Acta Scientiae Veterinariae. 35(1): 41-48, 2007.

ORIGINAL ARTICLE
Pub. 702

ISSN 1678-0345 (Print)
ISSN 1679-9216 (Online)

Use of a modified AFLP protocol to discriminate *Salmonella enterica* subsp. *enterica* serovar Enteritidis isolates

Utilização de um protocolo modificado de AFLP para discriminar isolados de *Salmonella enterica* subsp. *enterica* sorovar Enteritidis

Clarissa Silveira Luiz Vaz[1,2], André Felipe Streck[2], Tatiana Tramontina[3], Marisa Ribeiro de Itapema Cardoso[4] & Cláudio Wageck Canal[2]

ABSTRACT

Salmonella enterica subsp. *enterica* (*S.*) serovar Enteritidis is one of the main pathogens involved in food-borne diseases worldwide. In epidemiological investigations of food-related salmonellosis, subtyping is necessary to improve preventive and control measures. Single-enzyme amplified fragment length polymorphism (SE-AFLP) analysis is a modified AFLP that uses only one restriction enzyme to produce DNA fragments that are selectively amplified by PCR. In order to assess the applicability of SE-AFLP in *S.* Enteritidis typing, one hundred and eight strains isolated from poultry, swine and also from human salmonellosis outbreaks in Southern Brazil were analyzed. Strains from other countries and six different *S. enterica* serovars were also included as controls. SE-AFLP was able to distinguish *S.* Enteritidis from the other *S. enterica* serovars analyzed. However, most of *S.* Enteritidis strains isolated from poultry, salmonellosis outbreaks and most of the strains from other countries shared the same predominant pattern. The low genetic diversity identified in *S.* Enteritidis suggests that the strains analyzed are clonally related and one predominant SE-AFLP genotype is widely spread in Southern Brazil.

Keywords: *Salmonella* Enteritidis, SE-AFLP, genotyping, poultry.

RESUMO

Salmonella enterica subsp. *enterica* (*S.*) sorovar Enteritidis é um dos principais patógenos envolvidos em doenças de origem alimentar em todo o mundo. Em investigações epidemiológicas de salmoneloses relacionadas a alimentos, a subtipificação é necessária para aprimorar medidas de controle e prevenção. A análise através do polimorfismo de comprimento do fragmento amplificado utilizando uma única enzima de restrição (SE-AFLP) é um protocolo modificado de AFLP que utiliza uma única enzima de restrição para produzir fragmentos de DNA que são seletivamente amplificados por PCR. Para verificar a aplicabilidade da SE-AFLP na tipificação de *S.* Enteritidis, cento e oito cepas isoladas de galinhas, suínos e também de surtos de salmonelose humana do sul do Brasil foram analisadas. Cepas de outros paises e seis sorovares diferentes de *S. enterica* também foram incluídos como controles. A SE-AFLP foi capaz de distinguir *S.* Enteritidis dos outros sorovares de *S. enterica* analisados. Contudo, a maioria das cepas de *S.* Enteritidis isoladas de galinhas, de surtos de salmonelose e a maioria das cepas de outros paises compartilharam um mesmo padrão predominante. A baixa diversidade genética determinada nas cepas de *S.* Enteritidis sugere que as cepas analisadas são relacionadas clonalmente e que um genótipo predominante de SE-AFLP está largamente disseminado no sul do Brasil.

Descritores: *Salmonella* Enteritidis, SE-AFLP, genotipificação, frangos.

Received: August 2006 www.ufrgs.br/favet/revista Accepted: November 2006

[1,2]Programa de Pós-graduação em Ciências Veterinárias (PPGCV), Faculdade de Veterinária (FAVET), Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre/Brazil. [2]Laboratório de Virologia, FAVET, UFRGS. [3]Fundação Estadual de Produção e Pesquisa em Saúde (IPB/LACEN-RS), Porto Alegre/Brazil. [4]Laboratório de Medicina Veterinária Preventiva, FAVET, UFRGS. CORRESPONDENCE: C.W. Canal [claudio.canal@ufrgs.br; Fax: 55 51 3308 7305].

