DOI: 10.1590/1808-1657v75p0912008

SCIENTIFIC COMMUNICATION

RAPD/PCR AND PHAGE TYPING OF *SALMONELLA* ENTERITIDIS ISOLATED FROM POULTRY AND FOOD POISONING OUTBREAKS

L.R. dos Santos[1], A.R. Ribeiro[2], S.D. de Oliveira[3], L.B. Rodrigues[1], M.L. Flores[4], R.F.F. Lopes[5], V.P. do Nascimento[5]

[1]Universidade de Passo Fundo, BR 285, km 171, CEP 99001-970, Passo Fundo, RS, Brasil. E-mail: luruschel@upf.br

ABSTRACT

Salmonella Enteritidis (SE) is an important pathogen, causing both food poisoning outbreaks in humans and economic losses to the poultry industry, being also widely spread in the environment. This work aimed to identify SE phage types and to standardize the Random Amplified Polymorphic DNA (RAPD) for evaluating SE isolates obtained from different origins. To do so, 238 SE strains were selected, of which 104 were isolated from broiler carcasses, 106 from food samples and human biological materials involved in food poisoning outbreaks and 28 from different poultry materials. Among these 238 SE isolates, 111 were phage typed, and 57.7% (64/111) corresponded to phage type (PT) 4, 32.4% (36/111) to PT 4a, 3.6% (4/111) to PT 6a and 0.9% (1/111) to PT 7, whereas 5.4% (6/111) of the strains were not typeable (RDNC, reacts but does not conform). After the standardization of amplification conditions, all 238 SE isolates were submitted to RAPD/PCR. Among these, 91.8% (217/238) were classified as pattern A. Twenty-one isolates were differentiated into four patterns and into seven subtypes with the use of primer 1254, and into four patterns and ten subtypes using primer OPB 17. The combination of phage typing and RAPD/PCR proved to be a useful tool in epidemiological investigations. RAPD/PCR can be easily used as a routine laboratory method, thus helping with the monitoring of SE isolates and contributing to the establishment of effective *Salmonella* Enteritidis control and preventive programs.

KEY WORDS: *Salmonella* Enteritidis, phage typing, RAPD.

RESUMO

RAPD/PCR E FAGOTIPAGEM DE *SALMONELLA* ENTERITIDIS ISOLADAS DE AVES E DE SURTOS DE TOXINFECÇÕES ALIMENTARES. *Salmonella* Enteritidis (SE) é um patógeno de importância destacada como causa de toxinfecções alimentares em humanos, prejuízos ao setor produtivo e ampla disseminação no ambiente. Este trabalho objetivou identificar fagotipos e padronizar a RAPD/PCR (DNA polimórfico amplificado ao acaso) para a avaliação de isolados de SE. Foram selecionadas 238 amostras, sendo 104 oriundas de carcaças de frango, 106 de alimentos e material biológico de humanos isolados em episódios de toxinfecções alimentares e 28 de materiais de origem avícola. Foram fagotipadas 111 amostras sendo 57,7% (64/111) do fagotipo 4, 32,4% (36/111) fagotipo 4a, 3,6% (4/111) fagotipo 6a e 0,9% (1/111) fagotipo 7, enquanto 5,4% (6/111) não foram fagotipáveis (RDNC, *reagent do not conform*). Para a RAPD/PCR foram utilizados 238 isolados de SE. Destes, 91,8% (217/238) foram enquadrados no padrão A e 21 isolados (8,8%) foram diferenciados em quatro padrões e sete subtipos com o *primer* 1254 e em quatro padrões e dez subtipos com o *primer* OPB 17. A facilidade de execução da RAPD/PCR, após padronizada, habilita a sua implantação em uma rotina laboratorial, auxiliando na monitoria dos isolados de SE e, conseqüentemente, contribuindo para a elaboração de programas efetivos de controle e prevenção de *S.* Enteritidis.

