ONE-TUBE NESTED PCR FOR THE DETECTION OF SALMONELLA SP. IN SWINE FAECES

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Abstract

One-tube nested PCR assay based on the detection of Salmonella-specific invA gene using well defined primer sets was developed. Swine faeces spiked with S. Typhimurium and S. Choleraesuis were used as a sample model. The assay was able to detect 150 CFU/g faeces compared to 15 CFU/ml in saline solution. Simultaneously, the detection level of the applied single PCR tests as well as conventional nPCR reached 150 and 15 CFU/ml of saline solution and 15x10^7 and 15x10^2 CFU/g of swine faeces, respectively. The sensitivity of the test was increased up to 15 CFU/g using enrichment in buffered peptone water for 4 h. No serovar-dependent differences were observed. It is concluded that 4-step procedure including 4-h non selective pre-enrichment, DNA extraction, PCR amplification and electrophoretic detection of known molecular weight product (283 bp) can be used as a screening test for Salmonella detection in swine faeces.

Key words: pigs, faeces, Salmonella, PCR.

Salmonella sp. is recognized as an important pathogen for animals and humans. Salmonella infections are still a major problem in pig production and contaminated products of thereof put consumer’s health at risk. The bacteria shed in the faeces of asymptomatic swine are important sources of environmental contamination. Slaughtered asymptomatic pigs are also the important reservoirs of Salmonella Typhimurium which can be transmitted to humans through the food chain. Elaboration of reliable, efficient, and cost-benefit balanced diagnostic method is one of the efforts done to reduce animal and public health consequences of Salmonella infections. ELISA have been widely used for screening of swine herds but culture-based techniques give the possibility to detect the ongoing infection and identify the pathogen. The method, however, is laborious, relatively expensive and involves several enrichment steps lasting up to 5 d.

Material and Methods

Sample preparation and DNA extraction. Overnight agar cultures of S. Typhimurium and S. Choleraesuis field isolates were used to prepare solutions (0.5 McFarland scale) in 0.9% saline (1.5x10^8CFU/ml). Tenfold dilutions were used in further analysis. Salmonella-free swine faecal samples were mixed with 0.9% saline (1:10 w/v ratio) and spiked with the known number of Salmonella colony forming units. The samples were thoroughly mixed and left in room temperature for 15 min sedimentation before sub-sampling for DNA extraction. The sensitivity of PCR assays was assessed on: (1) Salmonella contaminated saline solutions, (2) spiked faecal samples and (3) faecal samples spiked with 15 CFU/g of Salmonella and pre-enriched in buffered peptone water according to ISO 6579:2002. Multi-spin commercial column (Genomic DNA Prep Plus, Helicionus, A&A Biotechnology) was
used for DNA extraction from 100 µl of saline or peptone water, depending on the experiment. In the third experiment, aliquots for DNA extraction were taken every hour during the first 8 h and then at the 12th, 18th, and 24th h of incubation. Spiked and blind faecal samples were tested simultaneously. Finally, 150 faecal samples were tested simultaneously. One-tube nested PCR assay.

Single PCR. The single PCR mixture (total volume of 50 µl) consisted of 5 µl of 10x PCR amplification buffer (100 mM Tris-HCl, pH 8.8, 500 mM KCl, 0.8% Nonidet), 12 µl of MgCl2 (25 mM), 1 µl of each four 10 mM deoxyribonucleoside triphosphates, 20 pmol (each) of the primer pairs 139/141 or SA07/09 (Table 1), 0.3 µl (1.3 U) of Taq DNA polymerase (all the reagents purchased from Fermentas), and 5 µl of extracted DNA. Thirty-eight amplification cycles (Table 1) were run in Programmable Thermal Controller (PTC-100; MJ Research Inc.).

Closed one-tube nested PCR. Own modification of the protocol described by McGoldrick et al. (9) was applied. In the first step, 5 µl of 22% trehalose was used to store and maintain the following nPCR mixture in the lid of 0.2 ml Eppendorf tubes: 20 pmol of each inner primers SA09 and SA10 (Table 1), 0.25 µl of each four 10 mM deoxyribonucleoside triphosphates and 0.25 µl (1.25 U) of Taq DNA polymerase. Prior to storage, the tubes were left open for 2 h at room temperature to dry the mixture. PCR was performed in the bottom of the tubes containing the dried reagents in the lid. Amplification was carried out in 50 µl mixture containing 5 µl of DNA extract and the following reagents: 5 µl of 10x PCR buffer (100 mM Tris-HCl, pH 8.8, 500 mM KCl, 0.8% Nonidet P40), 5 µl of MgCl2 (25 mM), 1 µl of each four 10 mM deoxyribonucleoside triphosphates, 5 pmol of each outer primers SA07 and SA08 (Table 1), 1 µl of 10% Triton X-100 (Sigma), 0.5 µl (2.5 U) of Taq DNA polymerase. Mineral oil was included as a vapour barrier between the first PCR reaction and the dried reagents within the lid. After the first amplification the tubes were inverted several times to dissolve the dried reagents in the lid, centrifuged briefly and subjected to nested amplification reaction (Table 1).

