(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
29 November 2001 (29.11.2001)

PCT

(10) International Publication Number
WO 01/90405 A1

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number: PCT/AU01/00557

(22) International Filing Date: 15 May 2001 (15.05.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PQ 7672 22 May 2000 (22.05.2000) AU

(71) Applicant *(for all designated States except US)*: **THE UNIVERSITY OF SOUTHERN QUEENSLAND** [AU/AU]; West Street, Toowoomba, QLD 4350 (AU).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **FARRELL, David, John** [AU/GB]; 2 Mortain Drive, Berkhamsted, Hertfordshire HP4 IJ2 (GB). **DAGGARD, Grant, Edward** [AU/AU]; 151 Long Street, Toowoomba, QLD 4350 (AU). **MUKKUR, Trilochan, Kanwaljit, Singh** [AU/AU]; 31 Ocean Street, Toowoomba, QLD 4350 (AU).

(74) Agent: **FISHER ADAMS KELLY**; LeveL 13, Amp Place, 10 Eagle Street, Gpo Box 1413, Brisbane, QLD 4000 (AU).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/90405 A1

(54) Title: SHARED PRIMER PCR COMBINED WITH HYBRIDISATION FOR THE DETECTION OF BORDETELLA PERTUSSIS AND B PARAPERTUSSIS

(57) Abstract: A method of detecting Bordetella pertussis and Bordetella parapertussis infection is provided. The method includes amplification of a Bordetella porin gene fragment from a sample such as a human nasopharyngeal aspirate using a Bordetella-specific primer, a Bordetella pertussis-specific primer and a Bordetella parapertussis-specific primer. The presence of the Bordetella gene fragment is then detected by hybridization to a Bordetella-specific probe. If positive, hybridization to a Bordetella pertussis-specific probe and/or a Bordetella parapertussis-specific probe is used to determine whether the porin gene fragment is indicative of Bordetella pertussis and/or Bordetella parapertussis infection. The method of the invention is designed so that errors introduced by amplification are minimized by generating amplification products of similar size and by incorporating amplification of a reference nucleic acid as an internal control.

TITLE

BORDETELLA DETECTION

FIELD OF THE INVENTION

THIS INVENTION relates to a method of detecting a *Bordetella* nucleic acid, and in particular, for distinguishing between *Bordetella pertussis* and *Bordetella parapertussis* nucleic acids. A particular application of the method of detection, although without limitation thereto, is the detection of a *Bordetella* nucleic acid in clinical samples as an indicator of *Bordetella* infection.

BACKGROUND OF THE INVENTION

The *Bordetella* genus of gram-negative bacteria includes disease-causing organisms which infect humans. Most notably, *Bordetella pertussis* and *Bordetella parapertussis* are organisms responsible for "pertussis syndrome" in humans, otherwise commonly referred to as "whooping cough". Whooping cough is primarily a disease in children which typically presents itself clinically as prolonged paroxysmal coughing seizures. Such coughing seizures are particularly severe on the respiratory system of infants, and in some cases can lead to death. This has led to mass immunization campaigns aimed particularly at infants and young children.

As a result of immunization, "typical" pertussis syndrome is readily diagnosed, particularly in unimmunized individuals. However, "atypical" pertussis syndrome presents particular challenges to clinical diagnosis. Atypical pertussis syndrome appears in two general scenarios:

(1) neonates and young children present with apnea and seizures without paroxysmal coughing;

(2) mild or absent symptoms occurring in adults or previously vaccinated children, such that the syndrome proceeds unrecognized.

A particularly dangerous consequence of (2) is that exposure of unimmunized children to asymptomatic individuals places these children at high risk of infection (Farrell *et al.*, 1999a, J. Clin. Microbiol. **37** 606).

Generally, the more severe forms of pertussis syndrome are caused by *Bordetella pertussis*, while *Bordetella parapertussis* causes a milder form of the illness. It should be noted that other agents such as adenovirus, chlamydiae, cytomegalovirus and respiratory syncytial virus have also been implicated in

pertussis syndrome (Farrell *et al.*, 1999a, *supra*), further complicating diagnosis and treatment.