PALAVRAS-CHAVE: *Salmonella* Enteritidis, fagotipagem, RAPD.

[2]Ministério da Agricultura, Pecuária e Abastecimento, Porto Alegre, RS, Brasil.
[3]Pontifícia Universidade Católica, Porto Alegre, RS, Brasil.
[4]Universidade Federal de Santa Maria, Santa Maria, RS, Brasil.
[5]Universidade Federal do Rio Grande do Sul Porto Alegre, RS, Brasil

Salmonellosis is one of the most important zoonotic diseases worldwide especially because of the difficulty in controlling its occurrence and the extraordinary number of infection sources, including several sources of animal protein for man. Birds are arguably one of the major vectors of this infection, spreading it with relative ease to other animal species, including humans (Hofer, 1997). Bacterial typing methods are based on phenotypic and/or genotypic analysis of a particular species, with the aim of identifying characteristics that allow its subdivision. Such analysis can be used for several purposes, such as epidemiological investigation, determination of recurrent infections, establishment of associations between specific clinical syndromes and characterization of pathogenic mechanisms (Power, 1996). For Ridley et al. (1998), differentiating *Salmonella* Enteritidis (SE) isolates is essential for the effective investigation of salmonellae sources of infection and phage typing can be a the method of choice for the primary differentiation of SE. However, the intraserovar differentiation by methods based only on phenotypic properties does not produce satisfactory outcomes. These methods are unable to detect changes in the bacterial genome, which can occur in the same serovar through plasmid acquisition or spontaneous genetic mutations, without presenting phenotype alterations (On; Baggesen, 1997). Consequently, such epidemiological markers have limited applicability for the subtyping of strains (Lin et al., 1996). Polymerase Chain Reaction have demonstrated good applicability for epidemiological studies such as Random Amplified Polymorphic DNA (RAPD/PCR) (Willians et al., 1990), Arbitrarily Primed PCR (AP-PCR), (Welsh; McClelland, 1990) and DNA-amplified fingerprinting (DAF), (Caetano-Anolles, 1993), where randomly chosen primers bind to multiple loci along the genome, without the need of any previous knowledge of the DNA sequence intended to be analyzed, producing characteristic amplified products of a strain (Meunier; Grimont, 1993). Amplification protocols, particularly annealing temperature, DNA polymerase and the composition of solutions are critical points of RAPD/PCR. Based on that, the standardization of the technique using reference strains would be the first step for the establishment of methodologies applied to each serovar (Hilton et al., 1997). Thus, the present work aimed to identify the phage types of *Salmonella* Enteritidis isolated from broiler carcasses, poultry and human food and biological material implicated in food poisoning outbreaks and to apply RAPD/PCR for the molecular analysis of these strains.

