

## PCR Analyses of tRNA Intergenic Spacer, 16S-23S Internal Transcribed Spacer, and Randomly Amplified Polymorphic DNA Reveal Inter- and Intraspecific Relationships of *Enterobacter cloacae* Strains

MAYSA M. CLEMENTINO,<sup>1\*</sup> IVANO DE FILIPPIS,<sup>1</sup> CARLOS R. NASCIMENTO,<sup>1</sup>  
REGINA BRANQUINHO,<sup>1</sup> CARMEM L. ROCHA,<sup>1</sup> AND ORLANDO B. MARTINS<sup>2</sup>

Department of Microbiology, National Institute of Quality Control in Health, FIOCRUZ,<sup>1</sup> and Department of Medical Biochemistry, ICB/CCS, Federal University of Rio de Janeiro,<sup>2</sup> Rio de Janeiro, Brazil

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**PCR analysis of tRNA intergenic spacer (tDNA-PCR) and of the 16S-23S internal transcribed spacer (ITS-PCR) and random amplified polymorphic DNA (RAPD) analysis were evaluated for their usefulness in characterization of *Enterobacter cloacae* strains isolated from both clinical origins and vaccine microbial contamination. tDNA-PCR presented specific and reproducible patterns for *Enterobacter sakazakii* ATCC 29004, *Enterobacter aerogenes* ATCC 13048, and *Enterobacter cloacae* ATCC 13047 and 23355 that presented the same profile for all 16 *E. cloacae* isolates, offering an alternative tool for species-level identification. ITS-PCR and RAPD analysis yielded completely different banding patterns for the 20 strains studied, except for *E. cloacae* strains isolated from different batches of vaccine that exhibited a unique pattern, suggesting contamination by the same strain. The combined use of tDNA-PCR and ITS-PCR in a one-step protocol allows accurate identification and typing of *E. cloacae* strains a few hours after the colony has been isolated.**

Medical microbiology is extremely reliant on the culture of bacteria from clinical specimens and their subsequent identification for the diagnosis of disease (17). Determining the relatedness of isolates of microorganisms has become increasingly important as the number and spectrum of nosocomial pathogens continue to expand (16). Generally, the etiologic agent causing an outbreak of infection is derived from a single cell whose progeny are genetically identical or closely related to the source organism (3). *Enterobacter cloacae*, found in the normal flora of the human gastrointestinal tract, has emerged as an important nosocomial pathogen (9), and cases of infection in surgical wards (29) and burn units (26) and of neonatal sepsis have frequently been reported.

The vaccines and sera destined for human use must be free from microbial contamination, which may originate from various sources during the manufacturing process. In order to verify the contamination-free status, the articles are subjected in batches to a sterility test (37), which must be conducted in a controlled environment to avoid accidental contamination (7, 30). An accurate identification to the genus and species level of the bacterial isolates provides invaluable information concerning the source of contamination (1). Traditional methods used for identification of bacterial contaminants are often based on phenotypic characteristics, including colonial morphology and biochemical reactions. However, most of these techniques are affected by physiological factors or are not sufficiently sensitive to distinguish between strains (29).

Recently, DNA analysis has become the preferred method of identification, since it provides a more stable determination of isolate identity (20). PCR-based fingerprinting has been used for assessing the genetic diversity of many microorganisms. Depending on the primers and amplification conditions employed, the results allow discrimination between organisms at the level of genera, species, or strains.

The genes for the three rRNA molecules (16S, 23S, and 5S) found in the ribosome are generally linked together and co-transcribed in a single operon in prokaryotes. The length of these genes and their sizes as well as sequences are conserved between different prokaryotic species (24). However, the number of operons for a given species largely depends on its growth rate (15) and can range from 1 to 11, generally dispersed throughout the prokaryotic genome (15). The 16S and 23S genes are separated by internal spacer regions (ITS), which exhibit a large degree of sequence and length variation at the levels of genus and species. The size of the spacer may vary considerably for different species, and even among different operons within a single cell in the case of multiple operons (11). The variation in length is mainly due to the presence of several functional units within them, such as tRNA genes; sequences for enzyme recognition, such as RNase III, involved in the process of splicing to yield the mature ribosome (6); and *boxA*, which has an antiterminator role during transcription (19). The rest of this region consists of nonessential sequences subject to frequent insertion-deletion events, such as *rsl* in some *Escherichia coli* operons (11). The tRNA genes occur in multiple copies dispersed throughout the genome in most species. The shared sequence motifs of tRNA genes imply that the use of primers that contain consensus tRNA sequences in the PCR are likely to result in a number of characteristic PCR products (34). Analysis of tRNA intergenic spacer (tDNA-