Thus, it has become imperative that diagnostic assays be devoloped which assist symptom-based clinical diagnosis in determining the causative organsim(s) underlying pertussis syndrome, whether typical or atypical.

Historically, diagnostic tests for *Bordetella* infection have employed culturing nasopharyngeal samples or serological detection of *Bordetella*-specific antibodies.

More recently, a number of diagnostic methods have been employed which utilize the polymerase chain reaction (PCR) as a means of detecting *Bordetella* nucleic acids. Usually, PCR has been used to detect *Bordetella* in samples obtained from nasopharyngeal specimens in the form of aspirates or swabs. However, such PCR methods have produced varying levels of efficacy, probably because a variety of different primers, sample collection methods, reaction conditions and target nucleic acids have been used. Of most importance is that PCR diagnosis and clinical diagnosis are consistent, in that there should be strong concordance between the results of the two diagnostic approaches.

A consensus was reached among groups involved in pertussis syndrome diagnosis (reported in Meade & Bollen, 1994, J. Med. Microbiol. **41** 51), and the following points were listed as being required of PCR-based *Bordetella* detection:

(i) sensitivity: demonstration that nearly 100% of "culture positives" are detected by PCR; demonstration that a variety of *Bordetella* species and strains are detectable, including laboratory strains and field isolates;

(ii) specificity: demonstration that *Bordetella pertussis* and *Bordetella parapertussis* can be distinguished;

(iii) suitable positive control to monitor PCR reaction and detection of amplification products; and

(iv) procedures to avoid "false positive" and in particular, "false negative" results.

Although the requirements of PCR-based *Bordetella* detection have

been well documented, achieving the necessary requirements has hitherto not been possible.

In this regard, the approach of Farrell *et al.*, 1999a, *supra*, was to use nested duplex PCR which employed a nested set of two pairs of primers specific for *Bordetella pertussis* and *Bordetella parapertussis* respectively. Detection of PCR amplification products was by visualization of ethidium bromide-stained gels. Specificity was achieved by detecting a 205 base pair (bp) amplification product indicative of a *B. pertussis* nucleic acid, and/or a 171 bp amplification product indicative of a *B. parapertussis* nucleic acid. However, the difference in amplification product sizes was considered sub-optimal, in that the efficiency of product amplification may well have been variable between *Bordetella* species. Further to this, a high proportion of "false positives" were detected, that is, PCR-positive samples which were obtained from "culture-negative" samples.

A shared primer approach was developed by Li *et al.*, 1994, J. Clin. Microbiol. **32** 783, which approach employed a primer capable of hybridizing to a 3' region of the porin gene conserved in *B. pertussis* and *B.parapertussis*. In order to achieve specificity, primers were designed according to a non-conserved 5' region of the porin gene in *B. pertussis* and *B. parapertussis* respectively. In a somewhat similar fashion to Farrell *et al.*, 1999, *supra*, size-based detection of amplification products was used to distinguish between *B. pertussis* and *B. parapertussis*. This afforded a simpler approach to that of Farrell *et al.*, 1999a, *supra*, in that fewer primers were required, although suffering from the same deficiency with regard to different amplification product sizes. Crucially, however, only 80% of culture-positives were scored as PCR-positive. It is to be noted that Li *et al.*, 1994, *supra* improved this proportion to 95% by using a subsequent step of immunoblot detection of digoxigenin-labeled PCR products.

An alternative approach was taken by Farrell *et al.*, 1999b, Abstract 1569 In: Abstracts of the 39th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco USA, where common primers specific for the *Bordetella* pertussis toxin gene (*ptx*) were used to amplify equal-sized products which were subsequently differentiated into *Bordetella pertussis* and *Bordetella parapertussis* products by a sequence-specific hybridization step. Furthermore, a

positive control reaction was used which utilized overlap extension PCR amplification of a mouse β actin gene segment to facilitate determination as to whether the PCR reaction had proceeded. This method was claimed to have resulted in an analytical specificity of 100%.

