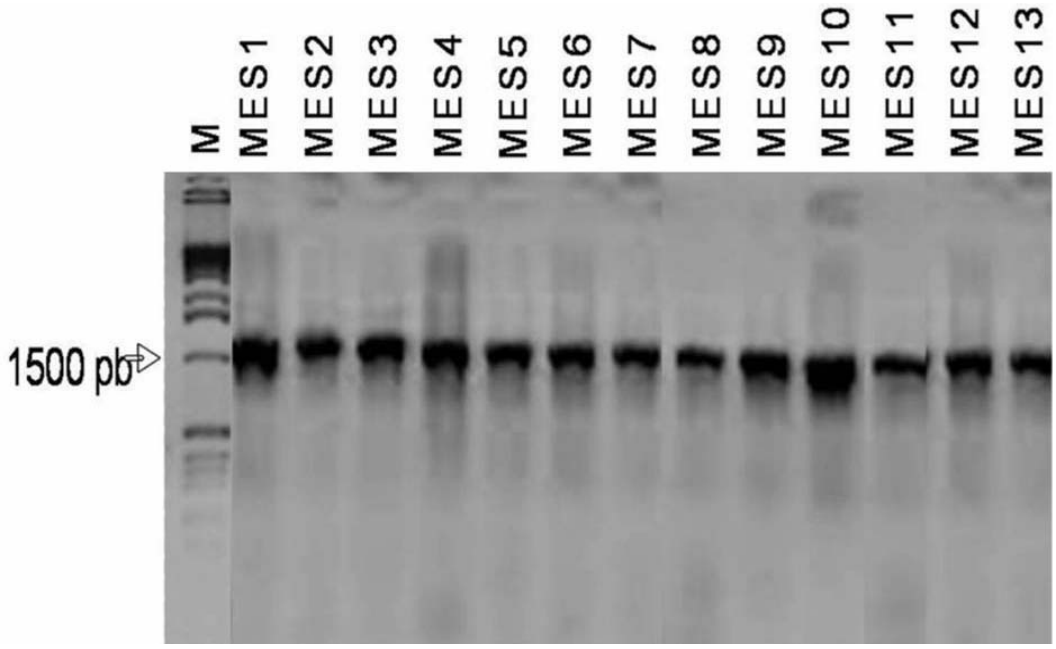


Figure 2. Amplification of rDNA 16S gene by polymerase chain reaction and resolved on 1,5% polyacrylamide gel. M denote the 1 bk DNA ladder.

The 35 subsequent cycles of amplification consisted of denaturation for 1 min at 94°C, annealing for 1 min at 40°C, and extension for 1 min at 72°C, with a final extension step of 10 min at 72°C. The products were electrophoresed on a 1.5% agarose gel and viewed by ethidium bromide staining.

Enzymatic restriction of 16S rDNA

ARDRA is a restriction fragment length polymorphism pattern of polymerase chain reaction amplified 16S rDNA gene (Heyndrickx et al., 1996). We applied ARDRA to rDNA 16S gene of isolated strains using *Rsa*I, *Alu*I, and *Msp*I enzymes. The final reaction was carried out in a final volume of 10 µl. 16 S rDNA

products were digested in an overnight mixture reaction in the presence of 1 µl of the appropriate buffer, 1 µl of the amplified DNA and 8 µl of restriction enzymes (5-10 U / µl): *Alu*I, *Msp*I and *Rsa*I, and resolved on 6% polyacrylamide gel (Difco Laboratories, Detroit, Mich) (Figure 3a, 4a and 5a).

Data analysis

GenBank database

We searched the sequences of 16S rDNA of different species of Enterobacteriaceae studied in the genomic bank: GenBank (www.ncbi.nlm.nih.gov) (Dennis et al., 2008).

DNAMAN

DNAMAN is a one-for-all software package for molecular biology applications. This package provides an integrated system with versatile functions for high efficiency sequence analysis. DNAMAN carries out all these tasks: restriction analyses, multiple sequence alignment, designing PCR primers, protein sequence analysis and drawing plasmids (http://www.lynnon .com/).