PCR amplicons were electrophoresed and compared visually against MassRuler DNA Ladder (Fermentas). A positive result was defined as the presence of band of expected size while no bands or unspecific weight band was regarded negative. Relative accuracy, specificity and sensitivity of PCR were calculated as described in ISO 16140: 2003. Ten Bachyspira hydysenteriae and six Lawsonia intracellularis field strains were used in specificity study.

Results

Salmonella detection limit of the single PCR assays in saline solution, at the sensitivity, specificity and accuracy of 100%, reached 150 CFU/ml and 250 CFU/ml using, respectively, 139/141 and SA07/08 primer pairs. Conventional nested PCR and the developed one-tube nPCR were able to detect ≤15 CFU/ml. Single PCR detected 150x106 CFU/ml whereas conventional or one-tube nPCR showed positive results in spiked faecal samples, with 1500 CFU/ml and 150 CFU/ml respectively. No serovar-depend differences were observed in one-tube nPCR at contamination level of ≥1500 CFU/ml (Table 2). No PCR products were observed with non-Salmonella strains resulting in 100% relative specificity of the test.

Salmonella was detected in all artificially contaminated faecal samples after 4 h of pre-enrichment (Fig. 1). The results obtained in shorter enrichment times differed significantly from the expected result (P<0.05). Salmonella serovar was not relevant for the pre-enrichment time. Single PCR assays, no matter what the primers were used (139/141 or SA07/08), were able to detect Salmonella after 18 h of pre-enrichment (data not shown).

Salmonella was detected by one-tube nPCR assay in 48 (32%) of naturally contaminated faeces compared to 36 (24%) of culture-confirmed samples (Table 3). The n-PCR assay failed to detect Salmonella in 3 positive samples. A total of 51 (34%) of the tested samples were found Salmonella-positive when data from culture-based and nested PCR tests were combined. Therefore, relative accuracy, specificity, and sensitivity of the assay reached 88%, 87%, and 92%, respectively.

Discussion

New diagnostic method used for diagnostic studies or routine testing should meet several specific demands such as accuracy, specificity, sensitivity or reproducibility. Several PCR assays based on Salmonella virulence gene invA, easy to perform on a large number of samples and cost-benefit balanced were described (2, 4, 6-8, 15). Although trehalose-based one tube nPCR was reported to be an efficient tool for virus detection (14), as far as we are concerned, such approach was not reported in Salmonella diagnostics.

The obtained results proved the usefulness of the developed closed one-tube nPCR assay for Salmonella detection in swine faecal samples. The major insufficiency of conventional nested PCR assays was high possibility for template carry-over (9). In our experiments closed one-tube PCR technique prevented cross-contamination of the samples. Moreover, any non-specific PCR product produced during the first PCR would not be amplified during the second step due to lack of complementarity to inner primer sequences. Hence, the need for further confirmation of the specificity of the PCR product by hybridization (4) or restriction enzyme digestion (2) was eliminated.
Table 1
Specific primer pairs targeting *invA* gene of *S.* Typhimurium SPI-1 published by Galan *et al.* (5) and PCR amplification details

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence 5' → 3' (reference)</th>
<th>position</th>
<th>product size</th>
<th>PCR conditions (temperature/time)</th>
<th>denaturation</th>
<th>annealing</th>
<th>extension</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single PCR (3, 13)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>139</td>
<td>GTGAAATTATCGCACCACGTTCGGGCAA</td>
<td>371-655</td>
<td>284 bp</td>
<td></td>
<td>95°C/30 s b</td>
<td>64°C/30 s</td>
<td>72°C/30 s c</td>
</tr>
<tr>
<td>141</td>
<td>TCATCGCACGTCAAAGGAACC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SA07</td>
<td>TGAATATCGTACTGGCGATTTGGTTTTA</td>
<td>212-756</td>
<td>544 bp</td>
<td></td>
<td>A: 95°C/5 s b</td>
<td>65°C/30 s</td>
<td>72°C/40 s c</td>
</tr>
<tr>
<td>SA08</td>
<td>GGACAAATCCATACCATGCGAGTCAT</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Nested PCR (3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SA09</td>
<td>GAAATTATCGCACCACGTTCGGGC</td>
<td>373-656</td>
<td>283 bp</td>
<td></td>
<td>B: 95°C/5 s</td>
<td>60°C/30 s</td>
<td>72°C/40 s c</td>
</tr>
<tr>
<td>SA10</td>
<td>TCATCGCACGTCAAAGGAACC</td>
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</tbody>
</table>

