**INTRODUCTION**

To date, eight different species are formally described in the genus *Bordetella*. Of them, *Bordetella pertussis* is the main causative agent of human pertussis (Kerr & Matthews, 2000). *Bordetella parapertussis* can also cause pertussis-like symptoms in humans (Heininger et al., 1994; He et al., 1998). *Bordetella holmesii* is a small Gram-negative coccobacillus first described in 1995, and it is oxidase negative, non-motile and produces soluble light-brown pigment when grown at 35°C on heart infusion tyrosine agar (Weyant et al., 1995). It has been cultivated from the blood, pleural fluid and sputum of immunocompromised patients (Lindquist et al., 1995; Tang et al., 1998; Morris & Myers, 1998; Russell et al., 2001; Shepard et al., 2004), but was also isolated from recultured nasopharyngeal (NP) specimens of patients with pertussis-like symptoms in MA, USA, in the years 1994–1998 (Yih et al., 1999; Mazengia et al., 2000). The prevalence of *B. holmesii* in European patients with pertussis-like symptoms is not known.

Since 1990, PCR has been used for the diagnosis of *B. pertussis* and *B. parapertussis* infections (Glare et al., 1990; van der Zee et al., 1993b, 1996; He et al., 1993, 1996, 1998; Muller et al., 1997; Lind-Brandberg et al., 1998; Loeffelholz et al., 1999; Heininger et al., 2000; Fry et al., 2004). Insertion sequences IS481 of *B. pertussis* and IS1001 of *B. parapertussis* have been the targets frequently used in PCRs that have proved to be sensitive and specific for *B. pertussis* and *B. parapertussis* (van der Zee et al., 1993b, 1996; He et al., 1993,
1996, 1998; Muller et al., 1997; Lind-Brandberg et al., 1998; Loeffelholz et al., 1999; Heininger et al., 2000; Fry et al., 2004). The genome of B. holmesii contains IS481- and IS1001-like elements (Reischl et al., 2001; Templeton et al., 2003). The copy numbers of the IS481- and IS1001-like elements are estimated to be 8–10 (Reischl et al., 2001) and 3–5 (this study). This raises the concern that B. holmesii can cause false-positive results in PCR assays based on the detection of IS481 and IS1001.

We investigated the prevalence of B. holmesii in Finnish and Dutch patients with pertussis-like symptoms to find out whether B. holmesii could confound IS481- and IS1001-based assays. Clinical specimens were NP swabs collected from 2804 Finnish patients from 2000 to 2003, and from 8515 Dutch patients from 1992 to 2003. The Finnish methods used were a conventional IS481 PCR (He et al., 1993, 1996) and a B. holmesii-specific real-time PCR (LightCycler, Roche) described in this study. The target gene used in the PCR was recA, a gene encoding the RecA protein involved in homologous recombination in bacteria and regarded as a housekeeping gene (Favre & Viret, 1990; Favre et al., 1991; Kuhl, 1996; Pietilä et al., 2000). The methods used in the Netherlands were IS481 and IS1001 PCR assays with conventional or real-time formats (van der Zee et al., 1993b, 1996; Templeton et al., 2003), and a newly developed B. holmesii-specific real-time PCR targeting the homologue of IS1001.

**METHODS**

**Bacterial strains and culture.** For development and validation of B. holmesii-specific real-time PCR, the 30 Bordetella strains used in Finland were 16 B. pertussis human isolates (Mooi et al., 1999; Gzyl et al., 2001; Wang et al., 2002), four B. parapertussis strains (ATCC 15311T and three human isolates) (He et al., 1998), one Bordetella bronchiseptica strain (Central Public Health Laboratory, Colindale, UK), seven B. holmesii strains (ATCC 51541T and six clinical isolates) (Yih et al., 1999), one Bordetella avium strain (ATCC 35086T) and one Bordetella hinzii strain (ATCC 51730T). In addition, 47 clinical isolates from 16 bacterial species and four clinical isolates of Candida albicans were tested. The 16 bacterial species included Haemophilus influenzae (4), Haemophilus parainfluenzae (1), Staphylococcus aureus (4), Streptococcus pyogenes (4), Streptococcus pneumoniae (4), Streptococcus agalactiae (3), Neisseria meningitidis (1), Moraxella catarrhalis (3), Escherichia coli (4), Proteus mirabilis (4), Corynebacterium diphtheriae (2), Enterococcus faecalis (4), Enterococcus faecium (1), Pseudomonas aeruginosa (3), Klebsiella pneumoniae (3) and Klebsiella oxytoca (3). Bordetella strains were grown on charcoal agar, as described previously (He et al., 1993). All other bacteria and the fungi were cultivated at the Department of Medical Microbiology, University of Turku, Turku, Finland, according to the methods described in the Manual of Clinical Microbiology published by the American Society for Microbiology.