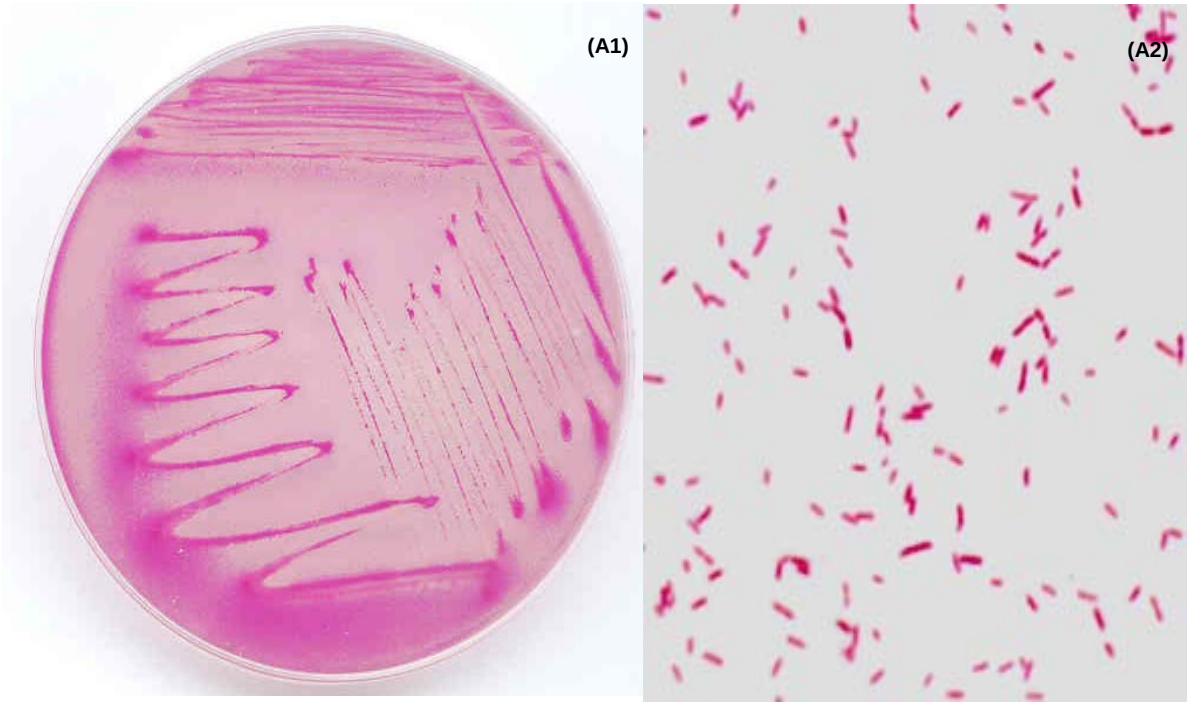
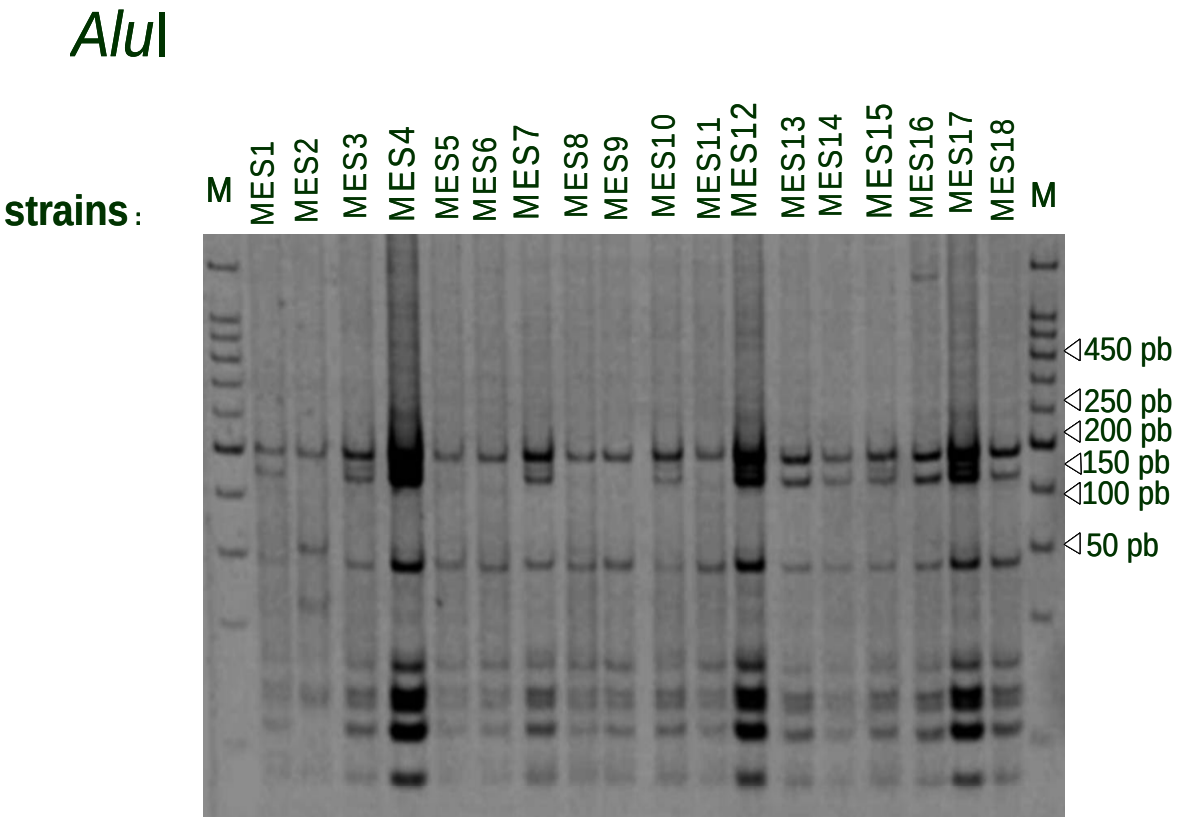


