Journal of Industrial Microbiology & Biotechnology (1998) 21, 141–144

http://www.stockton-press.co.uk/jim

Validation of ERIC PCR as a tool in epidemiologic research of *Salmonella* in slaughter pigs

M Swanenburg, HAP Urlings, DA Keuzenkamp and JMA Snijders

Department of the Science of Food of Animal Origin, Faculty of Veterinary Medicine, PO Box 80.175, 3508 TD Utrecht, The Netherlands

The purpose of this study was to test a protocol for a standardized ERIC PCR for its capability of genotyping *Salmonella*, isolated from pigs and their environment, in an epidemiologic approach. To test repeatability, four different *Salmonella* isolates were subjected to PCR three times. Furthermore, it was tested if the profiles on gel differed when a higher annealing temperature was used. Four *Salmonella* isolates were subjected to four different annealing temperatures (36, 40, 48 and 55°C). Moreover it was tested if the differentiation of *Salmonella* isolates, based on the genotypes, differed when a higher annealing temperature was used. Eight *Salmonella* isolates were tested at normal (36°C) and high (55°C) annealing temperatures. The results showed that this standardized ERIC PCR protocol was an efficient tool for typing many *Salmonella* isolates within a short period of time. The profiles were repeatable within one PCR reaction, but some profiles differed when they were compared between reactions. A higher annealing temperature resulted in profiles that contained more or fewer bands. The differentiation between isolates, when comparing profiles, remained the same. It was concluded that the standardized ERIC PCR protocol is useful for genotyping *Salmonella*.

Keywords: *Salmonella*; pigs; ERIC PCR; epidemiology

Introduction

Salmonella is an important cause of food-borne infections and some of these infections are caused by *Salmonella* originating from pork. The source of *Salmonella* contamination of pork is not always easy to discover. The source can be the farm, on which the pig is raised, or the pig may become infected or contaminated during further phases in the pork production chain (during transport or slaughter). Therefore, typing of *Salmonella* isolates, obtained during various points of the pork production chain could be an important tool to elucidate the main sources of contamination.

Many different typing methods for *Salmonella* have been used. Serotyping is one of the most commonly used typing methods, although it is a rather time-consuming and costly method. Genotyping methods for *Salmonella* (such as PCR, RAPD, RFLP, PFGE) are becoming more common [2–4,6,7]. Versalovic *et al* [8] described the presence of repetitive sequences of DNA in some eubacteria (ERIC). They showed that PCR amplification of *Salmonella* with primers containing the ERIC-sequence, followed by agarose gel electrophoresis, produced clearly resolvable bands. Van Lith *et al* [5] showed that ERIC PCR of *Salmonella* resulted in a typing of *Salmonella* to the serotype level although some variation in the profiles could occur within replicates.

The purpose of this study was to test a protocol for ERIC PCR in which some steps of the PCR reaction and the electrophoresis were standardized in order to avoid the disadvantage of varying profiles. This standardized ERIC PCR, which was based on the method of Versalovic, was tested for its capability of performing a quick and easy typing of *Salmonella* isolates originating from pigs and their environment, in an epidemiologic approach. The PCR reaction and electrophoresis were standardized by using preprepared polyacrylamide gels, Ready-to-go-beads and a silver staining kit. Furthermore, typing results were tested to determine if they were repeatable between different PCR reactions and if a higher annealing temperature of the reaction influenced the results.

Correspondence: M Swanenburg, Dept of the Science of Food of Animal Origin, Faculty of Veterinary Medicine, PO Box 80.175, 3508 TD Utrecht, The Netherlands
Received 26 January 1998; accepted 3 August 1998

Materials and methods

Bacterial isolates

A total of 744 *Salmonella* isolates were analysed. They were obtained from sampling in a pig slaughterhouse. Samples were obtained from slaughtered pigs (faeces, lymphonodes, tonsils, liver swabs, tongue swabs and carcass swabs) as well as from slaughter equipment (swabs). *Salmonellae* were isolated by standard procedures (preenrichment 24 h in buffered peptone water at 37°C, enrichment 24–48 h in Rappaport Vassiliadis at 42°C, 24 h on brilliant green agar at 37°C, confirmation of five suspected colonies per plate in triple sugar iron 24 h at 37°C). Isolated *Salmonella* strains were serotyped with group-sera.

