References:

SYBR Green Real-Time PCR for Salmonella detection in meat products

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Summary: The objective of this study was to develop a SYBR Green Real-Time PCR method for detecting salmonellae in meat samples. The study was conducted both on S. Typhimurium experimentally and naturally contaminated meat samples analyzed in parallel with the standard cultural method (ISO 6579/2001). After the pre-enrichment phase, a boiling DNA extraction procedure combined with SYBR-Green I Real Time PCR, using primers Styinva-JHO-2, was developed. The specificity of the reaction was confirmed by the Melting Temperature (Tm), which was consistently specific for the amplicon obtained (S. Typhimurium Tm=77.33±0.058). The standard curve constructed using the mean threshold cycle (Ct) and various concentrations of S. Typhimurium (ranging from 10^3 to 10^8 cfu/ml) showed a good linearity (R^2=0.9767) and a sensitivity limit of less than 10^3 cfu/ml. The comparison with the ISO method confirmed the effectiveness of the proposed method.

Keywords: rapid methods, pathogens detection, food.

Introduction: Salmonella continues to be one of the major causes of food poisoning in the western world. Different methods have been developed in order to reduce the time for the detection of the salmonella from food, since the ISO standard cultural method requires up of five days. Many of the PCR assays employ either visual scoring of ethidium bromide-stained agarose gels or post-PCR hybridisation-capture methods that are labour intensive, time consuming and difficult to automate. Recently, the use of double stranded DNA binding dye SYBR Green I for the detection of PCR product allows an early and simple approach to the Real-Time PCR and require less knowledge than classic Real-time PCR using fluorogenic oligoprobes (Hoofar et al., 2000). The objective of the present study was to develop a SYBR Green I Real-Time PCR method for the detection of salmonellae in meat products. The experiments were conducted on S. Typhimurium experimentally and naturally contaminated meat samples and the results were compared to the standard cultural method.

Materials and methods: a sample was experimentally contaminated as follows: twenty-five grams of pork meat homogenized with 225 ml of buffered peptone water in a Stomacher were incubated at 37 °C for 24 h. The pre-enrichment broth was then divided into three aliquots: the first aliquot was used to confirm the absence of salmonellae by means of standard cultural method (ISO 6579/2001);
the second aliquot was spiked with an appropriate quantity of S. Typhimurium suspension to obtain a final concentration of $10^7$ cfu/ml; the third aliquot was used as negative control. Thirty meat samples purchased from local retail outlets were analyzed both with the standard cultural method and with the SYBR Green I Real-time PCR. The extraction and purification of DNA was performed by boiling according to the procedure previously described (De Medici et al., 2003). Five µl of the supernatant, used as DNA template. The PCR was performed using the Styinva-JHO-2 primers (Hoorfar et al., 2000) at the concentration of 50nM. The amplification reactions were performed in a total volume of 50µl with an ABI Prism 7700 sequence detector 96-well micro-well plates. In each well, we placed 5µl of purified DNA, 25µl SYBR Green I PCR Master Mix (Applied Biosystems), 50nM primer Styinva-1, 50nM primer Styinva-2, and, to reach a total volume of 50µl per well, DNase-RNase-free distilled water. The reaction was run online at 50°C for 2 min and 95°C for 10 min, followed by 35 cycles at 95°C for 1 min, 55°C for 1 min and 72°C for 60 sec, with an extension phase of 1 cycle at 95°C for 1 min, 60°C for 1 min, and 95°C for 1 min (ramp time 19.59 min). The results were visualized using the software Sequence detector 1.7 provided with the ABI Prism 7700 system. The specificity of the reaction is given by the Tm of the amplification products immediately after the last reaction cycle.

**Standard curve**: it was obtained from the C_t values of the meat sample in pre-enrichment broth after incubation at 37°C for 24 h, supplemented with tapering concentrations of S. Typhimurium ($10^8$, $10^7$, $10^6$, $10^5$, $10^4$, and $10^3$ cfu/ml). One non-spiked sample was used as a negative control.

**Standard cultural method**: it was performed according ISO 6579/2001 method. Suspected salmonellae colonies were identified using API 20E system and commercially available specific antisera.

**Results**: the mean peak Tm obtained with the curves specific for S. Typhimurium obtained from various experiments was $77.33\pm0.058$. Of the 30 samples considered, 11 were found to be positive when analyzed both using SYBR-Green I Real-Time PCR (CT value ranging form $16.04\pm0.221$ to $18.892\pm0.183$) and the standard cultural method (table 1).