Figure 1. Culture of isolates on Mac Conkey media and microscopic observation after Gram staining (X 1000). (A1): Culture of isolates on MacConkey agar; **(A2):** Microscopic observation of cells shows Gram negative bacteria.

(A) Experimental ARDRA profile



(B) Simulated ARDRA profile

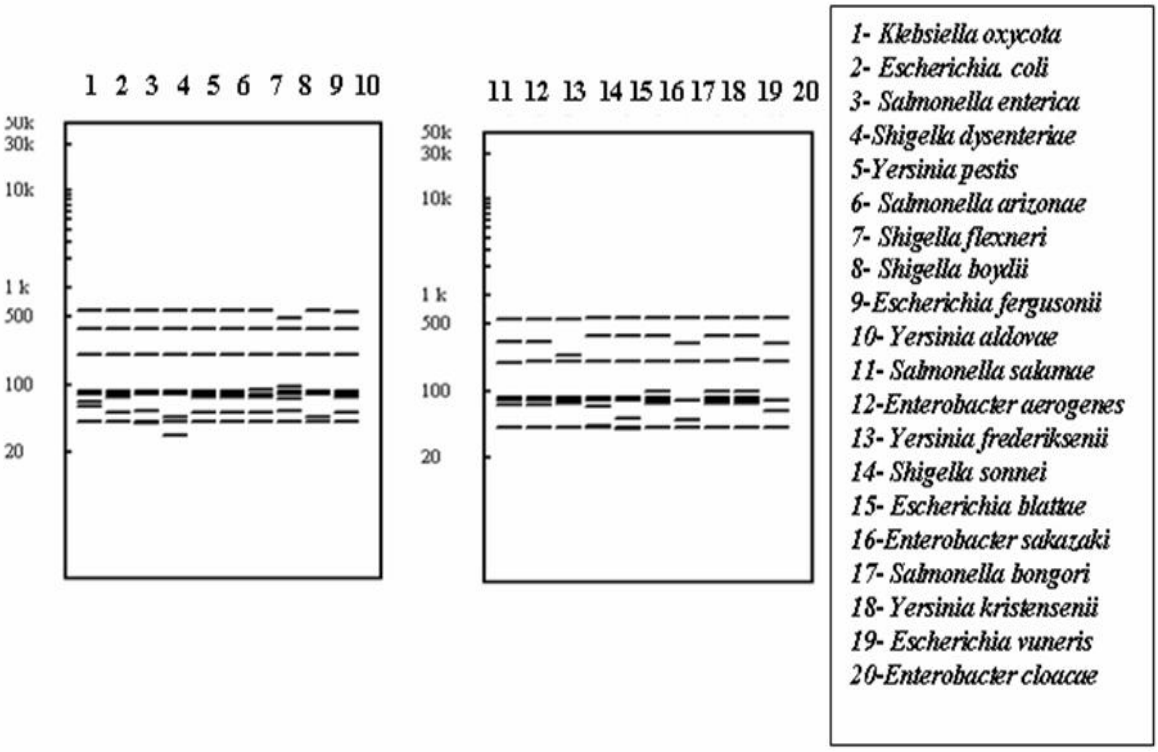


Figure 3. Experimental (A) and simulated (B) profile of 16S rDNA gene digested by the *Alu*I enzyme and resolved on polyacrylamide gel. M denote the 100 bp DNA ladder.

DNAMAN (5.2.10 version) software was used to select restriction enzymes to generate restriction profiles after the simulation of ARDRA technique.

Using GenBank database a collection of 16s rDNA sequence belonging to different genus of Enterobacteriacea family was generated. Then sequences obtained were digested using restriction enzymes for the simulation of the Restriction Fragment Length Polymorphism technique. This