Preparation of DNA

The *Salmonella* isolates were grown overnight at 37°C in brain heart infusion broth. Two millilitres of the bacterial culture were centrifuged for 10 min at 16 000 × *g* in an Eppendorf centrifuge. The pellet was washed in 900 μl of a physiological saline solution, centrifuged again for 10 min at 16 000 × *g* and then resuspended in 500 μl milliQ water. This suspension was heated for 10 min in a thermo-block

at 100°C and then cooled on ice, whereafter it was centrifuged for 5 min at 8100 × *g*. For the PCR reaction the supernatant fluid was diluted 20 times. The amount of isolated DNA of a representative number of samples was determined by measuring the optical density of the solution after DNA isolation. The yields of DNA isolated from the same strains or different strains did not differ significantly. For this reason it was decided to take the step of OD-measuring out of the protocol to save time.

PCR reaction

Reaction conditions were standardized by using Ready-to-go-beads (Pharmacia Biotech, Roosendaal, The Netherlands), containing the right amounts of dNTPs (0.4 mM each dNTP in a 25-μl reaction volume), polymerases (AmpliTAQ and Stoffel fragment), BSA (2.5 μg) and buffer (3 mM $MgCl_2$, 30 mM KCl and 10 mM Tris in a 25-μl reaction volume). The primers ERIC1R (3′-CACTTAGGGGTCCTCGAATGTA-5′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) were synthesized by Perkin Elmer (Nieuwerherk a/d Ÿssel, The Netherlands). For each reaction, 20 μl reaction mix, containing 1 μl ERIC1R (25×10^{-6} μmol μl^{-1}), 1 μl ERIC2 (25×10^{-6} μmol μl^{-1}) and 18 μl H_2O was added to one bead, together with 5 μl of the diluted DNA suspension. The PCR reaction mix was overlaid with mineral oil and centrifuged for a few seconds to ensure separation of the phases.

Amplification was performed in a Perkin Elmer thermal cycler 480 as follows: one cycle of 7 min at 95°C, followed by 40 cycles of 1 min at 94°C, 1 min at 36°C and 4 min at 72°C. The amplification ended with one cycle of 10 min at 72°C. The reaction products were stored at 5°C until they were electrophoresed.

DNA analysis

After PCR, 5 μl loading buffer was added to the 25-μl reaction mix. Five microlitres of this portion were loaded on a polyacrylamide gel (12.5%, 48S ExelGel, Pharmacia Biotech) for electrophoresis. Furthermore, on each gel, three molecular weight markers (100-bp ladder, Pharmacia Biotech) and a negative control sample were loaded routinely.

The DNA fragments were coloured by silver staining (DNA Silver Staining kit, Pharmacia Biotech). After drying, the gels were covered with a plastic layer. Profiles were compared to divide the *Salmonella* isolates into different genotypes. Isolates of the same serogroup were compared with each other in one PCR reaction and loaded on the same gel in order to group as many identical types as possible.

Serotyping

From each genotype and every gel a representative number of isolates (236 in total) was serotyped, according to the Kauffmann–White scheme, at the *Salmonella* reference centre RIVM at Bilthoven, the Netherlands. Serotyping was carried out with *Salmonella* anti-sera that were prepared at the RIVM.

Testing repeatability of profiles within and between different PCR reactions

Four different *Salmonella* serotypes were selected (*S. typhimurium*, *S. livingstone*, *S. panama* and *S. london*). From each isolate DNA was prepared by two technicians simultaneously. The resulting eight DNA samples and a negative control sample were amplified by ERIC PCR, loaded on a polyacrylamide gel for electrophoresis and thereafter stained by silver staining. This procedure (DNA preparation, PCR and electrophoresis) was repeated twice on consecutive days.

