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COMPARISON OF DNA-EXTRACTION METHODS AND SELECTIVE ENRICHMENT BROTHS ON THE DETECTION OF *SALMONELLA TYPHIMURIUM* IN SWINE FECES BY POLYMERASE CHAIN REACTION (PCR)

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ABSTRACT

The aim of this study was to compare different DNA-extraction methods and selective enrichment broths for their effectiveness to detect *Salmonella* Typhimurium in artificially inoculated swine feces samples (100 CFU/g) by polymerase chain reaction. After enrichment in Rappaport-Vassiliadis, selenite cystine or Müller-Kauffmann tetrathionate, aliquots were used for DNA extraction by three different methods: boiling-centrifugation, phenol-chloroform and salting-out. Aliquots of extracted DNA were then used as template in PCR. The selective enrichment broths had no effect on the efficiency of PCR when boiling-centrifugation and salting-out were used. On the other hand, phenol-chloroform was superior ($P<0.05$) when combined to Rappaport-Vassiliadis. Considering cost and efficiency parameters, we encourage the use of Müller-Kauffmann tetrathionate broth in combination with boiling-centrifugation DNA-extraction procedure.

Key words: diagnosis, DNA, feces, PCR, *Salmonella* Typhimurium

INTRODUCTION

Standard methods currently used to detect *Salmonella* clinical samples are laborious and require up to 7 days to obtain results. The polymerase chain reaction (PCR) assay represents a major advance in diagnostic methods in terms of speed and sensitivity. However, sample preparation prior to PCR is necessary, especially for feces, which contain high amount of compounds that are inhibitory for PCR (19). In spite of same enrichment broths to *Salmonella* are inhibitory for PCR (18), combination between different selective enrichment broths and PCR methods has been tested in order to improve sensitivity and dilute PCR inhibitory substances (6,15,18). The decision about the best methodology to be used to detect *Salmonella* in feces is very difficult due to the large amount of data that have been published. The aim of this study was to compare three DNA extraction methods (boiling-centrifugation, salting-out, phenol-chloroform) in combination with three different selective enrichment broths (Rappaport-Vassiliadis, Müller-Kauffmann tetrathionate and selenite cystine) for their effectiveness to detect *Salmonella* Typhimurium in artificially inoculated porcine fecal samples by PCR.

MATERIALS AND METHODS

Bacteria

Salmonella enterica subspecies *enterica* serovar Typhimurium (accession number 1007/00) previously isolated from swine feces was obtained from the culture collection of Instituto Adolfo Lutz, São Paulo, Brazil.

Inoculation of feces samples

Feces samples were collected from 10 sows of a farm where no *Salmonella* had been isolated before (12), stored in sterile plastic bags and taken to the laboratory under refrigeration to be processed on the same day. Each sample was subdivided in

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two 10-gram sub-samples. A fresh *Salmonella* Typhimurium culture prepared in tryptone soya broth was serially diluted and the number of *Salmonella* cells in each dilution was determined using the drop-counting technique (10). An aliquot (1 mL) of the dilution containing approximately 10^3 CFU/mL of *Salmonella* was added to one 10-gram sub-sample to obtain a final concentration of 10^2 CFU/g. The second 10-gram sub-sample was inoculated with 1 mL of phosphate-buffered saline (pH 7.4) as negative control.

Examination of spiked feces samples

Feces samples (10g) were pre-enriched in 90 mL of Hajna-GN broth (GN) at 37ºC for 6 hours. After pre-enrichment, 0.1 mL aliquot were transferred to 9.9 mL of Rappaport-Vassiliadis (RV), which were incubated at 42ºC for 24 hours. One mL aliquots of pre-enrichment broths were transferred to 9.0 mL of Müller-Kauffmann tetrathionate (MK) and 9.0 mL of selenite-cystine broth (SC), which were incubated at 37ºC for 24 hours and at 37ºC for 48 hours, respectively. After incubation, enrichment cultures were sub-cultured onto xylose-lysine-tergitol 4 (XLT4) agar plates, which were incubated overnight at 37ºC. Presumptive *Salmonella* colonies were inoculated on triple-sugar-iron agar and lysine-iron agar slants. All strains presumptively identified as *Salmonella enterica* were confirmed by slide agglutination test using poli-O and poli-H antiserum, as described by Poppoff and Le Minor (14).

