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Evaluation of PCR-based Fingerprinting Comparatively to the RFLP-PFGE for Discrimination of *Salmonella* sp. Isolated from Slaughtered Pork

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ABSTRACT

Effective epidemiological surveillance and control of *Salmonella* sp. requires accurate and expeditious genetic typing methods. In the present study, rapid PCR-based methods (ERIC-PCR, M13-PCR and RAPDs) were applied to 73 *Salmonella* sp. isolates, and the results compared with those previously obtained by RFLP-PFGE (*Salmonella* gold standard genotyping method), in order to evaluate their discriminatory ability. Results were very diverse among the primers used and, for each primer, the performance level was variable among the different serotypes. ERIC-PCR and RAPD with OPC19 was inefficient for *Salmonella* sp. discrimination beyond the serotype level. In opposite, M13-PCR, OPC15-RAPD and OPB17-RAPD allowed intraserotype discrimination that, in general, were less discriminative than RFLP-PFGE, indicating that should not be used as a unique typing method in epidemiological studies. Nevertheless, in particular situations, these PCR methods, which are faster and less expensive than RFLP-PFGE, could offers an attractive choice as a preliminary screening of the isolates to reduce the number of suspicious isolates that should be subsequently typed with a more discriminative and accurate methods such as RFLP-PFGE.

Key words: PFGE, Rep-PCR, RAPD, *Salmonella*, swine, genotyping

INTRODUCTION

Salmonella sp. is one of the most important pathogens involved in human foodborne illness in the developed world[(1)].

Effective epidemiological surveillance and control of *Salmonella* sp. and other zoonotic pathogens requires accurate subtyping of strains for identification of potential sources of infection[(2,3)]. Many of the traditional methods used for typing bacteria such as the morphological, physiological, and biochemical markers in conjunction with traditional serology, while long-established, are time-consuming, laborious, expensive, and are often not able to discriminate between related outbreak strains[(2,4,5)].

During recent years, new molecular typing techniques have been developed, based on the genomic differences between strains[(5)]. The basic premise of these typing systems is that epidemiologically related isolates are derived from the clonal expansion of a single precursor and share characteristics that differ from those of epidemiologically unrelated isolates[(3)].

RFLP-PFGE (Restriction Fragment Lenght Polimorphism-Pulsed Field Gel Electrophoresis) typing is considered to be a highly discriminatory typing system for numerous *Salmonella* sp. serovars, and its validity as an accurate and sensitive typing tool has been demonstrated by several investigators[(3,6-9)]. According to Gautom[(2)], RFLP-PFGE has become a standard technique among public health agencies due to its accuracy and reproducibility among different laboratories. Unfortunately, time-consuming and tedious specimen processing is an inherent problem, which limits the use of this powerful technology as a real-time epidemic investigational tool[(2)]. Often there is a need to rapidly differentiate specific *Salmonella* subtypes from other related isolates, for instance, in the case of large-scale outbreak[(5,10)]. PCR-based methods such as Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR), M13-PCR and Random Amplified Polymorphic DNA (RAPD) are faster, easier

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and more economical than RFLP-PFGE, and could be used in any laboratory with PCR capability[11-13]. Several studies have employed the ERIC-PCR[12,14-16], M13-PCR[12] and RAPDs[17-20] for typing *Salmonella* spp., but the results were, in most of the cases, controversial and sometimes contradictory. Comparison of any genotyping method with PFGE is important because of its acceptance as the standard for *Salmonella* sp. genotyping[21].

In the present study PCR-based methods, such as ERIC-PCR, M13-PCR and RAPD were applied to 73 *Salmonella* sp. isolates, and the results were compared with those previously obtained by RFLP-PFGE[22]. The findings intent to provide accurate information about the capability of the faster PCR-based methods to perform an efficient, quick and easy typing of *Salmonella* isolates originated from slaughtered pigs in an epidemiological approach.