Effect of annealing temperature on profiles

Four different *Salmonella* serotypes were selected (*S. typhimurium*, *S. livingstone*, *S. panama* and *S. london*). DNA was prepared and amplified by ERIC PCR four times at the following annealing temperatures: 36°C, 40°C, 48°C and 55°C. Thereafter, the samples were loaded on the same polyacrylamide gel for electrophoresis and stained using the silver staining kit. The resulting profiles were compared concerning the number of bands per profile and the intensity of the individual bands.

Effect of annealing temperature on differentiation of isolates

Eight *Salmonella* isolates, appearing to have different profiles, were selected for this experiment: two *S. typhimurium* isolates, with different profiles, *S. brandenburg*, *S. derby*, *S. infantis*, two *S. livingstone* isolates with different profiles and *S. bovismorbificans*. DNA was prepared twice from each isolate and amplified by ERIC PCR with an annealing temperature of 36°C. The PCR reaction was repeated the next day with an annealing temperature of 55°C. All amplified products were loaded on the same polyacrylamide gel for electrophoresis and stained with the silver staining kit.

Results

Typing according to the standardized protocol for ERIC PCR appeared to be a useful method for differentiating *Salmonella* isolates in an epidemiologic approach. The resulting profiles on the gels consisted of bright, clear bands and were easy to distinguish visually.

Typing four *Salmonella* isolates on three separate days showed that profiles from the same isolates, subjected to different PCR reactions and loaded on different gels were not always consistent (Figure 1). Major bands were present in all the profiles from all isolates, however some minor bands were not consistent. Duplicate profiles from the same isolate that had been subjected to the same PCR reaction and loaded on the same gel were always identical, although the DNA had been prepared by different technicians. It was concluded that profiles can only be compared within one PCR reaction.

Typing four *Salmonella* isolates at increasing annealing temperatures (from 36°C to 55°C) showed that the major bands of the profiles were consistent. Some minor bands disappeared (*S. panama* and *S. london*, a 500-bp band, Figure 2) at higher annealing temperatures, but in some cases more bands appeared (*S. typhimurium* and *S. panama*, between 650 and 900 bp, Figure 2) or bands became more