DNA-extraction

The DNA extractions of enrichment broths were performed by following methods:

Boiling-centrifugation (M1) as described by Soumet *et al*. (17). One mL aliquots of enrichment broths were centrifuged 13,000 x *g*, 3 minutes. The pellets were resuspended in 100 µL of sterile bi-distilled water, heated to 95ºC in a dry block for 10 min, cooled in ice and centrifuged at 13,000 x g for 3 min. These supernatants were used for PCR assay.

Salting-out (M2) was carried out using a commercial kit (Puregene®, Gentra Systems) according to the instructions provided by the manufacturer.

Phenol-chloroform (M3) as described by Fadl *et al*. (4). Aliquots (1 mL) were centrifuged (2,000 x*g*, 4 minutes) and the pellet was resuspended in 474 µL of TE (10 mM Tris-HCL pH 8, 1 mM Na_2EDTA), 25 µL 10% SDS and 1.25 µL proteinase K (20 mg/mL). After incubation at 55ºC for 30 minutes, 500 µL of phenol-chloroform pH 8 (1:1) was added, mixed vigorously and the samples were centrifuged (10,000 x *g*, 4 minutes). The aqueous phase was transferred to a fresh microtube and the DNA was precipitated with 3M sodium acetate and ice-cold isopropanol for 30 minutes. Samples were centrifuged (16,000 x *g*, 10 minutes) and the pellet was washed with 80% ethanol. The final pellet was resuspended in 50 µL of TE and stored at 4ºC until PCR was performed.

Oligonucleotide primers

The oligonucleotide primers S18 and S19 (Table 1) were based on the DNA sequence of the *omp*C gene of *Salmonella enterica* (9), used to amplify a 159bp fragment. The PCR product corresponded to base positions 1076-1234 bp of *S.* Typhi *ompC* gene (GenBank Accession Number M31424).

Table 1. Sequence and orientation of PCR oligonucleotide primers.

Name	Orientation sequence (5' - 3')
S18	Forward - ACCGCTAACGCTCGCCTGTAT
S19	Reverse - AGAGGTGGACGGGTTGCTGCCGTT

DNA amplification

The PCR mixture contained 5 µL of DNA sample, 2.0 mM MgCl2, 50 µM of each nucleotide, 0.4 µM of each primer and 1 U of *Taq* DNA polymerase in a final volume of 25 µL. Amplifications were performed in a thermal cycler (Personal®, Eppendorf, Germany). After a 3-min denaturation at 96ºC, reaction mixtures were submitted to 10 cycles of amplification at 95ºC for 30 s, 62C for 30 s and 72ºC for 15 s, followed by 25 cycles at 95ºC for 30 s, 62ºC for 30 s and 72ºC for 30 s, and a final extension at 72ºC for 3 min. Amplified PCR products (10 µL) were then analysed by standard gel electrophoresis using 1.5% agarose gel and 1XTBE buffer (0.089 mol l^{-1} Tris-borate, 0.089 mol l^{-1} boric acid and 0.002 mol l^{-1} EDTA, pH 8.0), with a 100 bp ladder (Gibco-BRL, Gaithersburg, MD,USA) as molecular weight marker. Following electrophoresis, gels were stained with ethidium bromide (10 µg/mL) and visualized under U.V. light (254 nm).

PCR sensitivity

The PCR sensitivity was determined by using suspensions of *Salmonella* Typhimurium prepared in tryptone soya broth incubated overnight at 37ºC. Ten-fold serial dilutions were prepared of each selective enrichment broth and the numbers of bacteria were determined on plate count agar after incubation at 37ºC for 24 h. The same DNA-extraction procedures described above were carried out for each enrichment broth.

Statistical analysis

Each selective enrichment broth combined to DNA extraction method was repeated two separate times. The Binomial Test (16) was used to analyse the proportion of *Salmonella* positive samples in each selective enrichment broth associated with each DNA extraction method. Differences were considered significant at $P<0.05$.