MATERIALS AND METHODS

I. *Bacterial Strains*

Sixty nine *Salmonella enterica* strains were obtained from the ileum (I), the ileo-colic lymph nodes (IM), the mandibular lymph nodes (ML), the tonsils (T), and from the corresponding carcass (C) of 101 pigs slaughtered in a Portuguese abattoir from June 2003 to September 2004. Serotyping of *Salmonella* isolates, performed according to the Kauffmann-White scheme, identified eight different serotypes: Typhimurium (n = 33), Rissen (n = 19), Enteritidis (n = 4), Tennessee (n = 5), 4,[5],12:i:- (n = 3), Anatum (n = 2), Give (n = 2), and Derby (n = 1)[23]. Two reference strains from international culture collections (*Salmonella enterica* Enteritidis CECT 4300 and *Salmonella enterica* Typhimurium ATCC 14028) and two other strains from the Laboratório Nacional Investigação Veterinária (LNIV) collection (*Salmonella enterica* Tennessee and *Salmonella enterica* 4,[5],12:i:-), that were not related to this work, were included in the study as unrelated strains. A total of 73 *Salmonella* sp. isolates were analysed.

II. *Genetic Typing of Salmonella sp. Isolates*

DNA extraction was performed by the guanidium thiocyanate method[24], and subsequently quantified using a UV1101 Biotech Photometer.

For PCR reaction the primers ERIC1, ERIC2, M13 and OPB17, synthesized by Invitrogen™, were selected based in previous research studies developed respectively by Weigel *et al*.[21], Grundmann *et al*.[25] and Soto *et al*.[18]. The OPC15 and OPC 19 primers were selected (from a set of four primers, that were selected by random) because of the clear and distinct banding patterns obtained (data not shown). The primer sequence used for PCR fingerprinting of *Salmonella* sp. isolates were listed in Table 1

Optimization of some parameters such as the concentrations of DNA template and primers was carried out to obtain stable and reproducible results (data not shown).

For each set of primers a negative control tube was included in which the DNA template was replaced by sterile ultra pure water[26].

Assays were performed in 25 μL reaction mixtures containing an Invitrogen™ 1X amplification Buffer, 3 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphates (Invitrogen™), 1 μM of each primer, 1U of *Taq* DNA Polymerase (Invitrogen™) and 10 ng of template DNA.

PCR amplification was performed in an Uno II Thermal Cycler (Biometra) and the reactions were as follows for ERIC-PCR and M13-PCR: an initial denaturation period of 2 min at 95°C, followed by 40 cycles consisting of 94°C for 45 sec, 50°C for 1 min, and 72°C for 1.5 min and a final extension for 6 min at 72°C. For RAPD-PCR with OPC15 and OPC19 the annealing temperature was changed to 36°C. RAPD-PCR with OPB17 was followed according to the protocol as described previously by Soto *et al*.[18].

The resolution of the PCR products was developed by electrophoresis in 1.2% (w/v) agarose gel (Invitrogen™) in 0.5X TBE (Tris-borate-EDTA) buffer at 90 V for 2 hr. In each gel the 1 Kb Plus DNA Ladder (Invitrogen ™) was used as molecular marker.

After electrophoresis, the gels were stained with ethidium bromide (0.2 μg/mL), photographed (Kodak Digital Science EDA 120 System) using the Kodak Digital Science EDA 120 System, under UV transiluminator and the image digitalization was processed using Kodak Digital Science 1D 2.0 Image Analysis Software.

Table 1. Primers used in the PCR fingerprinting of *Salmonella* sp. isolates

Primer	Sequence	Source
ERIC 1	5'ATGTAAGCTCCTGGGGATTCAC 3'	(21)
ERIC 2	5' AAGTAAGTGACTGGGGTGAGCG 3'	(21)
M13	5' GAGGGTGGCGGTTCT 3'	(25)
OPC 19	5' GTTGCCAGCC 3'	OPERON TECHNOLOGY, USA
OPB 17	5' AGGGAACGAG 3'	OPERON TECHNOLOGY, USA
OPC 15	5' GACGGATCAG 3'	OPERON TECHNOLOGY, USA

III. *Data Analysis*

The gel images were saved in TIFF format and imported into the computer software Bionumerics Software Version 4.0 (Applied Maths). All the images were normalized by aligning the reference tracks with a standard reference. Similarities among isolates were estimated using Pearson correlation coefficient and the clustering was based on the UPGMA method. PCR profiles were considered different when at least one polymorphic band was identified as previously defined by Soto *et al*.[18].