1. INTRODUCTION

Salmonella enterica subsp. *enterica* (*S.*) serovar Enteritidis has emerged worldwide as the most common bacteria isolated from human salmonellosis [1], and infections have occurred in Brazil since 1990s [2]. Since *S.* Enteritidis has a wide range of animal reservoirs and a high spread potential, improvements in epidemiological surveillance are currently necessary. In this sense, accurate characterization is crucial to differentiate pathogenic strains from less-pathogenic ones, as well as to study strain origin, evolution and major infection routes, which will direct preventive and control measures.

Phage typing is the method of choice for *S.* Enteritidis characterization. However, because of the predominance of some phage types [3-5], molecular typing became important for strain characterization and has been associated with the traditional phenotypic methods to improve discriminatory power. A range of these molecular approaches is available for such investigation [6-9] and there is consensus on the use of pulsed-field gel electrophoresis (PFGE) as a standard typing approach for genotyping [10]. However, PFGE is technically demanding and time-consuming.

Amplified-fragment length polymorphism (AFLP) typing presents a high power of strain discrimination [11], although the detection of DNA amplification fragments must be performed in polyacrylamide gels or by means of the analysis of fluorescent labeled fragments on automated DNA sequencer. A single-enzyme approach to AFLP (SE-AFLP) may constitute an alternative, since genomic DNA is digested with a restriction enzyme followed by selective amplification of a subset of fragments. These resulting PCR-amplified DNA fragments are less complex and can be analyzed by agarose gel electrophoresis [12-13]. SE-AFLP has been widely used for typing of different bacterial pathogens [14-17] and some *Salmonella* serovars [18-19], but there are no reports of SE-AFLP use to analyze comprehensive *S.* Enteritidis strain collections. In the present study, an SE-AFLP protocol was standardized and evaluated for the characterization of *S.* Enteritidis isolates from different sources.

2. MATERIALS AND METHODS

2.1 Bacterial isolates

A total of 114 strains of *S. enterica* were analyzed in this study (Table 1). *S.* Enteritidis from poultry, swine, food and humans were isolated from 1995 up to 2003 in Southern Brazil (State of Rio Grande do Sul). Food and human strains were isolated from salmonellosis outbreaks reported in Rio Grande do Sul and were kindly supplied by the government surveillance service (FEPPS, IPB, LACEN/RS). We also included some *S.* Enteritidis strains isolated from unknown sources and year of isolation in other countries. *S.* Enteritidis ATCC 13076 was used as SE-AFLP pattern control.

2.2 DNA extraction

Bacterial cultures grown overnight in brain and heart infusion at 37°C were used for genomic DNA extraction by cetyltrimethylammonium bromide (CTAB) method [20]. The DNA was precipitated and resuspended in 100 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and the concentration and quality of the samples were determined by absorbance readings at 260 and 280 nm.

2.3 Restriction endonuclease digestion and ligation of oligonucleotides adapters

An aliquot containing 4 mg of DNA was digested at 37°C for 3 h with 2 U of *Hin*dIII[1] in 10 mM Tris-HCl pH 8.5, 10 mM $MgCl_2$, 100 mM KCl, 0.1 mg/mL BSA in a final volume of 50 mL. DNA fragments were precipitated and resuspended in TE buffer. A 5 mL digested DNA aliquot was used in a ligation reaction containing 1 U of T4 DNA ligase[1], ligase buffer (40 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP, pH 7.8) and 0.2 mg of each oligonucleotide adapters[2] [21] in a final volume of 20 mL at 16°C for 4 h. The ligated DNA was precipitated with 2.5 mM ammonium acetate and absolute ethanol, washed with 70% ethanol and resuspended in 50 mL of TE buffer.

2.4 PCR amplifications

The PCR assays were performed in a DNA thermal cycler[3]. Primers[2] [21] were complementary to the adapter sequence, differing in the 3' final' base (A, T, C or G). These primers were called HI-A, HI-T, HI-C and HI-G, respectively. The PCR mixture contained the reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.0), 2.5 mM or 3.0 mM $MgCl_2$[5], 20 pmol of a single primer, 200 mM of each dNTP[4], 1 U of *Taq* DNA polymerase[5], 5 mL of ligated DNA as template and distilled water in a total volume of 25 mL. The reaction mixture was amplified under the following conditions: an initial denaturing step of 94°C for 4 min, followed by 36 cycles of 94°C for 1 min, 57°C for 1 min and 72°C for 2.5 min. Amplified fragments were separated

Table 1. Source, number of isolates tested, country of origin and SE-AFLP genotypes of the *Salmonella enterica* strains analyzed.

