Bordetella holmesii DNA is not detected in nasopharyngeal swabs from Finnish and Dutch patients with suspected pertussis

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Bordetella holmesii is a Gram-negative bacterium first identified in 1995. It can cause pertussis-like symptoms in humans. *B. holmesii* contains insertion sequences IS481 and IS1001, two frequently used targets in the PCR diagnosis of *Bordetella pertussis* and *Bordetella parapertussis* infections. To investigate the prevalence of *B. holmesii* in Finnish and Dutch patients with pertussis-like symptoms and whether *B. holmesii* has caused any false-positive results in diagnostic PCRs, *B. holmesii*-specific real-time PCRs were developed. The Finnish methods were conventional IS481 PCR and *B. holmesii*-specific real-time PCR (LightCycler, Roche) targeting the *B. holmesii* *recA* gene. The Dutch methods were IS481 and IS1001 PCRs with conventional or real-time formats and *B. holmesii*-specific real-time PCR targeting the homologue of IS1001. Of 11 319 nasopharyngeal swabs, 2804 were collected from Finnish patients from 2000 to 2003, and 8515 from Dutch patients from 1992 to 2003. *B. holmesii* DNA was not found in the samples analysed. The results suggest that *B. holmesii* is not among the causative agents of pertussis-like symptoms in Finnish and Dutch patients and thus does not in practice confound IS481 and IS1001 PCRs.

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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,

Abbreviations: NP, nasopharyngeal; PhHV, phocine herpes virus.

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 15311^T and three human isolates) (He *et al.*, 1998), one *Bordetella bronchiseptica* strain (Central Public Health Laboratory, Colindale, UK), seven *B. holmesii* strains (ATCC 51541^T and six clinical isolates) (Yih *et al.*, 1999), one *Bordetella avium* strain (ATCC 35086^T) and one *Bordetella hinzii* strain (ATCC 51730^T). 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 51541^T and two clinical isolates), and one strain each of *B. hinzii*, *Bordetella trematum* 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 ($\sim 10^9$ 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 0.2×10^6 to 0.2×10^2 cells per reaction. The number of cells was assessed 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 (T_m) 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

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

Country	Primer/probe	Sequence (5'–3')	Source or reference
Finland			
	<i>B. pertussis</i> specific (IS481)		
	BP1	GAT TCA ATA GGT TGT ATG CAT GGT T	Glare <i>et al.</i> (1990)
	BP2	TTC AGG CAC ACA AAC TTG ATG GGC G	He <i>et al.</i> (1993)
	<i>B. holmesii</i> specific (<i>recA</i>)		
	HrecAF	CCG AAT CCT CGG GCA AGA C	This study
	HrecAR	GGT GTC CGG TTG GGA GAT	This study
	Sequencing primers for <i>B. pertussis</i>, <i>B. paraptussis</i>, <i>B. bronchiseptica</i>, <i>B. avium</i>, <i>B. hinzii</i> and <i>B. holmesii</i> (<i>recA</i>)		
	RA1F	GGA GCT TGC CCA CTC AGG	This study
	RA2R	GAC CAC ATT GGC TTG CAC	This study
	RA3R	TTG CCC TGG CCG ATG CGG TT	This study
Netherlands			
Tilburg			
	<i>B. pertussis</i> specific (IS481)		
	Bp-F1	GCC GGA TGA ACA CCC ATA AG	This study
	Bp-R1	GCG ATC AAT TGC TGG ACC AT	This study
	Bp1-FAM	ATG CCC GAT TGA CCT TCC TAC GTC G	This study
	<i>B. paraptussis</i> specific (IS1001)		
	Bpp-F1	ATG CTG GAT CGC AAG TTG ATG	This study
	Bpp-R1	ACG CGT TCG ACG CCA TA	This study
	Bpp1-TET	CGC TGG GAG GCT GGC AGG G	This study
	PhHV inhibition control		
	PhHV-F1	GGG CGA ATC ACA GAT TGA ATC	This study
	PhHV-R1	GCG GTT CCA AAC GTA CCA A	This study
	PhHV1-VIC	TTT TTT ATG TGT CCG CCA CCA TCT GGA TC	This study
	<i>B. holmesii</i> specific (IS1001-like element)		
	Bh-F1	GCA ACT GGT TCG CTT GGT CTA	This study
	Bh-R1	CCA CGT CGT GGC CAA GTA C	This study
	Bh1-NED	ACG CAC CCG GTC TAT CAC TTC ACG G	This study
Amsterdam			
	<i>B. pertussis</i> specific (IS481)		
	BPIS-1 sense outer	GAC TTC GTC TTC GTG GCC AT	Farrell <i>et al.</i> (1999)
	BPIS-2 antisense outer	GTA CAG CGC GCC CGA TGC CT	Farrell <i>et al.</i> (1999)
	BP nest1 sense inner	CGC GTG GCC TTC ACC GAC AT	van der Zee <i>et al.</i> (1993b)
	BP nest2 antisense inner	GGG CGG TAA GGT CGG GTA AA	van der Zee <i>et al.</i> (1993b)
	<i>B. paraptussis</i> specific (IS1001)		
	BPPA sense outer	CGC CGC TTG ATG ACC TTG ATA	van der Zee <i>et al.</i> (1993a)
	BPPZ antisense outer	CAC CGC CTA CGA GTT GGA GAT ATA	van der Zee <i>et al.</i> (1993a)
	BPPB sense inner	CCA GGA TGC CGT GCA GAT AGC	This study
	BPPY antisense inner	CAT TGA TCG GGT GCG CGT GGA	This study