Diversity indices, for PCR fingerprints and RFLP-PFGE macrorestriction profiles, were calculated using Simpson's index[27]. This index reflects the probability that two individuals drawn at random from a population belong to the same species.

RESULTS AND DISCUSSION

In the present study the discriminatory capacity of three rapid PCR-based methods (ERIC, M13 and RAPDs: OPC19, OPC15, OPB17) was evaluated comparatively to RFLP-PFGE on 69 *Salmonella* sp. isolates of eight serovars: Typhimurium, Rissen, Tennessee, Enteritidis, 4, [5], 12:i:-, Give, Anatum and Derby[23]. Four unrelated strains were also included to evaluate the discriminatory ability of the methods, as it was previously suggested by Tenover *et al*.[28] and Hilton *et al*.[29].

All PCR-based methods used were successfully applied to all the isolates allowing their interserotype discrimination, since each different serotype was grouped into distinct clusters (data not shown). An exception was observed with primer OPC15 for *S*. Typhimurium and *S*. 4,[5],12:i:- isolates, that were included in the same cluster, revealing a higher interserotype genetic similarity, which can be seen during Figure 1 analyses that presents the banding profiles among the serotypes for each different technique. In this Figure it is possible to observe the similarity between the banding pattern, with OPC15 primer, of Ty3 genotype (from *S*. Typhimurium) and F9 genotype (from *S*. 4, [5], 12:i:-). Similar results were previously observed with the same isolates using RFLP-PFGE analysis[22].