Bacterial colonies on the plates were harvested for isolation of DNA at the Pertussis Reference Laboratory, National Public Health Institute, Turku, Finland.

In Tilburg, the Netherlands, bacterial strains used for the development and validation of B. holmesii-specific real-time PCR were four B. pertussis strains, two B. parapertussis strains (human and sheep isolates), three B. bronchiseptica strains (human, dog and pig isolates), three B. holmesii strains (ATCC 51541T and two clinical isolates), and one strain each of B. hinzii, Bordetella tremetatum and Bordetella petrii. Isolates were cultured on charcoal agar as described previously (van der Zee et al., 1993b).

**Isolation of DNA from bacterial cultures.** In Finland, the High Pure PCR Template Preparation kit (Roche Applied Science) was used for isolation of DNA. Bacteria (~10⁶ cells ml⁻¹) harvested from the culture plates were placed in 300 µl PBS and DNA was extracted according to the manufacturer's instructions. DNA concentrations were measured by a spectrophotometer (SmartSpec 3000, Bio-Rad). DNA concentrations were adjusted to 5 ng µl⁻¹ and the samples were stored at −20°C.

In Tilburg, the Netherlands, for the isolation of DNA from bacteria, cells were suspended in 500 µl physiological saline supplemented with 1 mM EDTA (van der Zee et al., 1993b) and heated for 10 min at 95°C to lyse the cells. A volume of 5 µl was used for PCR. Serial dilutions of bacterial cells were made, from 0·2 × 10⁶ to 0·2 × 10⁷ cells per reaction. The number of cells was measured by measuring OD₆₀₀.

**Sequence analyses of the Bordetella recA gene and the IS1001 homologue in B. holmesii.** Because IS481 associated with B. pertussis was also found in the B. holmesii genome, PCR that targets IS481 can detect B. holmesii. The Bordetella recA gene was chosen as the target for a specific PCR for the detection of B. holmesii, and its sequence was studied by PCR-based sequencing. Primers were designed based on the recA sequence of B. pertussis (Favre & Viret, 1990). The primers RA1F and RA2R (Table 1) were used for B. pertussis, B. parapertussis and B. bronchiseptica. For B. avium, B. hinzii and B. holmesii, primers RA1F and RA3R (Table 1) were used. The purified PCR products were sequenced by the ABI PRISM BigDye Terminator v3·0 Cycle Sequencing Kit (Applied Biosystems) with the same primers that were used in the PCR. The nucleotide sequences were analysed with BioEdit 5·0·9 Sequence Editor, and compared to sequences in GenBank using BLASTN search algorithms (Altschul et al., 1997; Jones et al., 2005).

In the Netherlands, with primers BppA and BppZ based on IS1001 of B. parapertussis in the conventional PCR assay (van der Zee et al., 1996) (Table 1), a DNA fragment was amplified from B. holmesii, using a lowered annealing temperature of 55°C. Fragments obtained from three B. holmesii isolates were sequenced (Baseclear), analysed with BioEdit 5·0·9 Sequence Editor, and compared to sequences in GenBank using BLASTN search algorithms.