a Accession No. M90846); b initial denaturation was applied for 95°C/1 min and 94°C/3 min. for 139/141, SA07/SA08, respectively; c final extension step was applied for 72°C/4 min and 72°C/3 min for 139/141, SA07/SA08, respectively, d initial denaturation was applied for 94°C/3 min.; e followed by final extension step for 72°C/3 min.
Table 2
The number of positive results obtained by one-tube nPCR found in faecal samples experimentally spiked with S. Choleraesuis and S. Typhimurium serovars, by contamination level

<table>
<thead>
<tr>
<th>Contamination level (CFU/ml)*</th>
<th>Salmonella</th>
<th>Choleraesuis</th>
<th>Typhimurium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>150 000</td>
<td>15 000</td>
<td>1 500</td>
</tr>
<tr>
<td>Choleraesuis</td>
<td>16</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>Typhimurium</td>
<td>16</td>
<td>16</td>
<td>11</td>
</tr>
</tbody>
</table>

Negative control*

blank faecal samples 0 0 0 0 0 0

* 16 samples were tested at every contamination level.

Table 3
The prevalence of *Salmonella* sp. in swine faecal samples and farms by using one tube nested PCR assay and conventional culture techniques

<table>
<thead>
<tr>
<th>Sample type</th>
<th>No. of samples tested</th>
<th>Number of samples confirmed positive by using</th>
<th>One-tube nested PCR</th>
<th>Culture-based</th>
<th>Combined (nested PCR and culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tested swine faecal samples</td>
<td>150</td>
<td>48 (32%)</td>
<td>36 (24%)</td>
<td>51 (34%)</td>
<td></td>
</tr>
<tr>
<td>Tested swine farms</td>
<td>25</td>
<td>11 (44%)</td>
<td>8 (32%)</td>
<td>12 (48%)</td>
<td></td>
</tr>
</tbody>
</table>

All faecal samples were 4 h enriched in BPW.

Fig. 1. Number of positive results obtained by one-tube nPCR in faecal samples spiked with 15 CFU/g, by pre-enrichment time and *Salmonella* serovar (faeces were diluted in BWP in 1:10 v/w ratio and 0.1 ml was used for DNA extraction).
The detection limits of ≤1500 CFU/ml achieved in spiked faecal samples (Table 2) was better than commonly achieved by single PCR (1, 4). Combination of pre-enrichment followed by PCR showed high sensitivity in samples contaminated with few salmonellae (15). Different selective media can influence the efficacy of DNA amplification (6, 7, 15, 16), therefore pre-enrichment according to well established standards (EN-ISO 6579:2002, 2) were used to test samples containing low Salmonella numbers undetectable either by available PCR assays or by culture. As shown in Fig. 1, 4 h incubation was enough to achieve an optimal sensitivity, no matter what Salmonella serovar was present in the sample. It allowed the detection of S. Typhimurium and S. Choleraesuis in faecal samples within a working day (10 – 12 h) that is considerably faster compared to culture-based method taking up to 5 d to produce the results, as well as to some other conventional PCR assays (6). High indexes for relative accuracy, sensitivity, and specificity could lead to conclusion that a specific, rapid, and cost-effective diagnostic tool, efficient to detect Salmonella serovars prevalent in Polish and Lithuanian swine herds was developed. That statement was verified on a limited number of faecal samples. Further studies are needed but, as already reported by others (1, 4, 6, 15, 16), the frequency of positive results obtained in n-PCR was higher than in culture (Table 3). Since we found lower Salmonella prevalence than observed by others (4, 15), an introduction of internal positive control to detect false negative results should be considered (1, 8). Salmonella infections in swine are economic and public health concern. Therefore, reliable and rapid technique such as one-tube nPCR is needed. Data from the current investigation suggests that due to high sensitivity, specificity, rapidity, and hampered cross-contamination risk, the developed one-tube nPCR assay could be applied in either routine diagnosis or as a screening test for symptomless, chronically infected animals or herds when low number of Salmonella is present in the tested faecal sample.

References