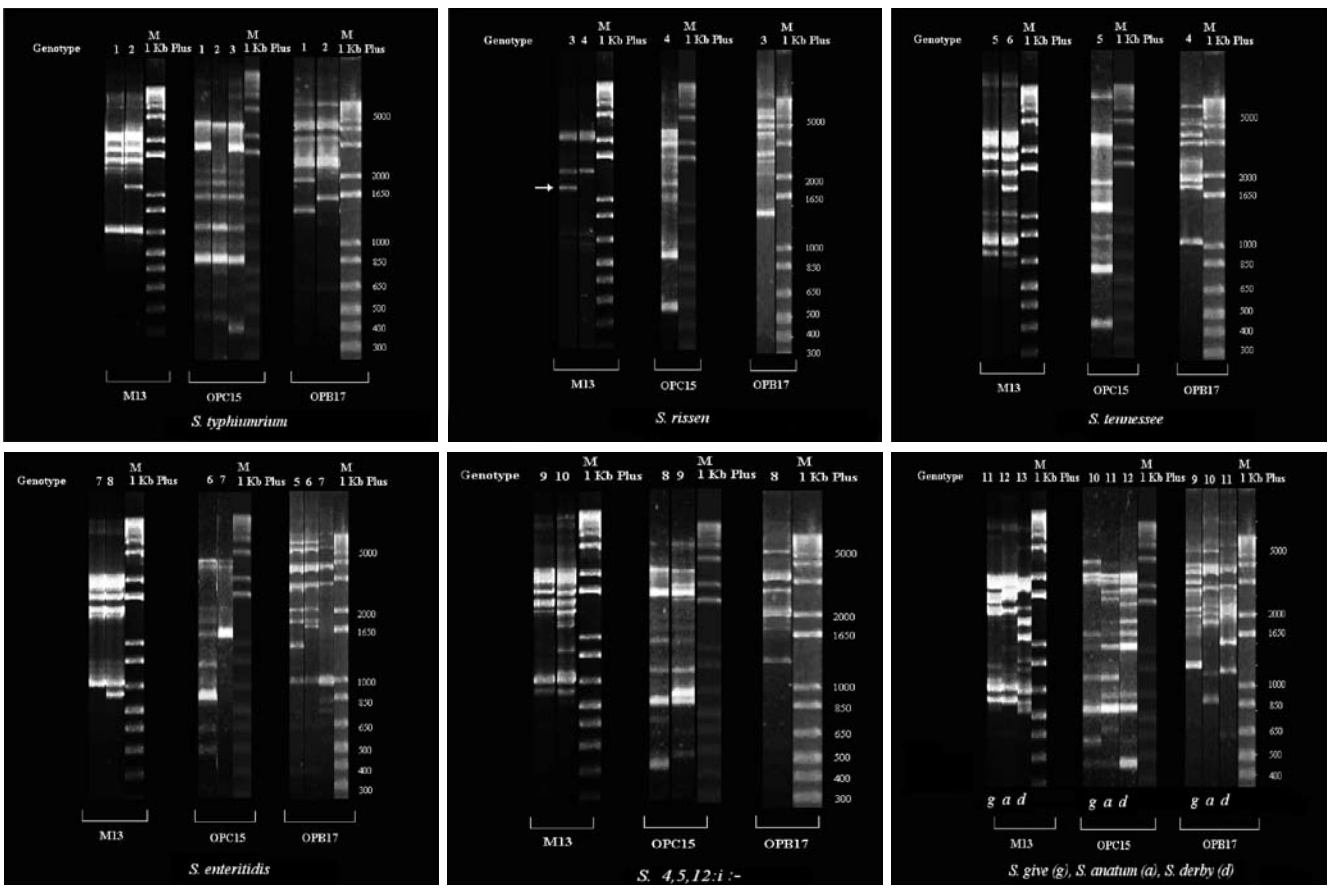

Figure 1. Representative banding profiles among the *Salmonella* serotypes for each different technique. The genotypes number identification is according with the ones described in Table 2. The white dart presented in the M13 PCR fingerprint of *S*. Rissen indicates the band that is presented in the 2003 isolates and absent in the 2004 isolates. **M 1 Kb Plus**: The Invitrogen ^{TM} DNA Ladder used as molecular marker (**M**). The numbers in the right lanes of the different panels represents the molecular weight of the correspondent band from the 1 Kb Plus DNA Ladder.

The high similarities detected among these two serotypes can be explained by the fact that *S*. 4,[5],12:i:- is considered a monophasic variant of *S*. Typhimurium[30]. Nevertheless, the remaining primers allowed the discrimination between these two serotypes which could be advantageous since *S*. 4,[5],12:i:- serotype is considered an emergent food borne pathogen revealing, in the last decade, a rapid increase in its occurrence frequency. Furthermore, this serotype is characterized by the presence of virulence invasion genes, production of enterotoxin and cytolysin, multidrug-resistant profile and presenting resistance within macrophages[31-33].

Using ERIC-PCR and RAPD-PCR with OPC19 primer, all the isolates from the same serotype, including the unrelated strains, presented similar genotypes, which point to the potential of these methods as *Salmo-*

Table 2. Distribution of the different genotypes for each serotype

Serotype	Date[a]	Isolates	Genotypes				
			M13	OPC15	OPB17	Combined[b]	PFGE[c]
S. Typhimurium (n = 34)	18 Jun 03	IL27	1	1	1	1,1,1	Ty1
	2 Jul 03	I40 to C47[d]	1	1	1	1,1,1	"
	19 Sep 04	IL99	1	2	2	1,2,2	Ty2
		IL101	1	3	1	1,3,1	Ty3
	-	ATCC 14028	2	2	1	2,2,1	Ty4
S. Rissen (n = 19)	5 May 03	[I1, T1, ML1]	3	4	3	3,4,3	R1
	2 Jun 03	I18	3	4	3	"	"
	11 Jun 03	C22, [I24, T24]	3	4	3	"	"
	12 Jul 04	C76, I77	4	4	3	4,4,3	"
	19 Jul 04	[I81, IL81, ML81], [T83, C83]	4	4	3	"	"
	13 Sep 04	C94, [ML95, C95]	4	4	3	"	"
	12 Jul 04	C75	4	4	3	"	R2
		C77	4	4	3	"	R3
S. Tennessee (n = 6)	24 May 04	[I55, IL55, T55]	5	5	4	5,5,4	Te1
	5 Jul 04	[I69, IL69]	5	5	4	5,5,4	"
	-	LNIV collection	6	5	4	6,5,4	Te2
S. Enteritidis (n = 5)	17 May 04	[I50, IL50]	7	6	5	7,6,5	E1
	17 Jun 04	IL65	8	6	5	8,6,5	"
	24 May 04	I57	8	6	6	8,6,6	E2
	-	CECT 4300	7	7	7	7,7,7	E2
S. 4,[5],12:i:- (n = 4)	5 Jul 04	I66	9	8	8	9,8,8	F1
		IL68	9	9	8	9,9,8	F2
	6 Sep 04	IL84	9	8	8	9,8,8	F3
	-	LNIV collection	10	9	8	10,9,8	F4
S. Give (n = 2)	5 Jul 04	[I71, IL71]	11	10	9	11,10,9	G1
S. Anatum (n = 2)	19 Jul 04	[IL79, ML79]	12	11	10	12,11,10	A1
S. Derby (n = 1)	11 Jun 03	ML24	13	12	11	13,12,11	D1
Total of different genotypes			13	12	11	18	18