RESULTS

Significant differences ($P<0.05$) were found among the selective enrichment broths used for recovering *Salmonella* Typhimurium from feces samples. All samples were positive by culture using Müller-Kauffmann tetrathionate, whereas only 40% of the samples were positive using either RV or SC (Table 2).

No effects of selective enrichment broth on the efficiency of PCR were seen when the DNA-extraction methods M1 and M2 were used. However, RV was superior to SC and MK when DNA was extracted by M3 (Table 3). The samples non-*Salmonella* inoculated (negative controls) were all PCR negative and also negative by culture.

The PCR sensitivity was dependent of DNA-extraction methods and selective enrichment broths used. Higher sensitivity was obtained with M3, which detected $3.6x10^2$ and 3.6x10 CFU/g of *Salmonella* Typhimurium in samples enriched in SC and RV, respectively. PCR sensitivity was markedly lower for all DNA-extraction methods when samples were enriched in MK ($3.6x10^8$ CFU/g).

DISCUSSION

Feces contain high amounts of compounds that are inhibitory for PCR. The effectiveness of PCR assay to detect *Salmonella* in feces depends not only on the DNA-extraction method and the selective enrichment broth used but also on the interaction between them. The extraction and purification of DNA can decrease the amount of inhibitory substances and the selective enrichment can increase the number of viable cells. In this study, three DNA-extraction methods in combination with three different *Salmonella* selective enrichment broths were tested. Müller-Kauffmann tetrathionate broth was significantly ($P<0.05$) more efficient than SC and RV in detecting *Salmonella* from culture and PCR when the DNA-extraction was performed by boiling-centrifugation or salting-out.

The RV broth has showed excellent results on the isolation of *Salmonella* in swine feces (5), become the only selective enrichment broth used at many laboratories that process this samples. Therefore, our results didn't corroborate with theses authors, since RV broth had a lower efficiency in the microbiological isolation. On the other hand, its efficiency was higher when associated to the PCR in any three DNA-extraction techniques. The sensitivity difference may be partially explained because the PCR detects both viable and nonviable *Salmonella* cells, whereas culture detects only viable organisms (2).

Soumet *et al*. (17) observed that SC was less inhibitory to PCR than MK and RV. However, the results this study indicate that either of three broths wasn't inhibitory to the PCR when the DNA-extraction method used was boiling-centrifugation or salting-out. Our results corroborate previous reports in which RV was non-inhibitory to PCR (5,11). In fact, similar results between selenite cystine and RV broths have been

Table 2. Recovery of *Salmonella* Typhimurium from 10 artificially inoculated porcine fecal samples (10^2 CFU/g).

Samples	Microbiological isolation		
	SC	MK	RV
A 1	-	+	-
A 2	-	+	+
A 3	+	+	-
A 4	-	+	-
A 5	+	+	-
A 6	+	+	+
A 7	-	+	-
A 8	-	+	-
A 9	+	+	+
A 10	-	+	+
Total	4	10	4

+: positive; - : negative; SC: selenite-cystine; MK: Müller-Kauffmann tetrathionate; RV: Rappaport-Vassiliadis.

Table 3. Selective enrichment broths (SC, MK and RV) and method of DNA extraction (M1, M2 and M3) effects on the detection of *Salmonella* Typhimurium in artificially inoculated porcine fecal samples (10^2 CFU/g) by polymerase chain reaction (PCR).

Samples	M1			M2			M3		
	SC	MK	RV	SC	MK	RV	SC	MK	RV
A1	+	+	+	-	+	+	+	-	+
A2	+	+	+	+	+	+	+	-	+
A3	+	+	+	-	+	+	-	-	+
A4	+	+	+	+	+	+	-	-	-
A5	-	+	+	+	+	+	-	-	-
A6	+	+	+	+	+	+	-	-	+
A7	+	+	+	+	+	+	-	-	+
A8	-	+	-	-	+	-	-	-	-
A9	+	+	-	+	-	+	-	-	+
A10	+	+	+	+	+	+	-	-	+
Total	8	10	8	7	9	9	2	0	7

+: positive; -: negative; SC: selenite-cystine; MK: Müller-Kauffmann tetrathionate; RV: Rappaport-Vassiliadis; M1: boiling-centrifugation; M2: salting-out; M3: phenol-chloroform.

reported (8). Schrank *et al*. (15) compared MK broth with SC broth and concluded that PCR combined with MK broth was more sensitive. These data are in accordance with our findings.