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

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. paraptussis*. 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 50 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, 1993b). In the multiplex PCR assay, an internal control consisting of PhHV (Niesters, 2001; Templeton *et al.*, 2003) was included (van der Zee, 1993b). 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. paraptussis* (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. paraptussis* 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. paraptussis* was used. The duplex PCR was performed with primers targeting IS481 of *B. pertussis* (Table 1; van der Zee *et al.*, 1993b; Farrell *et al.*, 1999). Primers for detection of *B. paraptussis* targeted IS1001 (Table 1; van der Zee *et al.*, 1993a). All primers were synthesized by Isogen Life Science, Maarssen, the Netherlands. The outer (BPIS-1, BPIS-2, BPPA, BPPZ) and inner PCR (BP nest1, BP nest2, BPPB, BPPY) mixtures contained 20 ng of each of the four primers, 200 µM dNTP, 2.0 mM 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 93 °C for 30 s, 58 °C for 30 s and 72 °C for 50 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. paraptussis* 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. paraptussis* infections, respectively. Some alternative detection methods for *B. holmesii* have also been published (Poddar, 2003; Vilemayer *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

Fig. 1. Partial *recA* sequences of *Bordetella* species and sequences of primers used for *B. holmesii*-specific *recA* PCR. The mismatches to the sequence of *B. holmesii recA* are highlighted.

(16 species) and fungal DNA. In addition, purified DNA preparations of *B. petrii* ($50 \text{ ng } \mu\text{l}^{-1}$) and *B. trematum* ($60 \text{ ng } \mu\text{l}^{-1}$) 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 T_m 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 ($0.005 \text{ pg } \mu\text{l}^{-1}$ – $5 \text{ ng } \mu\text{l}^{-1}$) were used to analyse sensitivity. The effect of DNA concentration on T_m was examined by analysing 10-fold dilutions of *B. holmesii* DNA ($0.005 \text{ pg } \mu\text{l}^{-1}$ – $5 \text{ ng } \mu\text{l}^{-1}$) in six parallel reactions in the same run (Fig. 2). The mean T_m was

87.70°C and the coefficient of variation was 0.16% . The mean T_m value obtained by testing aliquots of the same sample ($50 \text{ pg } \mu\text{l}^{-1}$) in five separate runs was 87.69°C , and the corresponding inter-assay coefficient of variation was 0.18% . The mean T_m value obtained by testing aliquots of the same sample ($50 \text{ pg } \mu\text{l}^{-1}$) 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 ansorpii* (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. (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 T_m 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 2003 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.*

Table 2. Number of NP samples tested by the PCR assays in the Netherlands

Place	Year	No. of samples	No. PCR positive (%) for:		
			<i>B. pertussis</i>	<i>B. parapertussis</i>	Both
Tilburg	1992	34	5 (14.7)	0	0
	1993	158	25 (15.8)	0	0
	1994	197	21 (10.7)	0	0
	1995	276	36 (13.0)	2 (0.7)	0
	1996	1139	178 (15.6)	14 (1.2)	1
	1997	838	162 (19.3)	5 (0.6)	0
	1998	520	137 (26.3)	1 (0.2)	0
	1999	866	315 (36.4)	5 (0.6)	0
	2000	544	137 (25.2)	1 (0.2)	0
	2001	1043	295 (28.3)	8 (0.8)	3
	2002	662	185 (27.9)	2 (0.3)	0
	2003	616	85 (13.8)	2 (0.3)	0
Amsterdam	Total	6903	1581 (22.9)	40 (0.6)	4
	2001	774	144 (18.6)	4 (0.5)	3
	2002	506	73 (14.4)	6 (1.2)	0
	2003	332	58 (17.5)	0	0
	Total	1612	275 (17.1)	10 (0.6)	3

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 cephalaxin 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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