[a]Date: refers to the date of sample collection from where *Salmonella* sp. was isolated.
[b]Combined: a combination of the results obtained by PCR-based methods.
[c]PFGE-RFLP macrorestriction profiles identified and described in Vieira-Pinto *et al*.[22].
[d]Thirty samples belonging to porks from farm A, and collected in the same day: [I40, IL40, ML40, C40], [I41, IL41, T41, ML41, C41], [IL42, T42, ML42], [T43, ML43, C43], [IL44, T44, ML44, C44], [I45, IL45, T45, ML45, C45], [IL46, T46, ML46], [IL47, ML47, C47].
Strains from several samples of the same pig are in brackets.

nella typing tools at the serotype level rather than the strain level. A similar result was previously reported by Van Lith and Aarts[15] using ERIC-PCR with primers ERIC1+ERIC2, that also suggested its application for tyiping *Salmonella* up to serotype level. On the contrary, it was not found any bibliographic references with similar results for RAPD-PCR.

Since all the isolates from one serotype revealed the same genotype when analysed by ERIC-PCR and RAPD with OPC19 primer, these results were not included for further analyses concerned to intraserotype discrimination.

The remaining primers allowed the detection of variable genotypes within the same serotype. The distribution of the different genotypes for each serotype is described in Table 2.

In general, among the 69 pig isolates and 4 unrelated strains of *Salmonella* sp., all PCR methods analyzed in this study revealed a lower number of band patterns than RFLP-PFGE that identified 18 different *Xba*I macrorestriction profiles (MRPs)[22]. PCR-M13, RAPD-OPC15 and RAPD-OPB17 allowed the detection of 13, 12 and 11 different profiles, respectively, and these results are in accordance with those previously obtained by Tsen *et al*.[20] and Eriksson *et al*.[25].

The Simpson diversity indices (D) calculated for all the methods, including RFLP-PFGE, are presented in Table 3.

The diversity index was not calculated for serotype *S*. Give, *S*. Anatum and *S*. Derby, because only one genotype was observed for each serotype, by all the methods.

Considering the results expressed in Table 2 and Table 3, several comments should be made about the application of the genetic typing methods for *Salmonella* sp. discrimination.

M13-PCR was able to distinguish the unrelated strains included in this study, except in the case of *S*. Enteritidis. This could be an advantage of M13 that can be used during an outbreak investigation for investigate the relatedness of the isolates, before typing with a more discriminative method (e.g. RFLP-PFGE). In the case of *S*. Tennessee isolates, M13-PCR revealed equal performance as RFLP-PFGE (D = 0.333), and a better discriminative ability than OPC15 and OPB17-RAPD that were unable to discriminate the isolates.

Previously, Vieira-Pinto *et al*.[22] detected three different genotypes among the 19 *S*. Rissen isolates with RFLP-PFGE. These genotypes presented only 1 or 2 bands differences (Figure 1) and the hypothesis was stated that Type R1 (composed by the 17 isolates obtained during 2003) belonged to a resident strain of the slaughterhouse and that, the others two types (R2 and R3, that include the remaining 2 isolates), were clonal descendants from the resident strain referred above. The analyses based on M13-PCR fingerprinting also supports this hypothesis, since all the seven 2003 isolates presented identical profiles that differed by the presence of an additional band from the M13-PCR fingerprints obtained for the 2004 isolates (all of the 2004 isolates also originated identical patterns among them) (Figure 1). The results suggest that an evolutive/adaptation process could occur during this two-year period. The remaining primers were incapable to discriminate these isolates.