\* Corresponding author. Mailing address: Departamento de Microbiologia, Instituto Nacional de Controle da Qualidade em Saúde, FIOCRUZ, Avenida Brasil, 4365-Manguinhos, 21045-900 Rio de Janeiro-RJ, Brazil. Phone: 55-21-598-4290. Fax: 55-21-290-0915. E-mail: maysa@alpha.incqs.fiocruz.br.

TABLE 1. tDNA-PCR, ITS-PCR, and RAPD patterns of the 20 strains analyzed

Species	Strain or strain origin	Pattern		
		tDNA-PCR	ITS-PCR	RAPD (primer 1)
<i>E. cloacae</i>	Type strain ATCC 13047	1	A	I
<i>E. cloacae</i>	Reference strain ATCC 23355	1	B	II
<i>E. sakazakii</i>	Reference strain ATCC 29004	2	C	III
<i>E. aerogenes</i>	Type strain ATCC 13048	3	D	IV
<i>E. cloacae</i>	Clinical strain IAL-125	1	E	V
<i>E. cloacae</i>	Clinical strain IAL-131	1	F	VI
<i>E. cloacae</i>	Clinical strain IAL-134	1	G	VII
<i>E. cloacae</i>	Clinical strain IAL-127	1	H	VIII
<i>E. cloacae</i>	Clinical strain IAL-124	1	I	IX
<i>E. cloacae</i>	Clinical strain IAL-132	1	J	X
<i>E. cloacae</i>	Clinical strain HU-UFRJ 10281	1	K	XI
<i>E. cloacae</i>	Clinical strain HU-UFRJ 4807	1	L	XII
<i>E. cloacae</i>	Clinical strain HU-UFRJ 10232	1	M	XIII
<i>E. cloacae</i>	Clinical strain HU-UFRJ 2303	1	N	XIV
<i>E. cloacae</i>	Clinical strain HU-UFRJ 1135	1	O	XIV
<i>E. cloacae</i>	Vaccine contaminant 1	1	P	XV
<i>E. cloacae</i>	Vaccine contaminant 2	1	Q	XVI
<i>E. cloacae</i>	Vaccine contaminant 3	1	Q	XVI
<i>E. cloacae</i>	Vaccine contaminant 4	1	Q	XVI
<i>E. cloacae</i>	Vaccine contaminant 5	1	Q	XVI

PCR), based on PCR amplification of spacers between tRNA genes, was proposed by McClelland et al. (27), to distinguish at the species level streptococcal strains of groups A, B, and G and *Streptococcus mutans*. This technique has also been applied successfully for the identification of *Staphylococcus* (25, 34, 35), *Acinetobacter* (14), *Listeria* (31), and *Streptococcus viridans* (13) strains.

Another important PCR-based fingerprinting technique used for typing of a wide range of bacteria is the random amplified polymorphic DNA (RAPD) analysis developed in 1990 by Williams et al. and Welsh and McClelland (33, 36). It has been widely adopted in gene mapping (22), phylogenetic analyses (21), population studies (10), and molecular typing of various microorganisms (12, 28, 38). Several investigators

found poor reproducibility with this method; however, it is reliable if the PCR conditions are optimized (4) and can potentially be used to screen for genetic similarities and differences in whole genomes (23).

#### MATERIALS AND METHODS

**Bacterial strains.** All the strains used in this study are listed in Table 1. The reference strains were obtained from the American Type Culture Collection (ATCC). Clinical samples were isolated from patients of the Federal University of Rio de Janeiro (HU-UFRJ) and Adolpho Lutz Institute (IAL), São Paulo, Brazil. The five contaminant strains of *E. cloacae* were isolated from three batches of the same vaccine at the National Quality Control Institute, Rio de Janeiro, Brazil. Contaminant 1 was isolated from a batch produced in 1998. Contaminants 2, 3, and 4 were isolated from another batch produced in 1998, and contaminant 5 was from a batch produced in 1999.

