A combined molecular and cultural method for the detection of the *Mycobacterium tuberculosis* complex (MTBC) and *Mycobacterium avium* subsp. *paratuberculosis* was developed and tested with artificially contaminated milk and dairy products. Results indicate that the method can be used for a reliable detection as a basis for first risk assessments.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’–3’)</th>
<th>Modification</th>
<th>Gene (GenBank accession no.)</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>Myco_tub_fw</td>
<td>GAA CCC GCT GAT GCA AGT</td>
<td></td>
<td><em>M. bovis</em> helicase gene, species-specific sequence (U87961.1)</td>
<td>This study</td>
</tr>
<tr>
<td>Myco_tub_re</td>
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<td></td>
<td><em>M. bovis</em> helicase gene, species-specific sequence (U87961.1)</td>
<td>This study</td>
</tr>
<tr>
<td>Myco_tub_S_</td>
<td>CTG ACG GTG GTG ACC TTC TT</td>
<td>FAM-TAMRA</td>
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<td>Myco_para_F57_fw</td>
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<td></td>
<td><em>M. avium</em> subsp. <em>paratuberculosis</em> F57 (X70277)</td>
<td>14</td>
</tr>
<tr>
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<tr>
<td>Myco_para_F57_S</td>
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<tr>
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<td></td>
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<td>TGA GCC GGT GTG ATC ATC TT</td>
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<td><em>M. avium</em> subsp. <em>paratuberculosis</em> ISMav2 (AF286339)</td>
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<td>pUC_19_S</td>
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<td>ROX-TAMRA</td>
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</table>

*FAM, 6-carboxyfluorescin; TAMRA, 6-carboxytetramethylrhodamine; HEX, 5’-hexachlorofluorescein; ROX, carboxy-X-rhodamine.*
berrcolusis described by Schönbrücher et al. (14) (Table 1). A
temperature-time profile, with an initial denaturation step at 95°C
for 10 min and 45 cycles with a denaturation step at 95°C for 15 s
and an annealing step at 55°C for 60 s, was adapted for the Strat-
agene MX 3000P and MX 3005P thermocyclers (Agilent Tech-
ologies). The primers were used with a concentration of 300 nM and
the probes with a concentration of 100 nM per reaction in com-
bination with the Brilliant Multiplex MasterMix (Agilent Tech-
ologies). DNA extraction from isolates was done with thermal
lysis (95°C for 10 min). For the DNA extraction from enrichment
broths, the Maxwell purification system (Promega, Germany) was
used after centrifugation at 10,000 × g for 5 min and lysis of the
resulting pellet at 37°C for 30 min using a 10 mM Tris-EDTA
buffer (pH 8) with a concentration of 3 mg/ml of lysozyme (Carl
Roth, Germany). The specificity of the system was tested with 47
denatured, yeast, and fungal strains (DSMZ, ATCC, and field
strains, isolated and characterized at our laboratory). For the
determination of robustness and sensitivity, standard curves using
DNA serial dilutions were generated. The quantification for fur-
ther tests was done with a most probable number (MPN) tech-
nique in accordance with the U.S. Food and Drug Administra-
tion Bacteriological Analytical Manual (1). The MPN was calcu-
lated depending on the presence of typical colonies. Real-time
PCR directly from the enrichment broths was done before incu-
bation and after 7, 14, and 56 days.