Some authors award the inhibitory effects of RV and MK for the presence of $MgCl_2$ and biliary salts, respectively, on it formulation (1,17). The commercial formulations of RV can differ greatly considering their magnesium chloride concentrations. Indeed, Peterz *et al.* (13) demonstrated that RV containing 40 g/L of $MgCl_2$ was more inhibitory to the recovering of *Salmonella* than those containing 29 g/L. RV broth used in our laboratory (Oxoid, CM 866) contains 13.58 g/L of $MgCl_2$, what can explain its low inhibitory effect.

PCR efficiency was lower (P<0.05) when either MK or SC was used for DNA template preparation by phenol-chloroform technique. Phenol-chloroform extraction eliminates biological contaminants in stool suspensions that may inhibit PCR, but this method appears to be unable to remove non-biological substances (18,19). Wilde *et al*. (19) reported that phenol-chloroform extraction failed to remove the inhibitors unless the specimens were further purified with chromatography medium CF11.

The sensitivity of PCR for pure *Salmonella*-cultures was high when the DNA-extraction method by phenol-chloroform was associated to samples enriched in the RV and SC broths. Müller-Kauffmann tetrathionate (MK) was highly inhibitory to PCR when it was performed on pure cultures, which corroborates previous findings (17). However, MK was not inhibitory when PCR was performed using artificially inoculated feces samples (Table 3), probably due to the higher number of salmonella organisms recovered by MK when compared to SC and RV (Table 2).

The efficiency of PCR associated to the boiling-centrifugation DNA-extraction method was very similar to those achieved by using the commercial kit (Table 2). Therefore, DNA samples prepared by boiling-centrifugation were more easily degraded and could not be stored for long periods at 4ºC. Commercial kits for DNA-extraction may be valuable tools when long-term storage of the extracted DNA is required (7). However, the speed and low cost of the boiling-centrifugation technique must be emphasized. In this study, we showed that RV, SC and MK can be successfully used as source of DNA for PCR when combined to boiling-centrifugation or salting-out. This information is valuable since more complex DNA extraction methods are expensive, time-consuming and laborious. Finally, SC and MK broths must be avoided when DNA-extraction is performed with phenol-chloroform.

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RESUMO

Comparação entre métodos de extração de DNA e caldos de enriquecimento seletivo na detecção de *Salmonella* Typhimurium em fezes de suínos pela reação em cadeia da polimerase (PCR)

O objetivo do presente estudo foi comparar diferentes técnicas de extração de DNA, realizadas a partir de três diferentes caldos de enriquecimento seletivo, na sua eficiência em detectar *Salmonella* Typhimurium em amostras de fezes suínas artificialmente inoculadas (100 UFC/g), pela técnica de reação em cadeia da polimerase (PCR). Após enriquecimento em Rappaport-Vassiliadis, selenito-cistina e tetrationato Müller-Kauffmann, alíquotas destes caldos foram utilizadas para extração do DNA, empregando três métodos diferentes, (a) fervura-centrifugação, (b) fenol-clorofórmio e (c) precipitação por sal. A eficiência dos métodos de extração de DNA por fervura-centrifugação e precipitação por sal foi a mesma, independentemente do caldo de enriquecimento seletivo utilizado. O caldo Rappaport-Vassiliadis apresentou maior eficiência (P<0,05) quando foi empregada a extração de DNA pelo método fenol-clorofórmio. Considerados os parâmetros custo e eficiência, os resultados do estudo indicaram que a partir de amostras fecais suínas a utilização do caldo tetrationato Müller-Kauffmann combinado a técnica de extração do DNA por fervura-centrifugação devam representar a melhor opção, relativamente às demais técnicas testadas.

Palavras-chave: diagnóstico, DNA, fezes, PCR, *Salmonella* Typhimurium.

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