simulation was made by the software DNAMAN. At first these sequences were amplified and saved in the database software, thereafter we simulated the restriction of all these sequences by the restriction enzymes contained in the database software Restrict.enz (180 enzymes), in order to select the more discriminative restriction enzymes.

Gel pro

Band profiles of electrophoresis gels were analyzed using Gel pro software (for Windows, N/T version 3.1, 95). MVSP software was used to estimate levels of similarity between strains based on ARDRA profiles (version 3.13I, Kovach, on 1995).

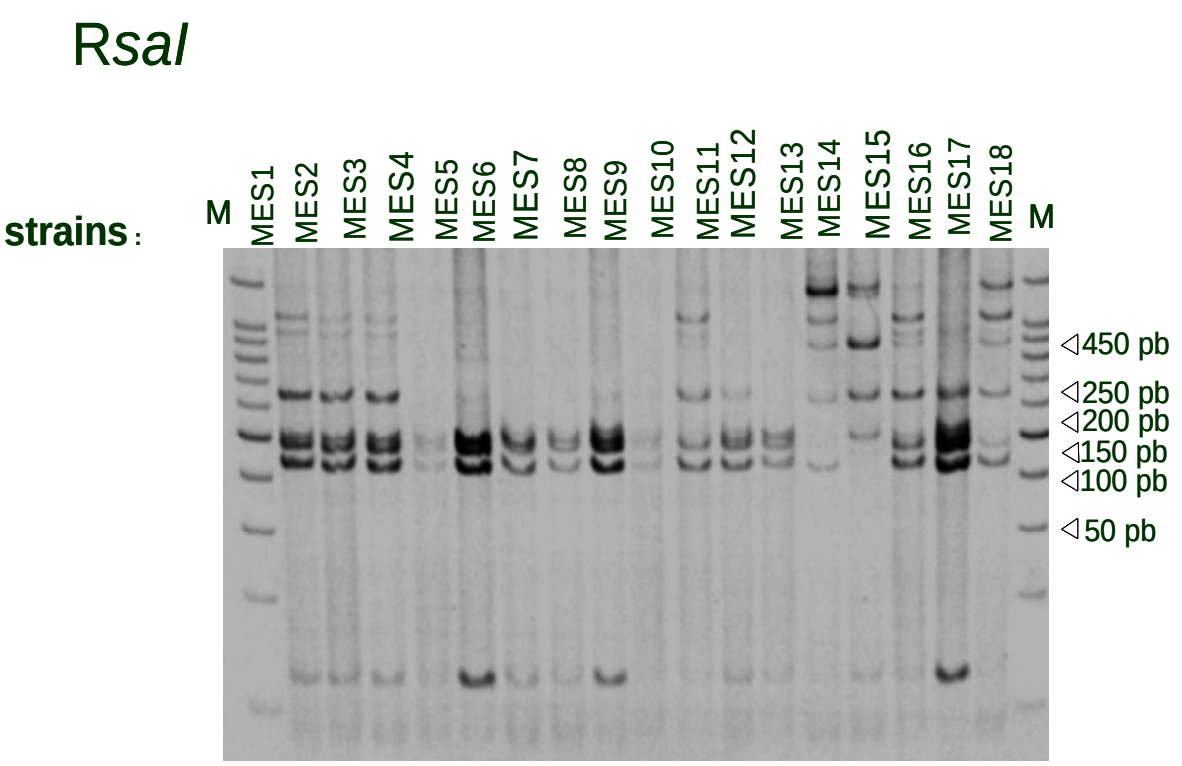
All bands acquired by the three restriction enzymes (theoretical and experimental bands) were treated by Gel - pro software to generate a matrix containing binary data. The clustering of strains was performed according to the Unweighted Pair Group Method with Arithmatic Mean method using MVSP software.