The sterility tests were performed in class 100 laminar airflow cabinets by different technicians on different dates by the direct inoculation method. The strains were identified by conventional biochemical tests (8) and the Vitek 32 system (bioMérieux Vitek, Inc.), and accompanying software (version 5.1) was used according to the manufacturer's instructions.

**DNA extraction.** All strains were grown on nutrient broth (Difco, Detroit, Mich.) for 24 h at 30°C and checked for purity on nutrient agar plates. Approximately two loops worth of biomass were scraped off the agar plates, suspended in 100  $\mu$ l of sterile distilled water, and boiled for 10 min. After centrifugation at 12,000  $\times$  g for 10 min at 4°C, the supernatants were recovered and 5  $\mu$ l was directly used as the template for PCR.

**tDNA-PCR and ITS-PCR.** For tDNA-PCR the reaction was carried out with the outwardly directed consensus primers T5A (5'-AGTCCGGTGCTAACC AACTGAG-3') and T3B (5'-AGGTGCGGGTTCGAATCC-3') described by Welsh and McClelland (34), which result in the amplification of regions between adjacent tRNA genes. ITS-PCR was carried out with primers L1 (5'-CAAGGC ATCCACCGT-3') and G1 (5'-GAAGTCGTAACAAGG-3'). These sequences are complementary to conserved regions in the 16S and 23S rRNA genes and result in the amplification of the variable spacer regions between these genes. The primers were obtained from DNAgency (Malvern, Pa.), and the amplifications were done on a Perkin-Elmer thermocycler in 25- $\mu$ l reaction mixtures consisting of 20 mM Tris-HCl, pH 8.4; 50 mM KCl; 3 mM MgCl<sub>2</sub>; a 200  $\mu$ M concentration (each) of dATP, dCTP, dGTP, and dTTP (Amersham-Pharmacia Biotech); a 0.5  $\mu$ M concentration of each primer; 1 U of *Taq* DNA polymerase (Gibco-BRL); and 5  $\mu$ l of DNA. The program consisted of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min for 30 cycles, with an additional extension at 72°C for 10 min.

**RAPD analysis.** For RAPD analysis we used arbitrarily chosen primers, which are able to anneal at multiple sites throughout the genome, producing a spectrum of amplified products characteristic of each template DNA. The primers used were primer 1 (5'-d[GGTGGCGAA]-3'), primer 2 (5'-d[GTTTCGCTC