**B. holmesii-specific real-time PCR.** In Finland, for the real-time PCR, the primers were designed based on the recA sequence of B. holmesii (Table 1), and the amplification reactions were carried out in a LightCycler (Roche Diagnostics). The 20 µl reaction mixture contained 2 µl LightCycler FastStart Reaction Mix SYBR Green I, 4 mM MgCl₂, 4% DMSO (Merck), 0·5 µM HrecAF and HrecAR primers, and 4 µl template DNA. The temperature profile included initial denaturation at 95°C for 10 min, followed by 50 cycles of denaturation at 95°C for 1 s, annealing at 64°C for 3 s, and extension at 72°C for 7 s. The temperature transition rate was 20°C s⁻¹. Fluorescence was measured at the end of each extension phase. After amplification, a melting curve was constructed by heating the product at 20°C s⁻¹ to 95°C, lowering the temperature at 20°C s⁻¹ to 40°C for 170 s, and then heating at 0·1°C s⁻¹ to 97°C. Fluorescence was measured through the slow-heating phase. Fluorescence data were converted to derivative melting curves by plotting the negative derivative of the fluorescence with respect to temperature against temperature [−(dF/dT)] against T, and a specific melting temperature value (Tm) was obtained. In each run, B. holmesii DNA (5 pg µl⁻¹) was used as positive control and the reagents, except template DNA, as negative control. The PCR product expected was
164 bp in length. A 16 μl sample of the PCR products was run on a 2% Agarose MP gel (Roche) to confirm the correct size of the amplicon. To monitor inhibition of PCR, purified *B. holmesii* DNA was spiked into five DNA solutions randomly selected from the NP samples submitted for routine diagnosis of pertussis.

In Tilburg, the Netherlands, the *B. holmesii* PCR was performed separately in a volume of 25 μl containing 12.5 μl PCR mastermix (Eurogentec), 7.5 pmol Bh-F1 and Bh-R1 primers, and 4.4 pmol *B. holmesii*-specific probe (Bh1-NED). Both reaction mixtures were incubated at 50°C for 2 min and preheated at 95°C for 10 min, followed by 50 cycles of 15 s at 95°C, and 1 min at 60°C. PCR was performed on a 7900 HT Sequence Detection System (Applied Biosystems) with 9600 emulation.

**Clinical specimens.** In Finland, PCR has been routinely used since 1993 for the diagnosis of pertussis at the Department of Medical Microbiology, University of Turku (He et al. 1993, 1996). NP swabs (dacron, Copan Diagnostics) were taken from patients with suspected pertussis at local health centres and hospitals, stored