OBJECT OF THE INVENTION

Notwithstanding the advances made in creating *Bordetella* detection methods, the method of Farrell *et al.*, 1999b, *supra* and others, suffers from several limitations. First, the PCR amplification method does not use primers specific for *Bordetella pertussis* and *Bordetella parapertussis*. Therefore, subtle differences between the *Bordetella pertussis* and *Bordetella parapertussis* target sequences with respect to annealing of the common primers may result in preferential amplification of one target sequence over another. Second, it has also become apparent that the *ptx* gene has relatively low clinical sensitivity. Third, polymorphisms in the *ptx* gene render doubtful it's use as an unambiguous target sequence for mass diagnostic screening. This polymorphism is believed to be driven, at least in part, by the use of the ptx protein in mass immunization regimes.

It is therefore an object of the invention to provide a method of detecting a *Bordetella* nucleic acid.

It is another object of the invention to provide a *Bordetella* detection kit.

SUMMARY OF THE INVENTION

In one aspect, the present invention resides in a method of detecting a *Bordetella* nucleic acid, said method including the steps of:

(i) subjecting a sample to a nucleic acid sequence amplification technique using a *Bordetella*-specific primer, a *Bordetella pertussis*-specific primer and a *Bordetella parapertussis*-specific primer; and

(ii) determining whether or not said sample includes a *Bordetella* nucleic acid according to whether or not one or more amplification products produced at step (i) hybridizes to a *Bordetella*-specific probe.

Preferably, if a *Bordetella* nucleic acid is detected at step (ii), a subsequent hybridization step is performed to determine whether said one or more

amplification products hybridize to a *Bordetella pertussis*-specific probe as an indication that said sample includes a *Bordetella pertussis* nucleic acid and/or to a *Bordetella parapertussis*-specific probe as an indication that said sample includes a *Bordetella parapertussis* nucleic acid.

Preferably, the *Bordetella* nucleic acid corresponds to a porin (*por*) gene or fragment thereof, in which case the *Bordetella pertussis* nucleic acid and the *Bordetella parapertussis* nucleic acid correspond to respective species-specific *por* genes or fragments thereof.

Preferably, probe hybridization is performed as a microwell-based assay.

Preferably, a reference nucleic acid is added to said sample before step (ii).

In another aspect, the present invention resides in a kit for detecting a *Bordetella* nucleic acid, said kit including:

(i) a *Bordetella*-specific primer, a *Bordetella pertussis*- specific primer and a *Bordetella parapertussis*-specific primer; and

(ii) a *Bordetella*-specific probe.

Preferably, the kit further includes a *Bordetella pertussis*-specific probe and a *Bordetella parapertussis*-specific probe.

Preferably, the kit further includes a reference nucleic acid.

Preferably, the kit further includes a reference nucleic acid-specific probe.

According to the first- and second-mentioned aspects, preferably the *Bordetella*-specific primer has a nucleotide sequence according to SEQ ID NO:4 or SEQ ID NO:13.

Preferably, the *Bordetella pertussis*-specific primer has a nucleotide sequence according to SEQ ID NO:1, SEQ ID NO: 2 or SEQ ID NO: 11.

Preferably, the *Bordetella parapertussis*-specific primer has a nucleotide sequence according to SEQ ID NO:3 or SEQ ID NO:12.

Preferably, the Bordetella-specific probe has a nucleotide sequence according to SEQ ID NO:7 or SEQ ID NO: 16.

Preferably, the *Bordetella pertussis*-specific probe has a nucleotide

sequence according to SEQ ID NO:5 or SEQ ID NO:14.

Preferably, the *Bordetella parapertussis*-specific probe has a nucleotide sequence according to SEQ ID NO:6 or SEQ ID NO:15.

Preferably, the reference nucleic acid-specific probe has a nucleotide sequence according to SEQ ID NO:10.

Throughout this specification, it will be understood that "*comprise*", "*comprises*" and "*comprising*" are used herein inclusively rather than exclusively, in that a stated integer or group of integers may include one or more other non-stated integers or groups of integers.

BRIEF DESCRIPTION OF THE TABLES

TABLE 1: Summary of recommendations for use of PCR in the diagnosis of *Bordetella pertussis* infections.