In this study, different performances were observed among the three primers used for RAPDs. As it was stated before, RAPD using primer OPC19 was inefficient for *Salmonella* sp. discrimination beyond the serotype level. In opposite, OPC15 and OPB17 revealed different capacity levels for discriminating *Salmonella* sp. isolates between and within serotypes, but with a scarce ability to distinguish the unrelated strains, which limit their use for epidemiological investigations. With respect to *S*. Enteritidis isolates, RAPD-PCR with primer OPB17 revealed a better discriminatory capacity (D = 0.700) than the other methods, including RFLP-PFGE (D = 0.667), revealing three genotypes, and allowing the discrimination of the unrelated strain. This result can be an important contribution to the success of discrimination between *S*. Enteritidis isolates, since this serotype has been proven to have a very homogeneous genotype[12,20,35]. In contrary, for *S*. Typhimurium and for *S*. 4,[5],12:i:- isolates, RAPD with primer OPB17 exhibited a worse discriminatory performance than using primer OPC15. Nevertheless, as stated before, primer OPC15 was unable to separate in different clusters *S*. Typhimurium and *S*. 4,[5],12:i:- isolates.

Table 3. Simpson's diversity index (D) calculated for all the genetic typing methods

Serotype	Genetic typing methods				
	M13	OPC15	OPB17	Combined primers	RFLP-PFGE
	(D)				
S. Ttyphimurium (n = 34)	0.059	0.169	0.059	0.171	0.171
S. Rissen (n = 19)	0.491	0	0	0.491	0.205
S. Tennessee (n = 6)	0.333	0	0	0.333	0.333
S. Enteritidis (n = 5)	0.667	0.400	0.700	0.900	0.667
S. 4,[5],12:i:- (n = 4)	0.5	0.666	0	0.833	1

In addition, all the PCR methods, except RAPD with primer OPC15, were unable to discriminate the 31 *S*. Typhimurium isolates group representative of an outbreak identified by RFLP-PFGE[22]. Ideally, the genotype patterns of isolates representing the outbreak strain would be indistinguishable from each other and distinctly different from those of epidemiologically unrelated strains.

The results obtained in the present study indicated that the choose of primers for *Salmonella* sp. PCR fingerprinting directly interferes in the epidemiological study success and orientation, suggesting that it must be carefully and rigorous made in order to select the more efficient to discriminate *Salmonella* isolates, since some primers are more efficient to discriminate specific serotypes than others. This concern was already expressed by Lin *et al*.[4], Burr *et al*.[36] and Shangkuan and Lin[37].

The combination of the genotype profiles generated by each of the three primers generated the most similar discriminatory results when compared with the RFLP-PFGE, both identifying 18 different genotypes (Tables 2 and 3). Among these genotypes: equal discriminative capacity was observed for *S*. Typhimurium, *S*. Tennessee, *S*. Give, *S*. Anatum and *S*. Derby. Furthermore, the isolates included in these five serotypes were grouped in the same way. Two PCR genotypes were identified for *S*. Rissen isolates but they were grouped differently, comparatively to the RFLP-PFGE. Among *S*. Enteritidis isolates, the three primers PCR fingerprinting improved the discriminative capacity comparatively to PFGE. On the opposite, for *S*. 4,[5],12:i:-, the combined PCR methods were insufficient to reach to the same discrimination obtained by RFLP-PFGE. However, the former allowed a better differentiation between *S*. Typhimurium and *S*. 4,[5],12:i:- isolates. Therefore, the combination of the results obtained with different primers improved the discriminatory capacity of PCR-based methods. Similar results were already reported by López-Molina *et al*.[12], Johnson *et al*.[13] and Liebana *et al*.[3].

In conclusion, PCR-based methods in spite of affording a quick and easy typing of *Salmonella* sp. isolates, provided lesser strain differentiation compared to RFLP-PFGE, thus not contributing to the improvement of the epidemiological study concerned to *Salmonella* sp. dissemination trough slaughtered pigs. For this reason, the authors would like to underline that, according to the results achieved in this study, PCR-based methods should not be used as exclusive method in studies that need to discriminate *Salmonella* sp. isolates epidemiological not related. Nevertheless, during a salmonellosis outbreak, PCR-based methods could represent an important tool to aggregate rapidly the related strains reducing the number of suspicious isolates that should be subsequently typed with a more discriminative and accurate methods such as RFLP-PFGE.

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