### Table 1. Primers and probes used in the sequencing and PCR

<table>
<thead>
<tr>
<th>Country</th>
<th>Primer/probe</th>
<th>Sequence (5’–3’)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finland</td>
<td><em>B. pertussis</em> specific (IS481)</td>
<td>GAT TCA ATA GGT TGT ATG CAT GGT T</td>
<td>Glare <em>et al.</em> (1990)</td>
</tr>
<tr>
<td></td>
<td>BP1</td>
<td>TTC AGG CAC ACA AAC TTG ATG GGC G</td>
<td>He <em>et al.</em> (1993)</td>
</tr>
<tr>
<td></td>
<td>BP2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>B. holmesii</em> specific (recA)</td>
<td>CCG AAT CCT CGG GCA AGA C</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>HrecAF</td>
<td>GGT GTC CGG TTG GGA GAT</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>HrecAR</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sequencing primers for <em>B. pertussis</em></td>
<td>GAT TCA ATA GGT TGT ATG CAT GGT T</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>B. avium</em> specific (recA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RA1F</td>
<td>GGA GCT TGC CCA CTC AGG</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>RA2R</td>
<td>GAC CAC ATT GGC TTG GAC</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>RA3R</td>
<td>TTG CCC TGG CCG ATG CGG TT</td>
<td>This study</td>
</tr>
<tr>
<td>Netherlands</td>
<td><em>B. pertussis</em> specific (IS481)</td>
<td>GAT TCA ATA GGT TGT ATG CAT GGT T</td>
<td></td>
</tr>
<tr>
<td>Tilburg</td>
<td>Bp-F1</td>
<td>GGC GGA TGA ACA CCC ATA AG</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>Bp-R1</td>
<td>GCG ATC AAT TGC TGG ACC AT</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>Bp1-FAM</td>
<td>ATG CCC GAT TGA CCT TCT TAC GTC G</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td><em>B. parapertussis</em> specific (IS1001)</td>
<td>GAT TCA ATA GGT TGT ATG CAT GGT T</td>
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</tr>
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<td></td>
<td>Bpp-F1</td>
<td>ATG CCT GAT CGC AAG TTG ATG</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>Bpp-R1</td>
<td>ACG CGT TCG ACG CCA TA</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>Bpp1-TET</td>
<td>CGC TGG GAG GCT GGC AGG G</td>
<td>This study</td>
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<td>PhHV inhibition control</td>
<td>GAT TCA ATA GGT TGT ATG CAT GGT T</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PhHV-F1</td>
<td>ATG CCT GAT CGC AAG TTG ATG</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>PhHV-R1</td>
<td>ACG CGT TCG ACG CCA TA</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>PhHV1-VIC</td>
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<td>This study</td>
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<tr>
<td></td>
<td><em>B. holmesii</em> specific (IS1001-like element)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Bh-F1</td>
<td>GCA ACT GGT TCG CTG GCT TGA</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>Bh-R1</td>
<td>CCA CGT CTG GGC CAA GTA C</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>Bh1-NED</td>
<td>ACG CAC CGG GTC TAT CAC TAC ATG G</td>
<td>This study</td>
</tr>
<tr>
<td>Amsterdam</td>
<td><em>B. pertussis</em> specific (IS481)</td>
<td>GAT TCA ATA GGT TGT ATG CAT GGT T</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BPIS-1 sense outer</td>
<td>GAC TTC GTC TTC GTG GCC AT</td>
<td>Farrell <em>et al.</em> (1999)</td>
</tr>
<tr>
<td></td>
<td>BPIS-2 antisense outer</td>
<td>GAC TTC GTC TTC GTG GCC AT</td>
<td>Farrell <em>et al.</em> (1999)</td>
</tr>
<tr>
<td></td>
<td>BP nest1 sense inner</td>
<td>GAC TTC GTC TTC GTG GCC AT</td>
<td>van der Zee <em>et al.</em> (1993b)</td>
</tr>
<tr>
<td></td>
<td>BP nest2 antisense inner</td>
<td>GAC TTC GTC TTC GTG GCC AT</td>
<td>van der Zee <em>et al.</em> (1993b)</td>
</tr>
<tr>
<td></td>
<td><em>B. parapertussis</em> specific (IS1001)</td>
<td>GAT TCA ATA GGT TGT ATG CAT GGT T</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BPPA sense outer</td>
<td>GAC TTC GTC TTC GTG GCC AT</td>
<td>van der Zee <em>et al.</em> (1993a)</td>
</tr>
<tr>
<td></td>
<td>BPPZ antisense outer</td>
<td>GAC TTC GTC TTC GTG GCC AT</td>
<td>van der Zee <em>et al.</em> (1993a)</td>
</tr>
<tr>
<td></td>
<td>BPPB sense inner</td>
<td>GAC TTC GTC TTC GTG GCC AT</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>BPPY antisense inner</td>
<td>GAC TTC GTC TTC GTG GCC AT</td>
<td>This study</td>
</tr>
</tbody>
</table>
dry in empty tubes and sent to the Department of Medical Microbiology, University of Turku. From 2000 to 2003, 2804 swabs were obtained.

In the Netherlands, NP swabs were tested at the Laboratory of Medical Microbiology, St Elisabeth Hospital, Tilburg, and at the GGD, Municipal Health Laboratory, Amsterdam. A total of 6903 NP swabs (dacron, Medical Wire) were collected from 1992 to 2003 and sent to the laboratory in Tilburg, and 1612 NP swabs were collected from 2001 to 2003 and sent to the laboratory in Amsterdam. At the time, the two laboratories received all specimens collected for PCR analysis of pertussis in the Netherlands.

**Isolation of DNA from NP swabs.** In Finland, the dry NP swabs were first placed in 300 μl autoclaved and UV-irradiated in-house distilled water at room temperature for 15 min, briefly mixed by vortex, and 200 μl of the fluid was then heated at 94 °C for 10 min. DNA was extracted by the Roche Kit and 10 μl was used for amplification. If not assayed immediately, the extracted DNA was stored frozen.