The real-time PCR systems showed 100% specificity for the
panel of strains tested. No cross-reaction was found between the
MTBC and the M. avium subsp. paratuberculosis detection sys-
tems. The sensitivity was assigned with 100 fg (about 20 genome
equivalents) for the detection of MTBC and M. avium subsp.
paratuberculosis. The robustness test showed a maximum differ-
ence of 1.2 cycles in different real-time PCR runs and on different
thermocyclers. When Dubos broth and Kirchner broth were
tested with pure cultures and artificially contaminated samples,
both broths showed no differences in the detection limits for BCG
and M. avium subsp. paratuberculosis. The detection limits using
pure cultures for the enrichment broths in combination with the
real-time PCR system are shown in Fig. 1. Using only cultural
methods, the detection limit was 1,000 CFU/ml for the BCG strain
and 410 CFU/ml for the M. avium subsp. paratuberculosis strain
after 14 and 57 days of enrichment. The results using artificially
contaminated samples are shown in Tables 2 and 3. The detection
with only cultural methods failed for samples contaminated with
less than 10^3 CFU/g sample material. But in every case the differ-
cences in the threshold cycle (C_T) values of the real-time PCR re-
results showed an increase of DNA during the incubation period
(more than 2.4 cycles, double the value of the robustness test).
Therefore, it can be assumed that there were viable and culturable
bacteria in the artificially contaminated samples. Our investiga-
tions showed that only a molecular screening combined with the
possibility of a cultural detection in a positive case can be used for
a reliable detection of MTBC and M. avium subsp. paratubercu-
losis in milk and dairy products on the retail level. The risk of MTBC
as a reemerging pathogen entering the food chain and causing
human infection is clearly demonstrated by Harris et al. (7).
Therefore, the need for reliable detection and risk-based investi-
gations of different matrices is obvious in the framework of the
official food control. Present reports about the development of
molecular methods for the detection and differentiation of MTBC

![FIG 1 Detection limit for MTBC and M. avium subsp. paratuberculosis using enrichment broths in combination with real-time PCR.](image)

| TABLE 2 Investigation of artificially MTBC-contaminated samples using an enrichment procedure in combination with real-time PCR |
|---|---|---|---|---|---|---|---|---|
| Enrichment time (no. of days) | Milk (3.8% fat) | Yoghurt | Sour cream | Curd cheese (40% fat) |
| 0 | 10^3 | 10^2 | 10^1 | 10^3 | 10^2 | 10^1 | 10^3 | 10^2 | 10^1 |
| 7 | 36.0 | 39.5 | – | 40.2 | 44.3 | – | 42.4 | 43.5 | – |
| 14 | 28.9 | 32.7 | 39.9 | 29.7 | 39.3 | – | 32.0 | 36.3 | 40.2 |
| 56 | 22.4 | 28.4 | 34.3 | 26.2 | 34.2 | 40.0 | 28.2 | 34.2 | 36.7 |

^a^ – negative value.
TABLE 3 Investigation of artificially *M. avium* subsp. *paratuberculosis*-contaminated samples using an enrichment procedure in combination with real-time PCR

<table>
<thead>
<tr>
<th>Enrichment time (no. of days)</th>
<th>Milk (3.8% fat)</th>
<th>Yoghurt</th>
<th>Sour cream</th>
<th>Curd cheese (40% fat)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^1</td>
<td>10^2</td>
<td>10^3</td>
<td></td>
</tr>
<tr>
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<td>38.8/40.5</td>
<td>42.4/44.3</td>
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<td>103</td>
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<td>7</td>
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<td>41.4/42.3</td>
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<td>14</td>
<td>35.7/37.3</td>
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<td>33.4/36.5</td>
<td>37.2/39.3</td>
<td>40.2/42.7</td>
<td>103</td>
</tr>
</tbody>
</table>

CT = values (avg of 3 PCR runs) for different contamination levels (CFU/g sample material)

a–, negative value. The first of each pair of values is from the F57 gene detection system; the second is from the ISMav2 gene detection system.

deal with clinical human and veterinary samples (3, 9, 11). They cannot be transmitted for the investigation of food, as low contamination levels per gram of sample material are to be expected in these matrices. In contrast to MTBC, many recent reports deal with *M. avium* subsp. *paratuberculosis* in milk and dairy products (2, 6, 8, 15). Not all studies were carried out with combined cultural and molecular methods; some of them dealt only with PCR or real-time-PCR–positive samples (8). For a solid risk assessment concerning, e.g., heat-treated dairy products like infant formula, data about the viability of *M. avium* subsp. *paratuberculosis* are decisive. RNA-based molecular methods developed for a differentiation between viable and dead cells (2a) might provide important information about the prevalence in milk and dairy products, but cultural data on the retail level are also needed. Therefore, the combined cultural and molecular method presented in our study can be useful for a routine screening of milk and dairy products for MTBC and *M. avium* subsp. *paratuberculosis* on the retail level as a basis for a preliminary risk assessment.

REFERENCES


13. Reference deleted.