Results and discussion

On the basis of morphological criteria, 47 isolates were selected from 10 meat samples from different environmental origins (Table 1). Strains were predominantly isolated from the samples of meat of fowl and sheep. The microscopic observation shows that all isolates were Gram-negative bacilli (Figure 1).

Morphological identification showed a higher proportion of strains of *Yersinia*, *Enterobacter* and *Salmonella spp*. This result agrees with studies of Michael et al. (1981) which focused on the biochemical identification of a collection of 2.200 isolates belonging to the family Enterobacteriacea isolated from various meat samples were phylogenetically closely related. Results showed that *Alu*I, *Msp*I and *Rsa*I are the more discriminative restriction enzymes. After restriction of amplified DNA by selected endonucleases, the fragments generated are separated by size in an electrophoresis gel (Figure 3b, 4b and 5b). Based on their ARDRA profiles a dendrogramme exhibiting 12 groups with 47 strains was obtained (Figure 5).

(A) Experimental ARDRA profile



(B) Simulated ARDRA profile

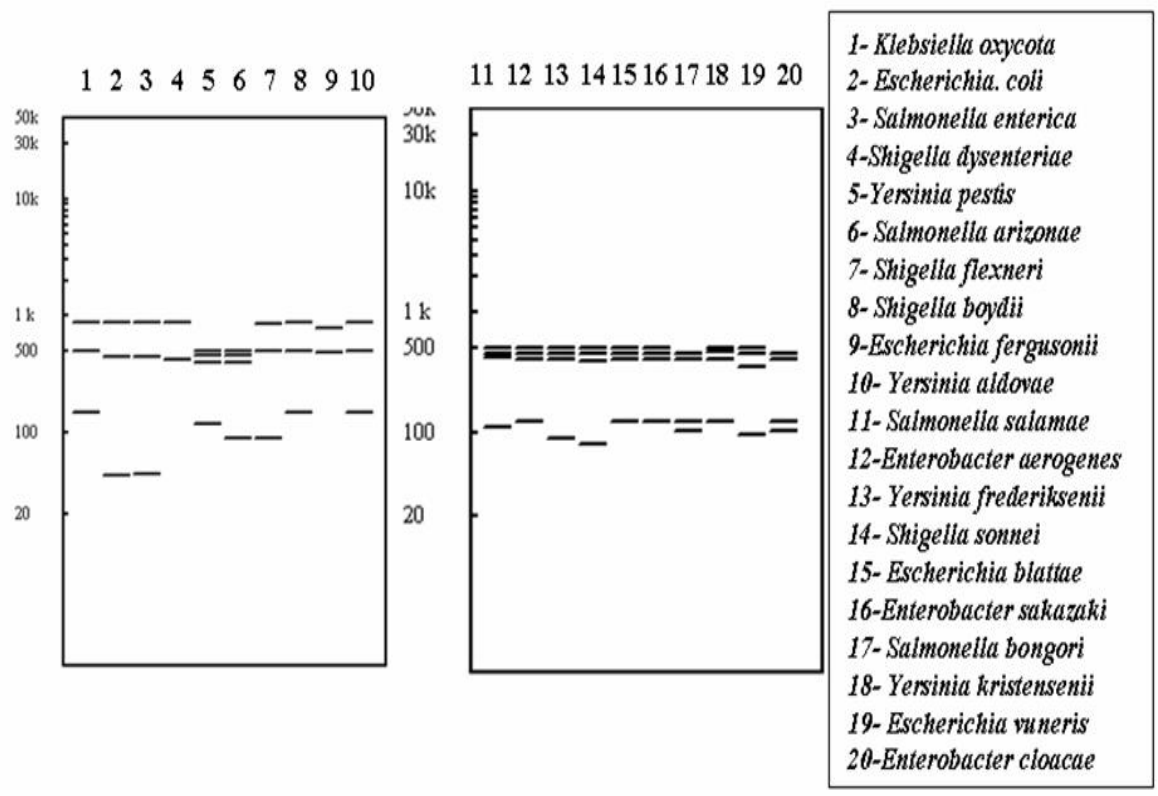
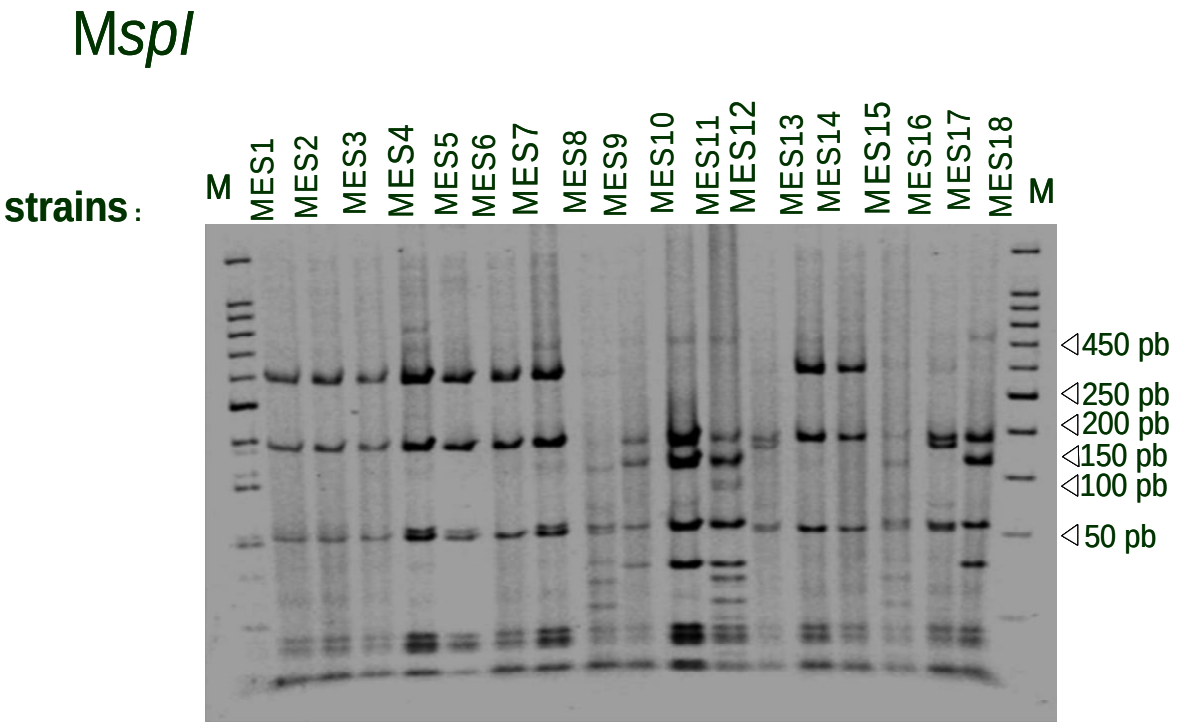


Figure 4. Experimental (A) and simulated (B) profile of 16S rDNA gene digested by the *Rsa*I enzyme and resolved on polyacrylamide gel. M denote the 100 bp DNA ladder.

