Comparison of four different PCR methods for the detection of Mycobacterium avium subsp. paratuberculosis in milk

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ABSTRACT
Emerging evidence suggests a role of Mycobacterium avium subs. paratuberculosis (MAP) in the development of human pathologies like Crohn’s disease and type I diabetes. For this reason, the need for rapid and robust tools to detect the presence of MAP in food is increasing. Polymerase Chain Reaction (PCR) techniques are able to give rapid and specific results, but their sensitivity is generally lower than traditional culture methods. The aim of this study was to compare four different PCR methods to detect MAP on cow bulk milk samples collected from presumably infected herds. MAP DNA was extracted by Adiapure kit (Adiagene, France). The PCR were: (a) IS900 Commercial end-point PCR (kit Adiavet, Adiagene, France); (b) IS900 Nested PCR; (c) IS900 TaqMan Real time PCR; (d) f57 house-made Sybr Green Real Time PCR. Both the commercial PCR (a) and the IS 900 Real Time PCR TaqMan (c) contained an internal amplification control in order to discriminate between negative or inhibited samples. Out of 37 milk samples tested we found only one positive sample (3%) using (a), (c) and (d) methods. The Nested PCR method (b) showed eight positive samples (22%). Although we used bulk milk samples coming from presumably infected herds, the prevalence of direct detection of MAP is low in all the “one round” PCR methods. As expected, the most sensitive method is Nested PCR, although it remains difficult to apply as regards to possibility of cross contaminations.

BACKGROUND
Emerging evidence suggests a role of Mycobacterium avium subs. paratuberculosis (MAP) in the development of human disorders like Crohn’s disease and Type I diabetes1, 2. For these reasons, the request for rapid and robust tools to detect the presence of MAP in food, especially milk, is increasing3. Molecular biology techniques, such as Polymerase Chain Reaction (PCR), are able to give rapid and specific results. These methods are also useful tools when the matrices are not appropriately conserved (compromised bacterial viability), or in presence of fastidious strains.

PCR assays developed for the detection of MAP have high specificity, while their sensitivity is generally lower than traditional culture methods. These assays amplify some typical MAP regions, such as f57 (one copy per genome of MAP4) and IS900 (12-18 copies per genome of MAP4). While the discovery of some IS900-like elements could make not conclusive a test based on IS900 targeting, the f57 sequence is highly specific for MAP5, 6. However, due to the higher number of copies, IS900 PCR tests are generally more sensitive than those based on f574. In order to compare the sensitivity of PCR tests, we applied a commercial end-point PCR, a Nested PCR and two Real Time PCR assays to bulk milk samples, previously positive to serological ELISA test.

MATERIALS AND METHODS
We tested about 3000 milk samples from different Italian regions using ELISA test (ID Screen® Paratuberculosis Indirect, Confirmation Test, ID Vet, MontPellier, France). Out of 82 positive samples, we extracted the DNA from 37 positive samples using Adiapure kit (Adiagene, France).

The PCRs were:
(a) IS900 Commercial end-point PCR (kit Adiavet, Adiagene, France), performed according to the kit manufacturing procedures. The reaction was carried out in Mastercycler ep gradient s (Eppendorf, Milan, Italy);
(b) IS900 Nested PCR, using p90-p91 primers\(^4\) for the first round followed by a second round with specific primers (see Table 1). The first round of Nested PCR was performed in 25 µl final volume with 0.1 U of TAQ (Qiagen, Milan, Italy) adding two µl of DNA. The second round was performed by adding two µl of the previous run in a final volume of 25 µl. The reaction was carried out in Mastercycler ep gradient s (Eppendorf);

(c) IS900 TaqMan Real time PCR, performed adding two µl of DNA in 25 µl final volume, using TaqMan® Gene Expression Master Mix (Applied Biosystems, Milan, Italy) in StepOnePlus™ Real-Time PCR System (Applied Biosystems). Fluorescence intensity was normalized with ROX dye;

(d) f57 SYBR Green Real Time PCR, performed adding two µl of DNA in 25 µl final volume using Power SYBR® Green PCR Master Mix (Applied Biosystems) in StepOnePlus™ Real-Time PCR System (Applied Biosystems). Fluorescence intensity was normalized with ROX dye. The amplification was followed by melting curve analysis, melting the amplicon from 60 °C to 95 °C and recording the fluorescence 3.3 times per degree with a temperature ramp rate of 2.2 °C/sec.

Both the commercial PCR (a) and the TaqMan (c) contained an internal amplification control in order to discriminate between negative or inhibited samples, while the other test did not contain any internal amplification control. Primers sequences are listed in table 1.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Oligo name</th>
<th>Sequences</th>
<th>Ta (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a Adiavet Kit primers</td>
<td>unknown</td>
<td>5-GAAGGGTGTTCCGGGACCCTGCTA-3’</td>
<td>62</td>
</tr>
<tr>
<td>b P90</td>
<td>5'-GAAGGGTGTTCCGGGACCCTGCTA-3'</td>
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<tr>
<td>P91</td>
<td>5'-GGCGTTGAGGTCGATCGCCCACGTGAT-3'</td>
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<tr>
<td>Nested Forward</td>
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<td>58</td>
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<tr>
<td>Nested Reverse</td>
<td>5'-CCGTAACCAGTCATTGTCAG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c IS900 Forward</td>
<td>5'-CCGTAAGGCGACCATTAT-3'</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>IS900 Reverse</td>
<td>5'-ACCCCGCTGAGAGCA-3'</td>
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<tr>
<td>IS 900 Probe</td>
<td>6-FAM-CATGTTTATTAACGAGCGCAGC-3'</td>
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<tr>
<td>d f57 Forward</td>
<td>5'-ATAGCCTTTCCCTCCTCGTC-3'</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>f57 Reverse</td>
<td>5'-CAGGGCAACACATATTTCGG-3'</td>
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</tbody>
</table>

**RESULTS**

Out of 37 milk samples tested we found only one positive sample (3%) using (a), (c) and (d) methods. The Nested PCR method (b) showed a total of eight positive samples (22%). No inhibited samples were found with (a) and (c) methods.

(a) Adiavet PCR. Note the internal amplification control in all samples. Only one sample (4) was positive. M: Marker; NE: negative extraction control; NN: no template control; 1-9: milk samples.
(b) Nested PCR. All positive samples (4, 7, 9, 10, 13) showed a 99 bp amplicon with the same size as the positive control. M: 100 bp Marker; NE: negative extraction control; 1°NN: no template 1st round PCR; 2°NN: no template 2nd round PCR; +: positive control (DNA from MAP ATCC 19698); 1-13: milk samples.

(c) IS900 TaqMan Real Time PCR. Note the internal amplification control. DNA from MAP ATCC 19698 was used as positive control. Data were in duplicate.
CONCLUSIONS
In order to compare the sensitivity of four PCR methods for the detection of MAP, we tested bulk milk samples derived from presumably infected herds (ELISA positive). As expected, the most sensitive method was Nested PCR, while conventional PCR and Real Time PCR showed lower sensitivity. Although more sensitive, Nested PCR method remains difficult to apply because it is time consuming and there are high risks of cross-contamination.

REFERENCES

(d) f57 Real Time PCR. The figure on the left shows the amplifications of the positive sample and the positive control. The figure on the right shows the melting analysis results of amplicons. Note the single peak at 84.6 °C corresponding to the single specific amplification product. DNA from MAP ATCC 19698 was used as positive control. Data were in duplicate.