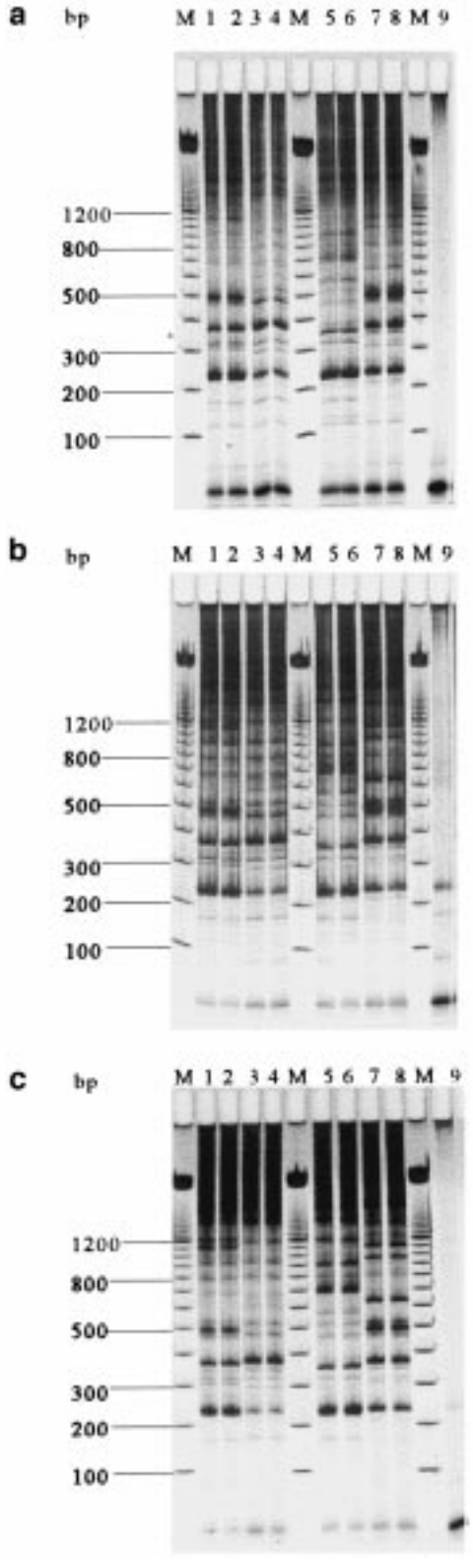

Figure 1 ERIC PCR profiles of *Salmonella* isolates. (a) Day 1; (b) day 2; (c) day 3. Lanes 1–2: *S. typhimurium*; lanes 3–4: *S. livingstone*; lanes 5–6: *S. panama*; lanes 7–8: *S. london*; lane 9: negative control sample; M: molecular weight marker; 100-bp ladder. The band near the bottom of the gel consists of the primers.

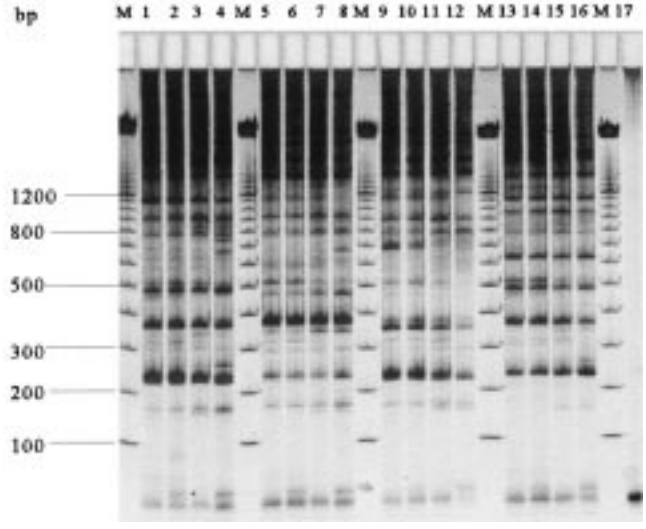

Figure 2 ERIC PCR profiles of *Salmonella* isolates using increasing annealing temperatures: 36°C, 40°C, 48°C, 55°C from left to right. Lanes 1–4: *S. typhimurium*; lanes 5–8: *S. livingstone*; lanes 9–12: *S. panama*; lanes 13–16: *S. london*; lane 17: negative control sample; M: molecular weight marker; 100-bp ladder. The band near the bottom of the gel consists of the primers.

intense. Typing eight isolates at annealing temperatures of 36°C and 55°C showed that the differentiation (based on genotype/profile) between these isolates was consistent when the annealing temperature was increased to 55°C, although each isolate showed different profiles at annealing temperatures of 36°C and 55°C. At both annealing temperatures the eight isolates showed eight different profiles. Duplicates (same isolate, same annealing temperature) showed identical profiles. It was decided to keep the annealing temperature at 36°C, as in the original protocol, as no differences were found in the interpretation of the results at annealing temperatures of 36°C and 55°C.

Genotyping 744 *Salmonella* isolates with this standardized protocol for ERIC PCR resulted in 15 different genotypes. Serotyping 236 isolates in total of these 15 genotypes resulted in 12 different serotypes (*S. typhimurium*, *S. derby*, *S. bredeney*, *S. brandenburg*, *S. infantis*, *S. livingstone*, *S. bovismorbificans*, *S. mbandaka*, *S. panama*, *S. give*, *S. london* and SI 4,5,12:d:2ef nat). Genotypes were serotype-specific: each serotype could be divided into one or more genotypes, while each genotype always had the same serotype.

Discussion

Our results show that the standardized method of ERIC PCR, which is based on the method of Versalovic [8], seems to be very useful for a quick typing of many *Salmonella* isolates in a well defined epidemiologic approach. DNA was prepared by heating, which is an easy and quick method when compared to chloroform-ethanol extraction. Although this method of DNA-preparation yielded no pure DNA, this did not seem to disturb the reaction. As the gel contains 48 slots, 44 isolates could be typed at once, together with three molecular weight markers and a negative control sample.