Serotype	Source	Number	Origin	SE-AFLP genotype
Enteritidis	Poultry meat	31	Brazil	HG1, HC1, HT1
Enteritidis	Broiler	22	Brazil	HG1, HC1 or HC2, HT1
Enteritidis	Swine	1	Brazil	HG3, HC3, HT3
Enteritidis	Humans	14	Brazil	HG1, HC1, HT1
Enteritidis	Food related to poultry	9	Brazil	HG1, HC1, HT1
Enteritidis	Food not-related to poultry	20	Brazil	HG1, HC1, HT1
Enteritidis	Unknown	1	Egypt	HG1, HC1, HT1
Enteritidis	Unknown	2	Italy	HG1, HC1, HT1 or HT2
Enteritidis	Unknown	2	Albania	HG1, HC1, HT1
Enteritidis	Unknown	2	Zimbabwe	HG1, HC1, HT1
Enteritidis	Unknown	2	Tanzania	HG1 or HG2, HC1, HT1
Typhimurium	Poultry	1	Brazil	HG4, HC4, HT4
Panama	Poultry	1	Brazil	HG5, HC5, HT5
Heidelberg	Poultry	1	Brazil	HG6, HC6, HT6
Senftenberg	Poultry	1	Brazil	HG7, HC7, HT7
Worthington	Poultry	1	Brazil	HG8, HC8, HT8
Orion	Poultry	1	Brazil	HG9, HC9, HT9
Infantis	Poultry	1	Brazil	HG10, HC10, HT10
Adelaide	Poultry	1	Brazil	HG11, HC11, HT11

by electrophoresis in a 1.5% (wt/vol) agarose gel[1] in Tris-borate buffer (0.045 M Tris-borate, 0.001M EDTA). Gel was stained with ethidium bromide[1] (0.5 mg/mL) and registered by digital image capturing system[6]. PCR reactions with each primer were repeated to assess the reproducibility of SE-AFLP patterns.

3. RESULTS

SE-AFLP analysis evaluated DNA segments obtained from *Hin*dIII digestion, which were distributed all over the *Salmonella* genome. The adapter oligonucleotides used were complementary sequences to *Hin*dIII fragments that were ligated to each end of the restriction fragment and were tagged to PCR amplification. Subsets of the ligated fragments were selectively amplified, since the primers used had one additional nucleotide (A, T, G or C) as the final 3' base, which extended into the restriction fragment.

For PCR analysis, $MgCl_2$ concentrations of 2.0, 2.5 and 3.0 mM were initially tested with each primer. Primers HI-C, HI-T and HI-A displayed better amplification patterns using 2.5 mM $MgCl_2$ in the PCR reaction, while primer HI-G gave better results with 3.0 mM $MgCl_2$. These PCR conditions were applied to the remaining *Salmonella* strains.

In an initial screening, randomly selected *S.* Enteritidis cultures were used to assess the suitability of the four selective primers according to quality of the amplified fragments. Each strain was analyzed using one single primer PCR-reaction and each primer tested produced reasonably well-defined banding patterns (data not shown). However, primer HI-A produced few bands, making comparative analysis difficult. So, the remaining primers were selected for further evaluations.

Although there were a number of shared bands, *S.* Enteritidis genotypes could be distinguished from other *Salmonella* serovars by the polymorphism in the number and length of the amplification products (Figure 1). Among the *S.* Enteritidis isolates tested, primer HI-G produced three different genotypes. A pre-

dominant pattern was found in the majority of the strains and designated HI-G1, while the remaining patterns were identified in a strain from Tanzania (HI-G2) and in an isolate from swine (HI-G3). Analysis with primer HI-T produced a predominant pattern (HI-T1) and also two alternative profiles: HI-T2 in a strain from Italy, and HI-T3 in an isolate from swine. Primer HI-C displayed a predominant pattern (HI-C1), the HI-C2 pattern in a poultry strain, and HI-C3 in one isolate from swine (Figure 2). All primers tested clustered the strains from human outbreaks in the predominant SE-AFLP pattern, as well as most of the strains from poultry. Furthermore, the alternative genotypes were identified only in the strains from the other sources and none in the strains related to human outbreaks. *S.* Enteritidis ATCC 13076 displayed the predominant SE-AFLP pattern found with each primer. Table 1 shows the SE-AFLP patterns found in the *Salmonella* strains analyzed.