(A) Experimental ARDRA profile



(B) Simulated ARDRA profile

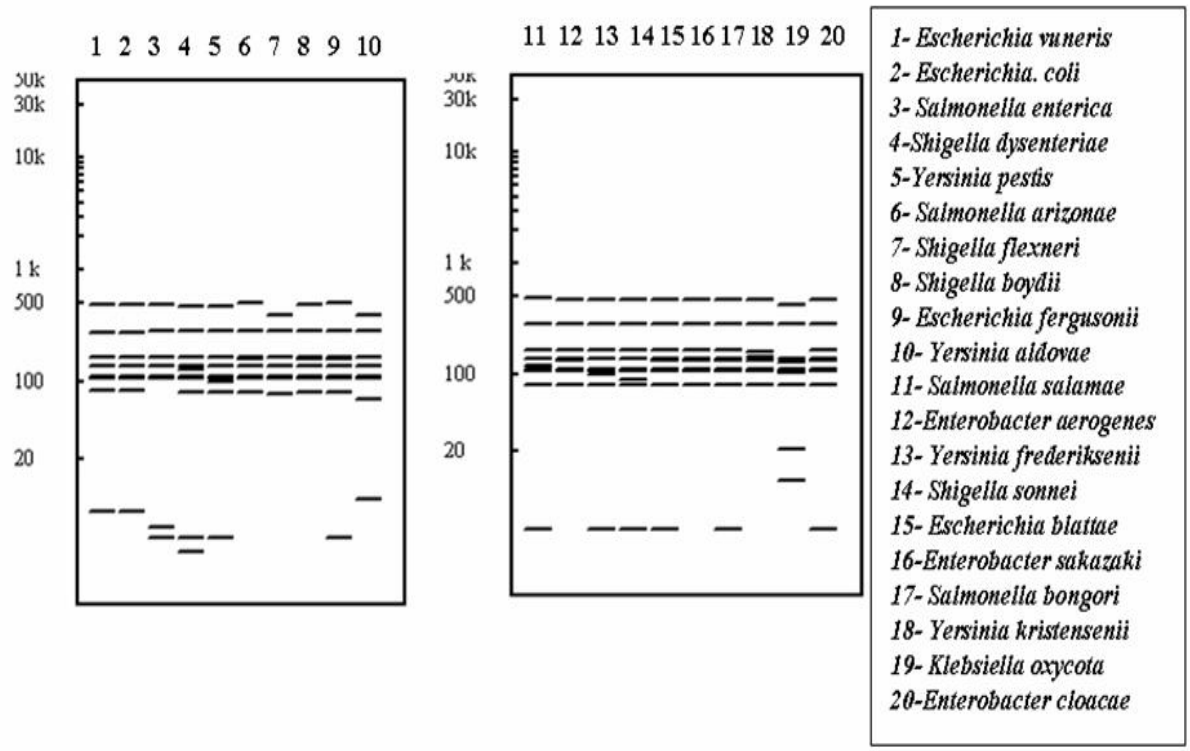


Figure 5. Experimental (A) and simulated (B) profile of 16S rDNA gene digested by the *Msp*I enzyme and resolved on polyacrylamide gel. M denote the 100 bp DNA ladder.