**Table 1. Serotypes identified by the standard cultural method and T_m values obtained by SYBR Green I real-time PCR**

<table>
<thead>
<tr>
<th>Sample</th>
<th>ISO 6579/2001 Serotype</th>
<th>SYBR-Green I Real-Time PCR T_m (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S. Newrochelle</td>
<td>77.03±0.231</td>
</tr>
<tr>
<td>2</td>
<td>S. Infantis</td>
<td>77.17±0.008</td>
</tr>
<tr>
<td>3</td>
<td>S. Typhimurium</td>
<td>77.27±0.031</td>
</tr>
<tr>
<td>4</td>
<td>S. Enteritidis</td>
<td>77.25±0.016</td>
</tr>
<tr>
<td>5</td>
<td>S. London</td>
<td>77.30±0.348</td>
</tr>
<tr>
<td>6</td>
<td>S. London</td>
<td>77.20±0.000</td>
</tr>
<tr>
<td>7</td>
<td>S. Infantis</td>
<td>77.27±0.153</td>
</tr>
<tr>
<td>8</td>
<td>S. Enteritidis</td>
<td>77.28±0.021</td>
</tr>
<tr>
<td>9</td>
<td>S. Typhimurium</td>
<td>77.30±0.081</td>
</tr>
<tr>
<td>10</td>
<td>S. Newrochelle</td>
<td>77.13±0.231</td>
</tr>
<tr>
<td>11</td>
<td>S. Typhimurium</td>
<td>77.36±0.062</td>
</tr>
</tbody>
</table>

The negative samples used as control did not show peaks with SYBR-Green I Real-Time PCR. The standard curve showed a good linearity of response ($R^2=0.9767$). The sensitivity limit of the reaction was less than $10^3$ cfu/ml.
Discussion: the proposed method seems to be effective, rapid and reproducible, the comparison with the standard method showed that neither false-positive nor false-negative results were obtained. The specificity of the reaction was confirmed by the determination of the Tm, specific for the amplicon obtained, that allows to eliminate the phase of electrophoresis, which is time-consuming and requires the use of ethidium bromide, a potent mutagenic agent, that is not suitable for routine use.

References


PD 12 Development of an ELISA test for Salmonella serological monitoring in Brazil

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Summary: EMBRAPA/CNPSA developed an ELISA test based on LPS antigens from Salmonella Typhimurium. After the optimal dilution determination of the test components, four sera were chosen as controls. The interplate variation was controlled by a coefficient correlation between standard and daily curves of control sera and the coefficient variation of sample sera triplicates. The cut-off was determined by a dispersion analysis in a nursery piglet population proved to be salmonellae negative. The test performance was evaluated in experimentally and naturally S. Typhimurium infected pigs and in animals vaccinated with other Salmonella serovars. The seroconversion was observed after two weeks post inoculation in experimentally infected and vaccinated animals. In naturally infected animals, which were sampled twice during the finishing period, at the first sampling 75 % of pigs were eliminating salmonellae in feces and 25 % were positive in the ELISA. At the second sampling 76.9 % became serologically positive. These results suggest that the developed test can be used for Salmonella Typhimurium monitoring programs in swine.

Keywords: Lipopolysaccharides, S. Typhimurium, swine, serology.

Introduction: Previous studies conducted in southern Brazil indicated a wide dissemination of salmonellae infection in swine herds (Bessa et al. 2001, Kich et al. 2001). The reduction in the number of carrier pigs at slaughter is one of the most important measures for pork contamination control. Most countries started intensive programs of Salmonella control on farms based on serological monitoring (Nielsen et al., 2001). In southern Brazil serovars Typhimurium, Agona, Derby, Bredney and Panama have proved to be the most prevalent in carrier pigs sampled at slaughter (Bessa et al., 2001). As these serovars have at least two common LPS antigens with Typhimurium, an ELISA test was developed based on LPS antigens from S. Typhimurium.

Material and Methods: Phenolic extraction of LPS from Salmonella Typhimurium was done as described previously (Vidal et al. 1999). Optimal dilution was determined for serum (1:400), antigen (1:2000) and conjugate (1:25,000). For control of intraplate and interplate variation reference sera, chosen from sera...