All SE-AFLP patterns were reproducible. DNA from strains that showed alternative patterns were re-extracted, ligated and amplified. Identical banding profiles were obtained from these strains.

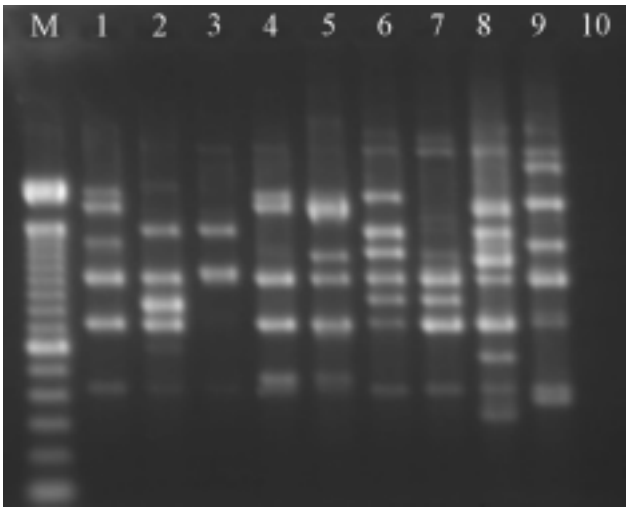

Figure 1. SE-AFLP patterns of the *Salmonella* serovars obtained with primer HI-C. Amplified PCR products were electrophoresed in 1.5% agarose gel stained with ethidium bromide[2]. M, molecular marker[2] (100-bp ladder); 1, *S.* Enteritidis ATCC 13076; 2-9, *S.* Typhimurium, *S.* Heidelberg, *S.* Senftenberg, *S.* Worthingtong, *S.* Orion, *S.* Infantis, *S.* Adelaide and *S.* Panama, respectively; 10, PCR-negative control containing no template DNA.

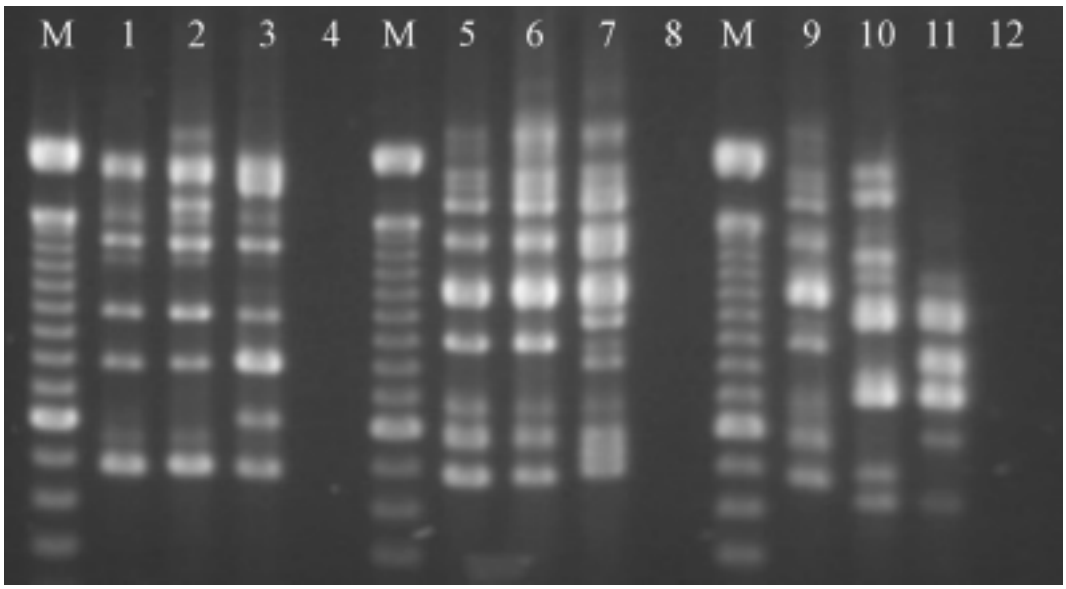

Figure 2. SE-AFLP patterns of the *S.* Enteritidis strains analyzed. Amplified PCR products were electrophoresed in 1.5% agarose gel stained with ethidium bromide[2]. M, molecular marker[2] (100-bp ladder); 1-3, patterns obtained with primer HI-G; 5-7, patterns obtained with primer HI-T; 9-11, patterns obtained with primer HI-C; 4, 8 and 12, PCR-negative control containing no template DNA.