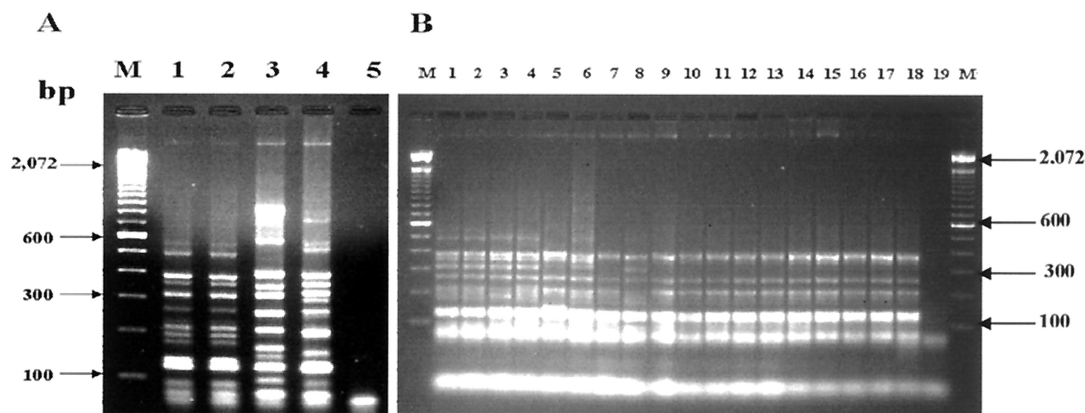


FIG. 1. tDNA-PCR patterns of reference, clinical, and vaccine samples of *Enterobacter* species by electrophoresis in a 2% agarose gel. Lanes M, molecular size marker (100 bp). (A) Lane 1, *E. cloacae* ATCC 13047; lane 2, *E. cloacae* ATCC 23355; lane 3, *E. sakazakii* ATCC 29004; lane 4, *E. aerogenes* ATCC 13048; lane 5, negative control with no added template DNA. (B) Lane 1, *E. cloacae* ATCC 13047; lane 2, *E. cloacae* ATCC 23355; lanes 3 to 8, clinical isolates of *E. cloacae* from IAL; lanes 9 to 13, clinical isolates of *E. cloacae* from HU-UFRJ; lanes 14 to 18, vaccine isolates of *E. cloacae*; lane 19, negative control with no added template DNA.

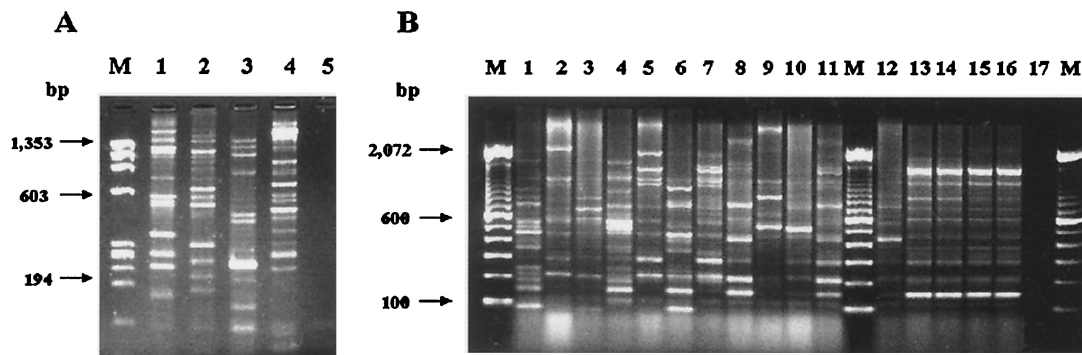


FIG. 2. ITS-PCR patterns of reference, clinical, and vaccine samples of *Enterobacter* species by electrophoresis in a 2% agarose gel. (A) Lane M, molecular size marker ( $\phi$ X174 RF DNA/*Hae*III); lane 1, *E. cloacae* ATCC 13047; lane 2, *E. cloacae* ATCC 23355; lane 3, *E. sakazakii* ATCC 29004; lane 4, *E. aerogenes* ATCC 13048; lane 5, negative control with no added template DNA. (B) Lanes M, molecular size markers (100-bp DNA ladder); lanes 1 to 6, clinical isolates of *E. cloacae* from IAL; lanes 7 to 11, clinical isolates of *E. cloacae* from HU-UFRJ; lanes 12 to 16, vaccine isolates; lane 17, negative control with no added template DNA.

C]-3'), primer 3 (5'-d[GTAGACCCGT]-3'), primer 4 (5'-d[AAGAGCCCGT]-3'), and primer 5 (5'-d[AACGCGCAAC]-3') obtained from the RAPD Analysis primer set (Amersham Pharmacia Biotech). Amplification was done in 25- $\mu$ l reaction mixtures consisting of 20 mM Tris-HCl, pH 8.4; 50 mM KCl; 3 mM MgCl<sub>2</sub>; a 200  $\mu$ M concentration (each) of dATP, dCTP, dGTP, and dTTP (Amersham-Pharmacia Biotech); 25 pmol of each primer; 2 U of *Taq* DNA polymerase (Gibco-BRL); and 5  $\mu$ l of DNA. The program consisted of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, and extension at 72°C for 2 min for 45 cycles, with an additional extension at 72°C for 7 min.

**Agarose gel electrophoresis.** A sample of 10  $\mu$ l of the PCR mixture was loaded onto a 2% (wt/vol) agarose gel, and the PCR products were separated by electrophoresis at 50 V for 3 h in 0.5 $\times$  Tris-borate-EDTA (pH 8.0) buffer with  $\phi$ X174 RF DNA/*Hae*III fragment size markers (GIBCO BRL) or a 100-bp DNA ladder; the gels were stained with ethidium bromide, and the gel images were digitized with the Video Documentation System and analyzed with ImageMaster software (Amersham Pharmacia Biotech).