Out of two hundred and thirty-eight cultures of *Salmonella* Enteritidis analyzed, 104 were isolated from chicken carcasses, 106 were from food and human biological materials involved in episodes of food poisoning, whereas 28 were of poultry origin. One hundred and eleven cultures of SE were phage typed (Santos et al., 2003) at the Oswaldo Cruz Institute, Rio de Janeiro, Brazil. Cultures of SE isolated from poultry meat were obtained from carcasses in southern Brazil, and submitted to microbiological examination between 1995 and 1996 at the Center for Diagnosis and Research on Avian Pathology (CDPA) of Federal University of Rio Grande do Sul (UFRGS). The cultures of SE isolated in food poisoning episodes were obtained from the Department of Bacteriology of the Central Laboratory of Public Health of the State of Rio Grande do Sul. The strains were collected in different regions of the state between 1995 and 1997, and were isolated from food (63 strains) and from human biological materials (43 strains). These isolates were characterized at the Adolfo Lutz Institute, São Paulo, Brazil. SE isolates from different poultry materials were processed between 1999 and 2000 at a privately owned laboratory in the state of Santa Catarina, southern Brazil, and characterized at the Oswaldo Cruz Institute, Rio de Janeiro. DNA extraction used phenol-chloroform protocol (Santos et al., 2001). RAPD/PCR primers used were OPB 17 (Lin *et al.*, 1996) and 1254 (Hilton et al., 1997). The reaction was performed in a 25µL volume containig 2.5µL 10X buffer (10 mM of $MgCl_2$, 500 mM of KCl, 100 mM of Tris HCl pH 8.3; Cenbiot Enzimas); 2µL of the dNTP (5 mM; GibcoBRL); 2 mL (20 pmol) of each primer; 0.2µL of *Taq* DNA polymerase 5 U/mL (Cenbiot Enzymes); 2 µL of DNA sample and water to the final volume of 25µL. The amplifications were performed in a Perkin Elmer Gene Amp PCR System 2400 (Perkin Elmer, USA) and in a PTC – 100™ thermocycler (MJ Research, INC., USA). The cycles were 94°C for 5 minutes, 40 cycles (94° C/1 minute), annealing (30° C/1 minute), extension (72° C/1 minute), extension of 72°C for 5 minutes and PCR products visualized in 1.2% agarose gel. The criteria for the interpretation of RAPD/PCR patterns are presented in Table 1. Isolates with the same pattern of the control were identified as related or possibly related and classified as pattern A and their subtypes as A1, A2, A3, and so on and so forth. Isolates with patterns that differ from those of the control were considered unrelated and categorized as pattern B, C, D, and so on and so forth.

In the phage typing of 111 isolates of SE, 57.7% of the samples corresponded to phage type 4, 32.4% to phage type 4a, 3.6% to phage type 6a and 0.90% to phage type 7, while 5.4% of the samples were non-phage typeable (RDNC: reacts but does not conform). Out of 238 SE samples submitted to RAPD/PCR, 91.8% (217/238) were classified as pattern A (related or possibly related). Twenty-one (8.8%) were differentiated into four patterns (A, B, C and D) and into seven subtypes (A1, A2, A3, A4, A5, A6 and A7) using primer 1254, and into four patterns (A, B, C and D) and into 10 subtypes (A1, A2, A3, A4, A5, A6, A7, A8, A9 and A10) using primer OPB 17.

Table 1 - Criteria for the interpretation of RAPD/PCR patterns.

Category	Number of genetic differences compared with the sample pattern	Number of different fragments compared with sample pattern
Indistinctive	Any one	Any one
A (related)	1	2-3
A (possibly related)	2	4-6
B, C,D (unrelated)	≥3	≥ 7

From the 43 isolates obtained from human 42 (97.8%) were classified as pattern A (related or possibly related). Among the 111 isolated strains of SE obtained from carcasses, 102 (91.9%) were classified as pattern A. Among the 28 SE strains obtained from different materials of poultry origin, 18 (64.3%) were categorized as pattern A, 10 as pattern A or as subtype A1 with primer 1254, and subtyped as A1, A2, A3 and A4 with primer OPB 17.

The *S.* Enteritidis selected for this work can be considered epidemiologically related, since 91.8% (217/238) of the strains were classified as pattern A. although the DNA amplification fragments seem to have the same molecular weight, in agreement with Hilton et al. (1996), they are not necessarily identical fragments. The present study is in consonance with Stanley; Baquar (1994), Olsen et al. (1994), Usera et al. (1994), Millemann et al. (1995), Thong et al. (1995) and Tassios et al. (1997), who reported low genetic variability among the strains of *S.* Enteritidis. The isolated strains of SE have demonstrated to be clonally related, even when they originate from distant geographic areas (Thong et al., 1995), from unrelated outbreaks (Usera et al., 1994) or from different periods of time (Tassios et al., 1997). The results of RAPD/PCR of this work are in agreement with the ones observed by Tassios et al. (1997), who analyzed 355 isolates of SE using PFGE, obtaining only two patterns, which represent 90 and 10% of the isolates, respectively.