4. DISCUSSION

The present study describes the application of SE-AFLP protocol for genotyping *S.* Enteritidis strains isolated from different sources in Brazil. *S.* Enteritidis infections are worldwide associated with the consumption of *S.* Enteritidis contaminated poultry-derived products [5,22]. In Brazil, *S.* Enteritidis outbreaks are probably related to the high incidence of this pathogen in broiler chickens [23], and most of the occurrences reported were associated with poultry products, mainly eggs [24-25]. This is why the majority of the strains analyzed in the present study were isolated from poultry. In order to test the ability of SE-AFLP in strain characterization, *S.* Enteritidis isolates from other countries were also included, since they are probably unrelated to the Brazilian strains. Although *Salmonella* serovars have been typed by this method in previous studies [18-19], few strains were tested. In the present study, we proposed the analysis of a large number of samples, including strains isolated from different sources and years.

Using SE-AFLP based on primers HI-G, HI-C and HI-T, most *S.* Enteritidis strains displayed a predominant genotype, found in 106 strains. In addition, some strains that shared the same pattern with one primer could be distinguished by another primer. Although previous studies choose one primer only to test bacterial strains by SE-AFLP [19,21], the present study verified that the analysis with different primers by SE-AFLP might produce alternative patterns from the same strain.

As expected, the SE-AFLP approach was able to distinguish *S.* Enteritidis from the other *Salmonella enterica* serovars analyzed. Differences between *S.* Enteritidis pattern and other *Salmonella* serovars have been found in a previous application of SE-AFLP [18], suggesting the reliability of this approach for *Salmonella* typing.

The *S.* Enteritidis strain isolated from swine had alternative SE-AFLP patterns (HI-T3, HI-G3 and HI-C3), which were different from those found in poultry strains. Although only one porcine strain was analyzed, a different species could spread clones different from those found in poultry. On the other hand, all *S.* Enteritidis strains from salmonellosis outbreaks displayed the same predominant pattern found in the strains isolated from other sources. Since strains from human outbreaks and the strains from the other sources shared the same genotype, probably the same clone was dispersed causing infections in both poultry and humans. Furthermore, the predominant SE-AFLP genotype identified in Brazilian strains was also found in the majority of the *S.* Enteritidis isolated from European and African countries analyzed. This observation can indicate that a predominant clone is spread worldwide. As previously reported, *S.* Enteritidis shows a highly clonal population structure [6,26], which may be the result of modern poultry farming practices and drove the clonal expansion of a single isolate following its introduction into breeding lines through international trade [27,28]. In Brazil, the increase of *S.* Enteritidis has been associated to the rise of phage type (PT) 4 [3], the clonal structure of which has been reported [6,26]. Since PT4 is also predominant in Southern Brazil [29], it is possible that the present SE-AFLP results confirmed this assumption. Overall, only very accurate approaches may be able to detect minor differences in these strains.

Different molecular approaches have been reported for *S.* Enteritidis typing. Currently, the most reliable and effective approach for *Salmonella* spp. characterization in epidemiological investigations is a combination of different methods [6-9,26,30]. PCR-based approaches are usually adopted by smaller laboratories, but random amplified polymorphic DNA (RAPD) analysis have displayed non-reproducible patterns and inconsistent band intensity [30], and limited discriminatory power has also been reported in repetitive sequence polymerase chain reaction (Rep-PCR) analysis [8]. AFLP has been recorded as highly discriminatory and reproducible [11], although the complexity of the patterns obtained needs to be resolved on acrylamide gels or analysis of fluorescent labeling on automated DNA sequencer. On the other hand, PFGE has been the most consistently standardized method for *S.* Enteritidis genotyping [10], although it is more expensive and time-consuming than PCR-based approaches, which has limited its use. The present study aimed to test SE-AFLP as an alternative approach to PFGE and the original AFLP methodology.