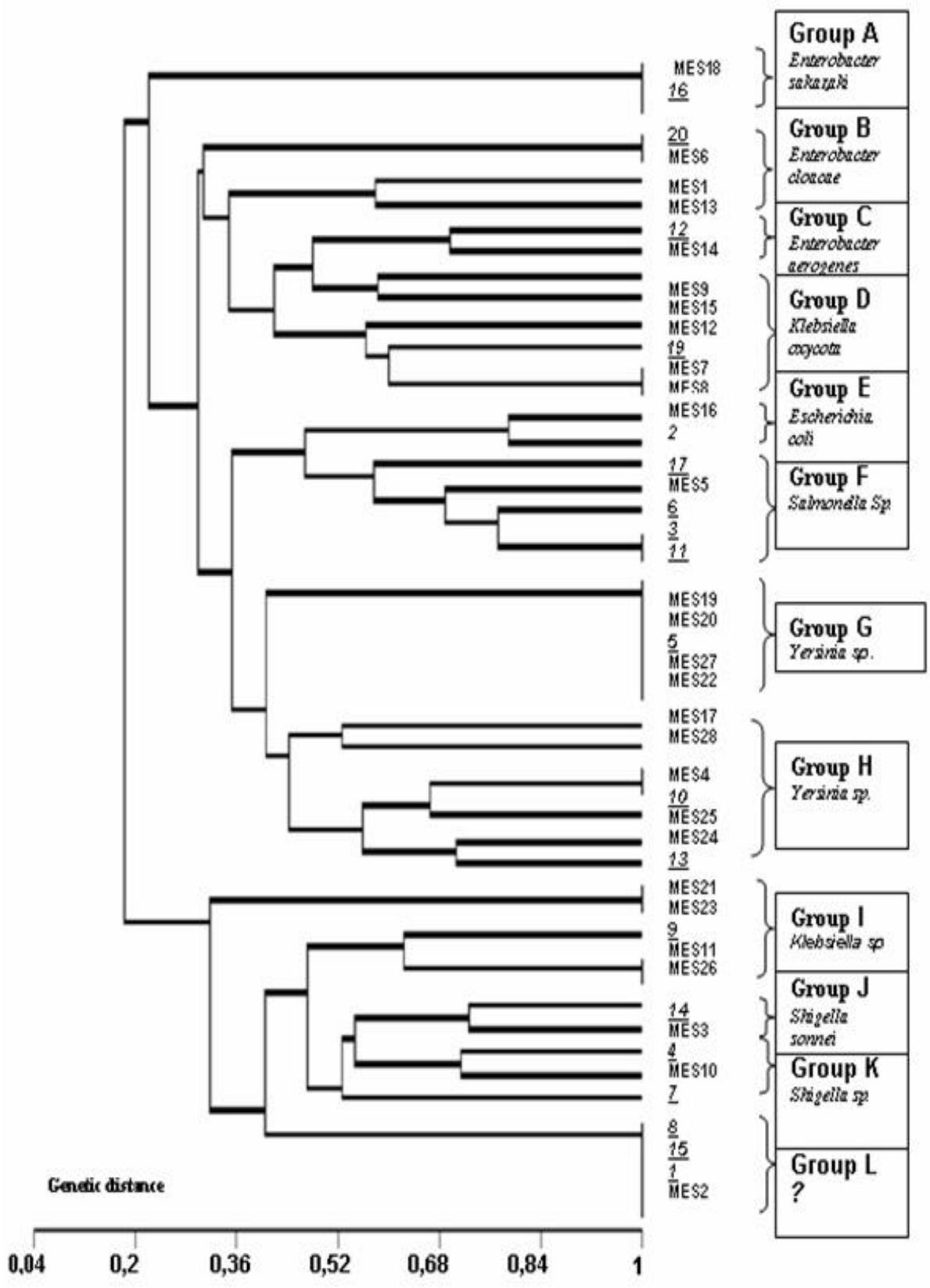


Figure 6. Dendrogramme of the percent genetic similarity estimated by comparison of experimental and theoretic ARDRA profiles.* Strains that begin with the prefix MES are strain test (experimental), strains with number are control strain (theoretical).

Table (3) shows the abundance of *Yersinia* and *Enterobacter* genus**,** they represent respectively 25 % and 17 % of the isolated stocks, the rest is divided in *Klebsiella oxytoca* (6 strains), *Escherichi coli* (2 strains), *Salmonella spp.* (5 strains) and *Klebsiella spp.* (5 strains), *Shigella sonnei* (2 strains) and *Salmonella spp.*(3 strain).

Table 3. Identification of strains according to dendrogramme of Figure 5.

ARDRA Group	Number of strains	Strains	Genus	Species
A	2	MES18, 16	*Enterobacter*	*Enterobacter sakazaki*
B	4	MES6, MES1, MES13, 20	*Enterobacter*	*Enterobacter cloacae*
C	2	MES14, 12	*Enterobacter*	*Enterobacter aerogenes*
D	6	MES9, MES15, MES12, MES7, MES8, 19	*Klebsiella*	*Klebsiella oxycota*
E	2	MES16, 2	*Escherichia*	*Escherichia. coli*
F	5	MES5, 2, 17, 6,3,11	*Salmonella*	*sp.*
G	5	MES19, MES20, MES27,MES22, 5	*Yersinia*	*sp.*
H	7	MES17, MES28, MES4, MES25 MES24, 10, 13	*Yersinia*	*sp.*
I	5	MES21, MES23, MES11, MES26, 9	*Klebsiella*	*sp.*
J	2	MES3, 14	*Shigella*	*Shigella sonnei*
K	3	MES10, 7, 4	*Shigella*	*sp.*
L	4	MES2, 4, 8, 15	*?*	*?*

Conclusion

Bacterial identification using molecular tools constitute an essential complement to classical methods, which are mainly based on morphological and biochemical analyses. The most efficient approach is 16S rDNA sequencing and the comparison to type strain sequences available in public databases. In order to optimize the cost and time necessary to identify different genus and species of the Enterobacteriaceae family, we performed the *In silico*-ARDRA (amplified rDNA restriction analysis) a new method for the identification of strains isolated from environmental origins.

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