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Comparison of 13 single-round and nested PCR assays targeting IS900, IS*Mav2*, f57 and locus 255 for detection of *Mycobacterium avium* subsp. *paratuberculosis*

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Abstract

For molecular biological detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP), PCR methods with primers targeting different regions specific for MAP are used worldwide. However, some uncertainties exist concerning the specificity of certain target regions and the sensitivity. To identify the methods which are best suited for diagnostics, 8 single-round and 5 nested PCR systems including 12 different primer pairs based on IS900 (9×), IS*Mav2* (1×), f57 (1×), and locus 255 (1×) sequences were compared regarding their analytical sensitivity and specificity under similar PCR conditions. Reference strains and field isolates of 17 *Mycobacterium* species and subspecies, 16 different non-mycobacterial bovine pathogens and commensals were included in this study.

Single-round PCR resulted in a detection limit of 100 fg to 1 pg, and nested PCR in 10 fg or below. Depending on the specific primer sequences targeting IS900, false positive results occurred with one of the five single-round and two of the four nested PCR systems. This also applied to the single-round PCR based on IS*Mav2* and the nested PCR based on f57. A high number of non-specific products were primarily detected for the single-round PCR assay based on IS*Mav2*, but also for a single-round PCR targeting the IS900 and the locus 255.

In conclusion, stringent selection of IS900-specific primers ensures that IS900 remains a favourite target sequence for amplification of MAP specific loci. The studied PCR systems based on f57, and locus 255 can also be recommended. Revision of IS*Mav2* primers is necessary. Single-round PCR systems are very reliable. Nested PCR assays were occasionally disturbed by contaminations, thus bearing a risk for routine diagnostics.

Keywords: Bovine paratuberculosis; PCR; Sequence dependent specificity; Cross-reactions; By-products; Analytical sensitivity

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1. Introduction

At present, faecal culture on solid media is still the most sensitive and specific method for direct diagnosis of paratuberculosis in animals, and thus the ''gold standard'' (Stabel, 1997). However, the method is too laborious and time consuming to be applicable in large-scale diagnostic programmes. Therefore, direct detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in faecal samples by PCR seems to be a practicable alternative. Different PCR methods including real-time and nested PCR are already in use for the detection of MAP, although valid data regarding the specificity and sensitivity of these assays are missing.

Several primers for single-round and nested PCR assays based on the first discovered MAP specific repetitive insertion segment IS900 have been published (Green et al., 1989; Moss et al., 1991; Sanderson et al., 1992; Collins et al., 1993; Vary et al., 1990; Millar et al., 1996; Englund et al., 1999; Marsh and Whittington, 2001; Bull et al., 2003; Vansnick et al., 2004; Ikonomopoulos et al., 2004). Their specificity was evaluated using different numbers of *Mycobacterium* species and other common bacteria (Collins et al., 1989; Vary et al., 1990; Englund et al., 1999; Ikonomopoulos et al., 2004; Vansnick et al., 2004; Tasara et al., 2005). The occurrence of IS900-like sequences in a non-MAP isolate (Cousins et al., 1999; Englund et al., 2002) and false positive results obtained by IS900 PCR for some *Mycobacterium avium* complex (MAC) strains and non-MAP isolates (Cousins et al., 1999; Motiwala et al., 2004; Tasara et al., 2005) caused uncertainties about the specificity of PCR systems targeting IS900 for scientific purposes and routine diagnosis. In the meantime, additional gene loci specific for MAP have been identified and suggested for use in diagnostics: IS*Mav2* (Strommenger et al., 2001), f57 (Poupart et al., 1993), IS*Map02* (Stabel and Bannantine, 2005), and 21 other MAP specific coding sequences (Bannantine et al., 2002).

The aim of the current study was to find out, which of the proposed primer pairs published and which MAP specific target region are most suitable for MAP detection. Thirteen single-round and nested PCR systems based on the target regions IS900, IS*Mav2*, f57, and locus 255 were involved. The specificity and analytical sensitivity of the primers was analysed under comparable conditions.

2. Materials and methods

2.1. Bacterial strains and DNA probes

Reference strains and field isolates of 17 mycobacterial species and subspecies, and of 16 other bacterial pathogens or commensals, which can be isolated from cattle, were included in this study and are listed in Table 1. The MAP field isolates recovered from faecal and organ samples were maintained on Loewenstein–Jensen-based paratuberculosis medium containing mycobactin as well as colistinsulfate, amphotericin, piperacillin and trimethoprim (PACT, Bioservice Waldenburg, Waldenburg, Germany). Non-MAP mycobacterial field isolates recovered from organ samples were maintained on Middlebrook 7H11 agar supplemented with oleic acid, albumin, dextrose and catalase (OADC). Other bacterial species, not belonging to *Mycobacterium*, were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and were cultivated on special culture media recommended by the DSMZ. *Salmonella* reference strains were cultivated on nutrient broth (Oxoid), *Escherichia coli* field isolates on Luria–Bertani agar, and *Coxiella burnetii* field isolates on Buffalo green monkey cells in Nephros LP or UltraCulture medium (BioWhittaker).

The identity of the bacterial species used was examined by different methods. The species of different *mycobacterium* strains was confirmed by PCR amplification of *hsp*65 and restriction enzyme analysis (Telenti et al., 1993). Field isolates of atypical mycobacteria were identified by sequencing of 16S ribosomal RNA genes. Additionally, isolates of the subspecies *Mycobacterium avium* (*M .a.*) *paratuberculosis*, *M. a. avium* and *M. a. hominissuis* were differentiated by PCR detection of the species-specific target regions IS900 (Englund et al., 1999), IS1245 (Guerrero et al., 1995), and IS901 (Kunze et al., 1992). Bacterial reference strains, not belonging to mycobacteria and purchased from DSMZ were identified by morphological characteristics. The identity of other species was confirmed by species-specific PCR.