SE-AFLP analysis provides a means for examining DNA segments distributed over the entire *Salmonella* genome, which is advantageous over other methods that evaluate specific genomic sites, as rep-PCR approach [8,31]. Furthermore, multiple bands obtained with this approach are derived from all over the genome, which prevents over interpretation due to point mutations or single-locus recombinations that may

affect other genotypic characteristics [11]. Reproducibility is an essential property of molecular approaches. In the present work, the *S.* Enteritidis strains analyzed were proven to exhibit identical SE-AFLP patterns following DNA re-extraction, which confirmed that the approach was reproducible. Furthermore, SE-AFLP used stringent PCR conditions that minimized the lack of reproducibility commonly observed in RAPD analysis.

In the present study, not all tagged fragments were amplified by PCR, allowing to visualize the results after electrophoresis in agarose gels. SE-AFLP was relatively simpler and cheaper than AFLP and PFGE, although it did not afford sufficient sensitivity to differentiate the *S.* Enteritidis strains analyzed. Other researchers previously demonstrated different *S.* Enteritidis strains sharing the same SE-AFLP pattern; however, the number of strains analyzed was small [10]. This approach displayed less diversity than PFGE in *Salmonella* Typhimurium analyses, although some of these strains were epidemiologically related [19]. It is important to remember that clonal populations are also found in *S.* Typhimurium associated to the appearance of antimicrobial resistance [28]. On the other hand, SE-AFLP was able to distinguish non-clonally related bacteria, as *Clostridium perfringens* [14], *Klebsiella pneumoniae* [15], *Pasteurella multocida* [16], *Campylobacter jejuni* [17] and *Helicobacter pylori* [21].

In order to improve the discriminatory power of SE-AFLP, a mixture of the four primers in the same PCR reaction has allowed a more complex and discriminative pattern of bands in *S.* Enteritidis and *S.* Typhimurium genotyping [32]. More detailed characterization may be obtained by fluorescent AFLP (FAFLP), using two restriction enzymes and fluorescent labeled primers, the amplification fragments of which are detected on automated DNA sequencers. This approach was able to generate distinct profiles in different phage types of *S.* Enteritidis [33] including PT4 [34]. FAFLP also displayed a high discriminatory power with different *Salmonella* serovars [35]. Furthermore, restriction enzymes other than *Hin*dIII might produce different fingerprinting. The fingerprintings obtained with DNA restriction by *Pst*I, *Nhe*I, *Eco*RI and *Xba*I followed by ligation with the appropriate adapters and PCR amplification maximized the SE-AFLP discriminatory power in *Klebsiella pneumoniae* [15].

Nowadays, epidemiological investigations of *S.* Enteritidis include subtyping by molecular approaches, since the phenotypic methods may be unable to find minor differences present in the same strain. In the present study, SE-AFLP displayed reproducible banding patterns, although most of the strains shared a predominant SE-AFLP genotype. The low genetic diversity suggests that the strains analyzed are clonally related. In order to definitively assess SE-AFLP use for *S.* Enteritidis genotyping, further studies using the same strains must compare the SE-AFLP results with the ones generated by other molecular approaches.

5. CONCLUSION

SE-AFLP analysis provided a simple approach for typing *Salmonella*, producing reproducible banding patterns. This approach was able to distinguish the *S. enterica* serovars tested. However, most of *S.* Enteritidis strains isolated from poultry shared a predominant SE-AFLP pattern, which was also found in strains isolated from salmonellosis outbreaks and from other countries. The present findings suggest that most of strains analyzed are clonally related and this predominant genotype is widely spread. Further analysis will have to compare SE-AFLP with other typing methods to assess the discriminatory power of the different methods for *S.* Enteritidis characterization.

Acknowledgements. C.S.L. Vaz and this work were supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, 140993/03-7) and Projeto de Apoio ao Desenvolvimento de Tecnologia Agropecuária para o Brasil (PRODETAB, 083-02/01).

SOURCES AND MANUFACTURERS

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[2]Invitrogen, Carlsbad (CA), USA.
[3]Applied Biosystems GeneAmp 2400, USA
[4]Ge Healthcare, Piscataway (NJ), USA
[5]CenBiot, Porto Alegre (RS), Brazil.
[6]UltraLum, Paramount (CA),USA.

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