In Tilburg, dry swabs were suspended upon receipt in 500 μl physiological saline supplemented with 1 mM Tris/EDTA buffer. DNA was released by boiling the samples at 96 °C for 15 min. Inhibition was measured by addition of phocine herpes virus (PhHV) at a concentration 10× above the detection limit. Inhibited samples were further purified using the Qiagen DNA kit. Samples received after the year 2000 were purified using MagNaPure extraction (Total Nucleic Acid kit, Roche Molecular Biochemicals), with a final elution volume of 50 μl. Five microlitres were used for amplification.

In Amsterdam, dry swabs (dacron or cotton) were used. The tips of these swabs were cut and placed in tubes containing 300 μl PBS. Cells and other material originating from the patient were eluted from the swabs by shaking the tubes at room temperature for 30 min. After a short spin, 30 μl of the eluate was heated at 95 °C for 15 min. Further purification was done by traditional isopropanol precipitation. Two microlitres were used for the outer PCR reaction of the nested PCR.

**PCR assays for detection of B. pertussis and B. parapertussis.** In Finland, the PCR used for routine diagnosis of pertussis was a conventional PCR described previously (He et al., 1996). In brief, the primers BP1 and BP2 chosen from IS481 of B. pertussis were used (Glare et al., 1990; He et al., 1993) (Table 1). The sensitivity of the PCR assay is ~5 bacteria per reaction tube (He et al., 1996). AmpliTaq Gold polymerase (Applied Biosystems) was used. The reaction mixture of 30 μl contained 50 mM KCl, 10 mM Tris/HCl (pH 8-3), 1.5 mM MgCl₂, 0.01% (v/v) gelatin, 200 μM deoxyribonucleotides, 20 pmol primers BP1 and BP2, 1 U polymerase, and 5 μl extracted DNA. Amplification was performed using a DNA Engine (MJ Research) with the following conditions: initial denaturation for 10 min at 95 °C, then 40 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 55 °C, extension for 1 min at 72 °C and a final extension step of 5 min at 72 °C. The PCR products were stored at 4 °C before gel electrophoresis was performed. The amplicons were separated by agarose gel electrophoresis and the bands were visualized after staining with ethidium bromide.

In Tilburg, PCR was performed as described previously with primers specific for IS481 and IS1001 (Table 1; van der Zee et al., 1999b). In the multiplex PCR assay, an internal control consisting of PhHV (Niestroy, 2001; Templeton et al., 2003) was included (van der Zee et al., 1999b). In 2001, the conventional PCR was changed to a real-time format that required shorter amplicons than the former PCR. New primers were designed based on IS481 of B. pertussis and IS1001 of B. parapertussis (Table 1). Different fluorescent probes (Table 1) were used with 6-carboxytetramethylrhodamine (TAMRA) quenchers. Real-time PCRs for the detection of B. pertussis and B. parapertussis were combined in a multiplex PCR that also included amplification of the internal PhHV control. Viral DNA was added to clinical material to enable monitoring of the efficacy of MagNaPure isolation of DNA as well as inhibition of the PCR. In addition, a real-time PCR was developed based on the IS1001-like element of B. holmesii (Table 1).

In Amsterdam, nested duplex PCR for B. pertussis and B. parapertussis was used. The duplex PCR was performed with primers targeting IS481 of B. pertussis (Table 1; van der Zee et al., 1999b; Farrell et al., 1999). Primers for detection of B. parapertussis targeted IS1001 (Table 1; van der Zee et al., 1999a). All primers were synthesized by Isogen Life Science, Maarsen, The Netherlands. The outer (BPIS-1, BPIS-2, BPPA, BPPZ) and inner PCR (BP nes1, BP nes2, BPPB, BPPY) mixtures contained 20 ng of each of the four primers, 200 μM dNTP, 2 μM MgCl₂, 0.5 U Taq polymerase, and 2.5 μl 10× Taq buffer (Silverstar, Eurogentec) and water. To the outer PCR mixture, 2 μl nucleic acid solution was added to obtain a final volume of 25 μl. The PCR programme consisted of 1 step at 94 °C for 3 min followed by 30 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, and a final extension step of 72 °C for 5 min, in the PTC200 DNA engine (MJ Research). After completion of the outer PCR, 2 μl was transferred to the inner PCR mixture, and the same PCR cycling programme was followed. The inner PCR products were separated on a 10% acrylamide gel and stained with ethidium bromide. The length of the inner PCR fragments was indicative of the putative pathogen (Table 1). Mixtures of culture-positive controls and a buffer sample as negative control were added in each test run.