Although the subtyping of SE strains is limited by the high homogeneity of the isolates of this serovar (Nastasi et al., 1997; Laconcha et al. 1998), molecular typing techniques can represent an additional discriminatory power. In the present study RAPD/PCR allowed the differentiation of only three isolates, all of which were characterized as phage type 4. Isolates 898, 936 and 872 were classified as pattern B, C and D, respectively, and were not considered related to pattern A of PT 4 (control strain) using primers 1254 and OPB 17. Millemann et al. (1995) report that molecular typing systems can be used for the investigation of outbreaks, confirmation and characterization of the transmission patterns of one or more clones, hypothesis tests on the origin and vectors of transmission of these clones, and monitoring of their reservoirs. According to the authors, molecular analysis would also be useful for epidemiological surveys and for the evaluation of control measures through the documentation of the occurrence of certain serovars over time and of the circulation of clones in infected populations.

The RAPD/PCR used this work can be an epidemiological tool for the typing of indistinct and related isolates, excluding unrelated ones. The exclusion criteria can be used for the evaluation of specific cases. A practical application of RAPD/PCR is given by Laconcha et al. (1998) through the analysis of a food poisoning outbreak in humans produced by SE. In this outbreak, cheese and egg-containing foods were pointed out as possible contaminating agents and RAPD/PCR demonstrated that the SE strains isolated from egg-containing foods belonged to pattern A, while the isolates obtained from cheese and from humans were classified as pattern B, excluding egg-containing foods as contaminating agents.

In agreement with Mendonza; Landeras (1999), a typing method should be proposed, using larger samples, reference strains, and sporadic or epidemic isolates, and then comparing the previous results with the patterns established. Therefore, RAPD/PCR can be used in a laboratory as an additional tool for the analysis of *Salmonella* Enteritidis.

The combination of phage typing and RAPD/PCR proved to be a useful tool in epidemiological investigations. RAPD/PCR can be easily used as a routine laboratory method, thus helping with the monitoring of SE isolates and contributing to the establishment of effective *Salmonella* Enteritidis control and preventive programs.

References

CAETANO-ANOLLES, G. Amplifying DNA with arbitrary oligonucleotide primers. *PCR Methods Application,* v.3, p.85-94, 1993.

HILTON, A.C.; BANKS, J.G.; PENN, C.W. Optimization of RAPD for fingerprinting *Salmonella*. *Letters in Applied Microbiology*, v.24, p.243-248, 1997.

Hofer, E. Prevalência de sorovares de *Salmonella* isolados de aves no Brasil. *Pesquisa Veterinária Brasileira,* v.17, p.55-62, 1997.

LACONCHA, I.; LÓPEZ-MOLINA, N.; REMENTERIA, A.; AUDICANA, A.; PERALES, I.; GARAIZAR, J. Phage typing combined with pulsed-field gel electrophoresis and random amplified polymorphic DNA increases discrimination in the epidemiological analysis of *Salmonella enteritidis* strains. *International Journal of Food Microbiology*, v.40, p.27-34, 1998.

LIN, A.W.; USERA, M.A.; BARRET, T.J.; GOLDSBY, R.A. Application of random amplified polymorphic DNA analysis to differentiation strains of *Salmonella enteritidis*. *Journal of Clinical Microbiology,* v.34, p.870-876, 1996.

MENDONZA, M.C.; LANDERAS, E. Molecular epidemiological methods for differentiation of *Salmonella enterica* serovar Enteritidis strains. *Salmonella enterica* serovar Enteritidis in humans and animals – epidemiology, pathogenesis and control. Ames: *Iowa State University Press*, 1999. p.125-140.

MEUNIER, J.R.; GRIMONT, P.A.D. Factors affecting reproducibility of random amplified polymorphic DNA fingerprinting. *Research in Microbiology*, v.144, p.373-379, 1993.