**Reproducibility evaluation.** The experiments were evaluated in triplicate with at least two bacterial lysates of each strain, and DNAs were coamplified in separate PCRs. The banding patterns were highly reproducible after visual and automated analysis.

**Phylogenetic analysis.** A phylogenetic tree was generated using NTSYSpc 2.02 h software. Similarity was determined on the basis of the simple matching coefficient, and the generated matrix was subjected to clustering by the unweighted pair group method with arithmetic average algorithm.

**RESULTS**

**Phenotypic characteristics.** All isolates were gram-negative rods, oxidase-negative, glucose fermenters with production of acid and gas, and no spores were observed. The strains were identified as *Enterobacter cloacae* after being subjected to stan-

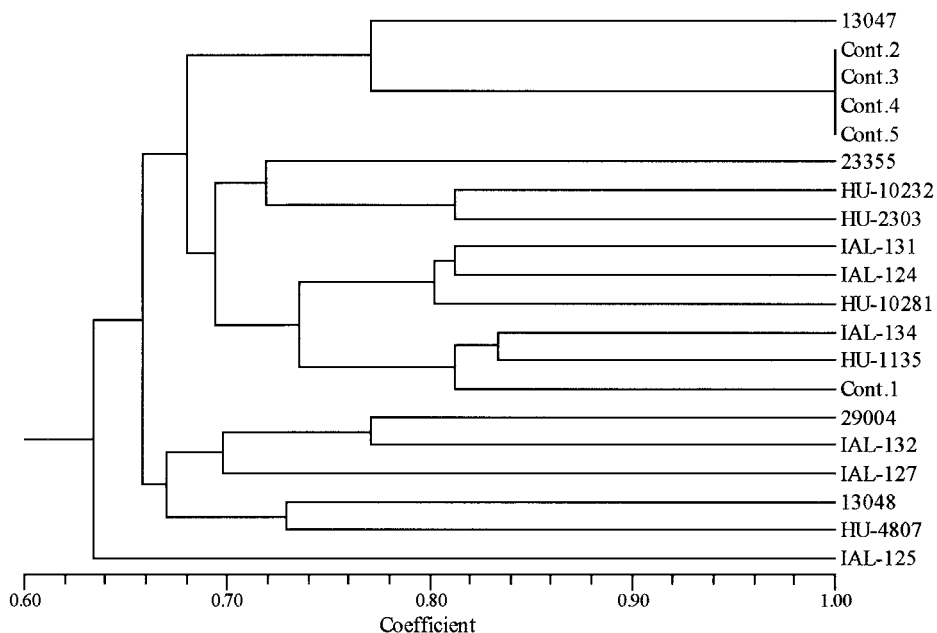


FIG. 3. Phenetic relationship of 20 strains of *Enterobacter* sp. by ITS-PCR. The dendrogram was constructed by unweighted pair group method with arithmetic average. The numbers 13047, 23355, 29004, and 13048 are the ATCC strains; IAL and HU strains are *E. cloacae* strains from clinical samples; and Cont. 1 to 5 are *E. cloacae* strains from vaccine contaminants (Table 1).