**RESULTS AND DISCUSSION**

In Finland and the Netherlands, the nationwide vaccination programmes against pertussis were started in 1952 and 1953, respectively, and the vaccination coverage has been high (He et al., 1998; Mooi et al., 1998). Despite the high coverage, pertussis has remained endemic and the incidence has increased in both countries. The two countries have also started to use IS481 PCRs for the diagnosis of pertussis, since 1992 and 1993, respectively (van der Zee et al., 1996; He et al., 1996). The diagnostic performance of the PCR assays is excellent and has proved to be stable. In the present study, the positivity rates obtained by IS481 PCR were similar in both countries.

*B. holmesii* has been isolated from the blood, pleural fluid and sputum of immunocompromised (Lindquist et al., 1995; Tang et al., 1998; Shepard et al., 2004) and immunocompetent patients (Ririe et al., 1997; Tang et al., 1998; Morris & Myers, 1998). The role of *B. holmesii* as a cause of pertussis is not known, but this organism was isolated from NP specimens of patients with pertussis-like symptoms in the USA from 1994 to 1998 (Yih et al., 1999; Mazengia et al. 2000). This has raised concerns about the role of *B. holmesii* as a cause of false diagnosis of *B. pertussis* and *B. parapertussis* infections, because *B. holmesii* also contains IS481. This sequence is almost identical to that in *B. pertussis*. *B. holmesii* also contains the IS1001-like element. The two IS elements are frequently used targets for the PCR diagnosis of *B. pertussis* and *B. parapertussis* infections, respectively. Some alternative detection methods for *B. holmesii* have also been published (Poddar, 2003; Vielmayer et al., 2004).
**Bordetella** species-specific recA sequences and the IS1001 homologue in *B. holmesii*

Alternative targets for detection of *Bordetella* species were identified in this study. The recA sequences of *Bordetella* species were aligned using BLASTN search algorithms, and a segment corresponding to 1046 bp of *B. holmesii* recA was analysed. The sequence similarity of *B. holmesii* recA was highest (90%) with *B. hinzii* recA (Fig. 1). The corresponding similarities of *B. holmesii* recA to those of other *Bordetella* species were 87% to *B. pertussis*, 87% to *B. parapertussis*, 87% to *B. bronchiseptica* and 86% to *B. avium* (Fig. 1). *B. parapertussis* and *B. bronchiseptica* recA had the highest sequence similarities to *B. pertussis* recA: 99.6% and 99.8%, respectively. The recA nucleotide sequences are available in the NCBI database and the accession numbers are AF399658 (*B. pertussis*), AF399659 (*B. parapertussis*), AF399657 (*B. bronchiseptica*), AY124330 (*B. avium*), AY124331 (*B. hinzii*) and AF399664 (*B. holmesii*).

The IS1001 fragments generated with primers BppA and BppZ (van der Zee et al., 1993a) from three isolates of *B. holmesii* were sequenced. The amplified fragments of the three isolates showed 97% similarity, whereas similarity with IS1001 of *B. parapertussis* was about 87%. By inverse PCR with primers directed outward of the obtained fragments, attempts were made to sequence the complete IS element. Analysis of flanking sequences revealed that the right inverted repeat was present and was almost identical to that of IS1001 in *B. parapertussis*. The left part of the IS element was lacking (data not shown). From the PCR reactions with outward primers, three to five DNA fragments were generated, suggesting that three to five copies of IS1001 are present in the genome of *B. holmesii*. The partial IS1001 nucleotide sequences of *B. holmesii* are available in the NCBI database and the accession numbers are AY786980–AY786982.