The method was standardized as much as possible to improve repeatability of the profiles. The use of Ready-to-go beads, which contain all ingredients for the reaction in the right amounts, avoids many pipetting steps, guaranteeing equal concentrations for each reaction. The use of pre-prepared polyacrylamide gels also has the advantage of equal concentrations. Moreover, these gels avoid the use of ethidium bromide, which is carcinogenic. Furthermore, these gels have the advantage that no photograph needs to be taken. The gels can be kept in plastic and do not lose their colour. The use of the silver staining kit guarantees equal concentrations of staining solutions for every gel.

Although the method was standardized as much as possible, the results showed that isolates that were amplified on three different days did not always have repeatable profiles. Minor bands appeared or disappeared. This is possibly due to small differences in DNA concentrations, as all other reaction circumstances had been standardized. It can be concluded that only profiles resulting from the same PCR reaction and loaded on the same gel can be compared, which demands reference isolates from each known genotype on every gel.

The results showed that the annealing temperature influences the resulting profiles. Although we expected that profiles would contain fewer bands when annealing temperature increased because annealing of the primers to the DNA will be more specific at higher annealing temperatures, this was not observed. In contrast, some of the resulting profiles consisted of more bands at higher annealing temperatures, although others consisted of fewer bands. We do not have an explanation for the fact that some profiles contained more bands at higher annealing temperatures. Gillings *et al* [1] found that profiles of eukaryota contained fewer bands at higher annealing temperatures, but they tested at higher temperatures (52–66°C) than we did (36–55°C). They concluded that the ERIC primers annealed at anonymous binding sites with only partial homology to the ERIC sequences. They concluded that ERIC PCR is a highly reproducible variant of randomly amplified polymorphic DNA methods (RAPD).

Genotyping 744 *Salmonella* isolates with the standardized method of ERIC PCR resulted in 15 different profiles and thus 15 different genotypes, whereas serotyping resulted in 12 different serotypes. Genotypes were serotype-specific, which had already been reported by van Lith *et al* [5], but we found that some serotypes (*S. typhimurium*, *S. brandenburg* and *S. livingstone*) could be divided in more than one genotype. These results make it possible to do a quick and cheap typing of *Salmonella*.

Acknowledgements

We thank Dr WJ van Leeuwen and the RIVM for serotyping the *Salmonella* isolates. We thank the staff and personnel of the participating slaughterhouse for their cooperation. This work was partially funded by the Dutch Product Boards for Livestock, Meat and Eggs, the Ministry of Agriculture, Nature Management and Fisheries and the European Union (FAIR contract FAIRI CT95–400).

References

1 Gillings M and M Holley. 1997. Repetitive element PCR fingerprinting (rep-PCR) using enterobacterial repetitive intergenic consensus (ERIC) primers is not necessarily directed at ERIC elements. Appl Microbiol Lett 25: 17–21.
2 Hilton AC, JG Banks and CW Penn. 1996. Random amplification of polymorphic DNA (RAPD) of *Salmonella*: strain differentiation and characterization of amplified sequences. J Appl Bacteriol 81: 575–584.
3 Hofstra H and K Wernars. 1993. Molecular biology as a tool in epidemiological research: the use of RAPD, RFLP and PCR methods. In: Interruption of Bacterial Cycles in Animal Production (Urlings HAP, ed), pp 35–42, VVDO, Utrecht.
4 Lin AW, MA Usera, TJ Barrett and RA Goldsby. 1996. Application of random amplified polymorphic DNA analysis to differentiate strains of *Salmonella enteritidis*. J Clin Microbiol 34: 870–876.
5 Van Lith LAJT and HJM Aarts. 1994. Polymerase chain reaction identification of *Salmonella* serotypes. Appl Microbiol Lett 19: 273–276.
6 Shah SA and TL Romick. 1997. Subspecies differentiation of *Salmonella* by PCR-RFLP of the ribosomal operon using universal primers. Appl Microbiol Lett 25: 54–57.
7 Suzuki Y, M Ishihara, M Matsumoto, S Arakawa, M Saito, N Ishikawa and T Yokochi. 1995. Molecular epidemiology of *Salmonella enteritidis*. An outbreak and sporadic cases studied by means of pulsed-field gel electrophoresis. J Infect 31: 211–217.
8 Versalovic J, T Koeuth and JR Lupski. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucl Acids Res 19: 6823–6831.