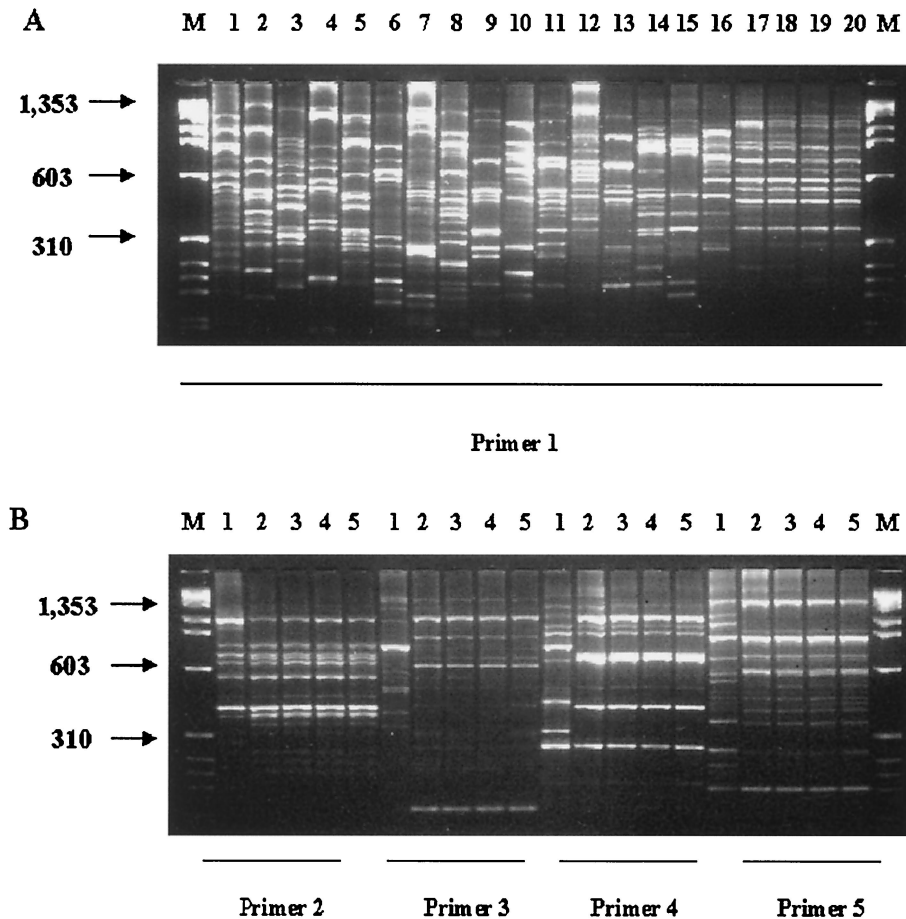


FIG. 4. RAPD patterns of reference, clinical, and vaccine samples of *Enterobacter* species by electrophoresis in a 2% agarose gel. Lanes M, molecular size marker ( $\phi$ X174 RF DNA/*Hae*III). (A) Lane 1, *E. sakazakii* ATCC 29004; lane 2, *E. aerogenes* ATCC 13048; lane 3, *E. cloacae* ATCC 13047; lane 4, *E. cloacae* ATCC 23355; lanes 5 to 10, clinical isolates of *E. cloacae* from IAL; lanes 11 to 15, clinical isolates of *E. cloacae* from HU-UFRJ; lanes 16 to 20, vaccine isolates. (B) Lanes 1 to 5, vaccine isolates analyzed with RAPD primers 2, 3, 4, and 5.

standard conventional biochemical tests (8) and the Vitek System (bioMérieux Vitek).

**tDNA-PCR.** The tDNA-PCR profiles of *Enterobacter* species showed patterns of 9 to 12 DNA fragments ranging in size from 70 to 1,000 bp that distinguished the species by a number of specific DNA fragments (Fig. 1A). The two reference strains of *E. cloacae* showed the same set of fragments (Fig. 1A, lanes 1 and 2), whereas *Enterobacter aerogenes* and *Enterobacter sakazakii* exhibited distinct profiles (Fig. 1A, lanes 3 and 4). As indicated in Fig. 1B, the 11 clinical (lanes 3 to 13) and 5 vaccine (lanes 14 to 18) isolates showed the same pattern obtained by *E. cloacae* reference strains (lanes 1 and 2).

**ITS-PCR.** As seen in Fig. 2, an ITS-PCR of each reference strain of *Enterobacter* showed each strain's own banding pattern, including those of the two strains of *E. cloacae* (lanes 1 to 2). All clinical samples (Fig. 2B, lanes 1 to 11) showed distinct patterns, indicating a very significant degree of intraspecies variation. However, the four *E. cloacae* strains isolated from two different batches of vaccine produced an indistinguishable banding pattern (Fig. 2B, lanes 13, 14, 15, and 16). Considerable variation was observed in *E. cloacae* isolated from another batch (Fig. 2B, lane 12).