MILLEMANN, Y.; LESAGE, M.C.; CHASLUS-DANCLA, E.; LAFONT, J.P. Value of plasmid profiling, ribotyping, and detection of IS200 for tracing avian isolates of *Salmonella typhimurium* and *S. enteritidis. Journal of Clinical Microbiology*, v.33, p.173-179, 1995.

NASTASI, A.; MAMMINA, C.; FANTASIA, M.; PONTELLO, M. Epidemiological analysis of strains of *Salmonella enterica* serotype Enteritidis from foodborne outbreaks occurring in Italy, 1980-1994. *Journal of Medical Microbiology*, v.50, p.379-382, 1997.

OLSEN, J.E.; SKOV, M.N.; THRELFALL, E.J.; BROWN, D.J. Clonal lines of *Salmonella enterica* serotype enteritidis documented by IS 200, ribo-pulsed-field gel electrophoresis and RFLP typing. *Journal of Medical Microbiology*, v.40, p.15-22, 1994.

ON, S.L.W.; BAGGESEN, D.L. Determination of clonal relationships of *Salmonella typhimurum* by numerical analysis of macrorestriction profiles**.** *Journal of Applied Microbiology,* v.83, p. 699-706, 1997.

POWER, E.G.M. RAPD typing in microbiology – a technical review. *Journal of Hospital Infection,* v. 34, p.247-265, 1996.

RIDLEY, A.M.; THRELFALL, E.J.; ROWE, B. Genotypic characterization of *Salmonella enteritidis* phage types by plasmid analysis, ribotyping, and pulsed-field gel electrophoresis. *Journal of Clinical Microbiology*, v.36, p.2314-2321, 1998.

SANTOS, L.R. DOS; NASCIMENTO, V. P. DO; OLIVEIRA, S.D.; FLORES, M.L.; PONTES, A.P.; RIBEIRO, A.R.; SALLE, C.T.P.; LOPES, R.F.F. Polymerase chain reaction (PCR) for the detection of *Salmonella* in artificially inoculated chiken meat. *Revista do Instituto de Medicina Tropical de São Paulo.* v. 43, p.247-250, 2001.

SANTOS, L.R. DOS; NASCIMENTO, V. P. DO; OLIVEIRA, S.D.; RODRIGUES, D.P., REIS, E.M.F.; SEKI, L.M.; RIBEIRO, A.R.; FERNANDES, S.A. Phage types of *Salmonella* Enteritidis isolated from clinical and food samples, and from broiler carcasses in southern Brazil. *Revista do Instituto de Medicina Tropical de São Paulo*. v.45, p.1-4, 2003.

STANLEY, J.; BAQUAR, N. Phylogenetics of *Salmonella enteritidis***.** *International Journal of Food Microbiology,* v.21, p.79-87, 1994.

TASSIOS, P.T.; MARKOGIANNAKIS, A.; VATOPUOLOS, A.; KATSANIKOU, E. Molecular epidemiology of antibiotic resistance of *Salmonella enteritidis* during a 7-year period in Greece. *Journal of Clinical Microbiology*, v.35, p.1316-1321, 1997.

THONG, K.; NGEOW, Y.; ALTEWEGG, M.; NAVARATNAM, P; PANG, T. Molecular analysis of *Salmonella enteritidis* by pulsed-field gel electrophoresis and ribotyping. *Journal of Clinical Microbiology*, v.33, p.1070-1074, 1995.

USERA, M.A.; POPOVIC, T.; BOPP, C.A.; Molecular subtyping of *Salmonella enteritidis* phage type 8 strains from the United States. *Journal of Clinical Microbiology*, v.32, p.194-198, 1994.

WELSH, J.; MCCLELLAND, M. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research*, v.18, p.7213-7218, 1990.

WILLIANS, J.G.K.; KUBELIK, A.R.; LIVAK. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, v.18, p.6531-6535, 1990.

Received on 8/1/07
Accepted on 6/3/08