The recA sequences in the genus *Bordetella* appear to be species specific. Furthermore, no intra-species variation of the recA sequence was found when *B. pertussis* strains isolated in different countries and at different times were studied (Mooi et al., 1998; Gzyl et al., 2001; Wang et al., 2002). The results agree with the earlier observation that *B. pertussis* is a homogeneous species and recently derived (van der Zee et al., 1997; Parkhill et al., 2003). In addition, the partial IS1001 homologue in *B. holmesii* was sequenced and probably showed sufficient sequence variation to warrant a new IS designation. Unfortunately, no complete IS copy was isolated.

**Specificity and sensitivity of real-time PCR assays**

In this study, a *B. holmesii*-specific real-time PCR based on recA was developed, and the melting-curve analysis was used for specific detection of the amplicons (Ririe et al., 1997; Pietilä et al., 2000). Also, a newly developed *B. holmesii*-specific real-time PCR targeting the homologue of IS1001 is described. The specificity of these PCRs proved to be high.

The specificity of the *B. holmesii* recA real-time PCR was tested by using serial 10-fold dilutions (0-005 pg μl⁻¹–5 ng μl⁻¹) of DNA extracted from strains of *B. pertussis*, *B. parapertussis*, *B. bronchiseptica*, *B. avium*, *B. hinzii* and *B. holmesii* and 3 ng μl⁻¹ of other non-*Bordetella* bacterial species.
(16 species) and fungal DNA. In addition, purified DNA preparations of *B. petrii* (50 ng μl\(^{-1}\)) and *B. trematum* (60 ng μl\(^{-1}\)) were examined. An amplification product was obtained from the DNA of *B. holmesii*, but not from those of other *Bordetella* species, except *B. hinzii*. Also, other non-*Bordetella* bacterial DNA and the fungal DNA gave a negative result. A small amount of amplification product was obtained from *B. hinzii* DNA, but the Tm of the product was 2 °C higher than that of *B. holmesii*. No primer dimers were detected when the 50-cycle amplification profile was used. Serial 10-fold dilutions of *B. holmesii* (ATCC 51541\(^T\)) DNA (0.005 pg μl\(^{-1}\)–5 ng μl\(^{-1}\)) were used to analyse sensitivity. The effect of DNA concentration on Tm was examined by analysing 10-fold dilutions of *B. holmesii* DNA (0.005 pg μl\(^{-1}\)–5 ng μl\(^{-1}\)) in six parallel reactions in the same run (Fig. 2). The mean Tm was 87.70 °C and the coefficient of variation was 0.16%. The mean Tm value obtained by testing aliquots of the same sample (50 pg μl\(^{-1}\)) in five separate runs was 87.69 °C, and the corresponding inter-assay coefficient of variation was 0.18%. The mean Tm value obtained by testing aliquots of the same sample (50 pg μl\(^{-1}\)) five times in the same run was 87.70 °C, and the corresponding intra-assay coefficient of variation was 0.03%. The sensitivity of the PCR assay was 20 fg of DNA per reaction tube (∼3 bacterial cells) (Fig. 2). The analytical sensitivity of this assay was comparable to that of the conventional IS481 PCR.

The specificity of the IS1001-based real-time PCR for detection of *B. holmesii* was tested with all available *Bordetella* isolates, except *Bordetella anisorpii* (Ko et al., 2005), which is a potential ninth species of *Bordetella*. Only *B. holmesii* was

![Fig. 2. Analytical sensitivity of the *B. holmesii*-specific recA PCR assay.](image)

(A) amplification profiles; (B) melting curves; (C) agarose gel obtained from LightCycler PCR products. Samples: 1, 2000 pg; 2, 200 pg; 3, 20 pg; 4, 2 pg; 5, 0.2 pg; 6, 0.02 pg *B. holmesii* DNA. (A) For each dilution, the starting point of amplification can be seen from the amplification profiles (F1/cycle number). (B) Melting-curve analysis of PCR products and Tm values from recA PCR, using SYBR Green I fluorescence detection on the F1 channel of the LightCycler. The melting peaks were created by plotting the negative derivative of fluorescence versus temperature. (C) The products obtained from PCR were run on a 2% agarose gel. The gel was stained with ethidium bromide and visualized under UV light. Lanes: L, 100 bp ladder; 1–6, samples.
found to be positive. The sensitivity of the assay was 10 fg DNA per reaction tube (~2 bacterial cells).