These results can be also observed by the dendrogram of Fig. 3, which was generated from ITS-PCR analysis, showing the genetic diversity of the *E. cloacae* strains analyzed. The four vaccine contaminant strains (contaminants 2, 3, 4, and 5) showed the same pattern, clustering at 100% similarity, and clustered at 77 and 72% with *E. cloacae* ATCC 13074 and ATCC 23055, respectively. Meanwhile, the vaccine contaminant (contaminant 1) clustered at 68% with the other contaminants. The group formed by the vaccine contaminant and the ATCC type strain clustered at 64% with the group formed by all the clinical samples and the two other ATCC species of *Enterobacter*. This dendrogram showed once more the high similarity (100%) of the contaminant strains, suggesting they belong to the same strain isolated from different batches of the same vaccine.

**RAPD analysis.** RAPD analysis with primer 1 demonstrated distinct fingerprints of *Enterobacter* reference strains (Fig. 4A, lanes 1 to 4) as well as all clinical samples of *E. cloacae* (Fig. 4A, lanes 5 to 15). It is noteworthy that the four vaccine isolates showed identical banding patterns after RAPD analysis (Fig. 4A, lanes 17, 18, 19, and 20); a different banding pattern was observed with the *E. cloacae* isolate from another

batch of the vaccine (Fig. 4A, lane 16). The amplifications with RAPD primers 2, 3, 4, and 5 presented the same results described with primer 1 (Fig. 4B)

## DISCUSSION

The most striking data presented in this study were that the use of tDNA-PCR and ITS-PCR together offered a hierarchical system for *E. cloacae* strain differentiation; tDNA-PCR resolved the cultures at the species level (Fig. 1), and ITS-PCR differentiated them at the intraspecies level (Fig. 2). The presence of microbial contamination in a sterility test of vaccine is a sporadic and random event requiring the analysis of large sample numbers and demands highly sensitive procedures due to a heterogeneous distribution of microorganisms in a given biological production batch (30). For analyzing sterility test results it is necessary to have an accurate identification and differentiation between the isolates of one species, in order to make a statement about the source and ways of contamination for effective quality control (1). In order to fulfill these requirements we checked the efficiency of the combined methods by using 18 samples of *E. cloacae*. In the tDNA-PCR 11 clinical samples and five vaccine isolates showed the same pattern exhibited by two *E. cloacae* reference strains (Fig. 1B), and ITS-PCR yielded distinct patterns for the strains, except for the four *E. cloacae* strains isolated from different batches of vaccine that presented a unique single pattern (Fig. 2B). With the aim of evaluating if the genetic variability of *E. cloacae* is confined only to the more variable part of the genome or is scattered over the whole genome, we compared DNA fingerprinting obtained with ITS-PCR and RAPD analyses. We observed an expressive agreement between ITS-PCR results and those of RAPD analysis with five different random primers (Fig. 2 and 4). Our results permitted us to conclude that all clinical and vaccine isolates were *E. cloacae* and the four vaccine isolates were actually the same strain, probably originating from a common source during the manufacturing process, instead of an accidental contamination during the sterility assays. These findings were essential for our quality control laboratory to perform a proper interpretation of sterility tests. It is important to point out that three isolates (Fig. 2B, lanes 13, 14, and 15) belonged to the same batch of the vaccine produced in 1998, for which a sterility test and retests were performed on different dates by different technicians; the other isolate (lane 16) was from a vaccine batch produced in 1999, and all of them showed the same set of fragments.

Recently, a number of molecular biology-based approaches directed at species identification have been described. Ribotyping (5) and pulsed-field gel electrophoresis have been used for typing of *E. cloacae*, with high discriminatory potential and good reproducibility (18); however, they are labor-intensive and time-consuming (29). Another method reported to discriminate bacteria of different genera to the species level is amplified ribosomal DNA restriction analysis (32). It is very sensitive, but compared to tDNA-PCR analysis several restrictions may be needed to obtain a final discrimination between species.

tDNA-PCR and ITS-PCR have been used successfully in our laboratory to identify and determine the genetic relationships between *Staphylococcus aureus* and *Bacillus* sp. strains

isolated from different sources (data not shown). The assays offer potential usefulness in the clinical laboratory and can be performed independent of concentration of DNA, genome size, or sequencing when, for example, rapid identification and typing with a high degree of specificity of clinical isolates are desired for choice of antibiotic therapy (2) or even for surveillance of outbreaks (5).

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