In the Netherlands, both the real-time and conventional PCRs used the same targets, IS481 and IS1001, for the detection of *B. pertussis* and *B. parapertussis*. The real-time multiplex PCR for detection of *B. pertussis* and *B. parapertussis* was validated by comparison with the conventional assay. A random subset of 588 of 8515 clinical samples was investigated by both assays, and results were 100% concordant. The sensitivity of the assay was 1 and 10 fg of *B. pertussis* and *B. parapertussis* DNA, respectively, per reaction tube (1–2 bacterial cells). In contrast to the conventional assay, primers based on IS1001 in the real-time assay did not detect *B. holmesii*.

The real-time PCR offers many advantages compared to conventional PCR, such as rapidity, and simultaneous amplification and detection. The post-amplification detection procedure is not usually needed, therefore the potential for contamination of amplicons is low (Exner & Lewinski, 2002; Teo et al., 2002).

### Detection of *B. pertussis*, *B. parapertussis* and *B. holmesii* in clinical specimens

This is the largest study of clinical material published to date to evaluate whether *B. holmesii* confounds PCR assays of IS481 and IS1001.

In Finland, 2804 NP swabs were tested between 2000 and 2002 by conventional *IS481* PCR and 459 (16·4%) were positive for *B. pertussis*. The yearly PCR positivity rates were 16·6% (125/754) in 2000, 7·5% (32/424) in 2001, 14·3% (59/413) in 2002 and 20% (243/1213) in 2003.

In Tilburg, 6903 NP swabs were tested from 1992 to 2003 by IS481 and IS1001 PCRs with conventional or real-time formats (Table 2). A total of 1581 (22·9%) and 40 (0·6%) were positive for *B. pertussis* and *B. parapertussis*, respectively. Four samples were positive for both. In Amsterdam, 1612 NP swabs were tested from 2001 to 2003 by IS481 and IS1001 nested duplex PCR, and 275 (17·1%) and 10 (0·6%) were positive for *B. pertussis* and *B. parapertussis*, respectively (Table 2). Three samples were positive for both.

In Finland, every other swab of the 459 NP swabs positive by conventional IS481 PCR was tested by *B. holmesii*-specific LightCycler *recA* PCR. In the Netherlands, *B. holmesii*-specific PCR was performed on all IS481-PCR-positive samples received from 2000 to 2004, both from Tilburg and Amsterdam (total 1856). No *B. holmesii*-positive specimens were found in either country.

Our results show that *B. holmesii* is not found in Finnish and Dutch patients with pertussis-like symptoms. This indicates that *B. holmesii* does not in practice confound IS481 and IS1001 PCRs in the diagnosis of *B. pertussis* and *B. parapertussis* infections in these Finnish and Dutch populations.

### Concluding remarks

To our knowledge, this is the first report to study the prevalence of *B. holmesii* infections in patients with pertussis-like symptoms in European countries. We conclude that *B.
holmesii is not among the causative agents of pertussis-like symptoms in Finland and the Netherlands. It is of note that B. holmesii does not grow in culture if cephalixin is used (Mazengia et al., 2000). The limited number of isolations from respiratory tract samples could be due to lack of awareness, because B. holmesii is not thought to be a common inhabitant of the respiratory tract. Although PCR is usually more sensitive than culture in the diagnosis of pertussis, both tests are usually positive in unvaccinated patients. The possibility of B. holmesii should be taken into account, especially when NP samples of unvaccinated patients are IS481 PCR positive but culture negative. These samples could be retested with PCR assays described in this study.

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REFERENCES


32–40. PCR product of the sensu stricto by LightCycler fluorescence melting curve analysis of a probe and probe-target melting analysis.