TABLE 2: Primer sequences for the method of detecting *Bordetella* nucleic acids. DFPOR1F (SEQ ID NO:1) and DFPOR1'F (SEQ ID NO:2) are *Bordetella pertussis*-specific primers; DFPOR2F (SEQ ID NO:3) is a *Bordetella parapertussis*-specific primer; DFPORRB (SEQ ID NO:4) is a *Bordetella*-specific primer; DFPORP1 (SEQ ID NO:5) is a *Bordetella pertussis*-specific probe; DFPORP2 (SEQ ID NO:6) is a *Bordetella parapertussis*-specific probe; DFPORP3 (SEQ ID NO:7) is a *Bordetella*-specific probe; DFPORFMB (SEQ ID NO:8) is a primer containing the SEQ ID NO:1 sequence used in constructing a β-actin reference nucleic acid; DFPORRMB (SEQ ID NO:9) is a primer containing the SEQ ID NO:3 sequence used in constructing the β-actin reference nucleic acid; and DFMBAP (SEQ ID NO:10) is a β-actin-specific probe for detecting amplification products PCR amplified from the reference nucleic acid.

TABLE 3: Alternative primers and probes. DFPOR3F (SEQ ID NO:11) is a *Bordetella pertussis*-specific primer; DFPOR4F (SEQ ID NO:12) is a *Bordetella parapertussis*-specific primer; DFPOR2R (SEQ ID NO:13) is a *Bordetella*-specific primer which is preferably 5' biotinylated; DFPORP4 (SEQ ID NO:14) is a *Bordetella pertussis*-specific probe; DFPORP5 (SEQ ID NO:15) is a *Bordetella parapertussis*-specific probe; DFPORP6 (SEQ ID NO:16) is a *Bordetella*-specific probe.

TABLE 4: Absorbance values (A450/620) for reactions containing different amounts of *Bordetella pertussis* (strain Tohama I) and *Bordetella parapertussis* (strain ATCC15237) expressed as colony forming units (CFU) per reaction. All values represent the average of three (3) readings.

TABLE 5: Specificity of the individual probes used in the *Bordetella* detection method.of the invention. The number of individual strains are in parentheses. *Tested by the modified POR method of the invention.§From Farrell *et al.*, 1999a, *supra.*

TABLE 6: Comparison of results from method of the invention (POR) and prior art (IS481) detection method. Number of samples =705.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention is predicated, at least in part, on the discovery that use of primers differentially specific for the porin gene of *B. pertussis* and *B. parapertussis* to produce similar-sized amplification products, together with a subsequent hybridization step, results in a diagnostic method having greater efficiency and specificity while having reduced likelihood of failing due to polymorphic variation within the bacterial population.

As used herein, an "*oligonucleotide*" is a single- or double-stranded nucleic acid having no more than seventy (70) nucleotides (bases) or nucleotide pairs (base pairs). A "*polynucleotide*" has more than seventy (70) nucleotides or nucleotide pairs.

As used herein, a "*probe*" is a single- or double-stranded oligonucleotide or polynucleotide, one and/or the other strand of which is capable of hybridizing to another nucleic acid, to thereby form a "hybrid" nucleic acid

As used herein, a "*primer*" is a single-stranded oligonucleotide which is capable of hybridizing to a nucleic acid "template" and being extended in a template-dependent fashion by the action of a suitable DNA polymerase such as *Taq* polymerase, RNA-dependent DNA polymerase or Sequenase™.

Probes and primers of the invention may be labeled, for example, with biotin or digoxigenin, with fluorochromes such as FITC, TRITC, Texas Red, TET, FAM6, HEX, ROX and Oregon Green and with radionuclides such as ^{125}I, ^{32}P, ^{33}P or ^{35}S to assist detection of amplification products by techniques are well

known in the art.

As used herein, a "*nucleic acid sequence amplification technique*" is any technique which employs a DNA polymerase in repeated cycles of template-dependent primer extension to thereby generate an amplification product. The most commonly used nucleic acid sequence amplification technique is polymerase chain reaction (PCR), although a variety of other amplification techniques have been produced over the years. These include ligase chain reaction (LCR), strand displacement amplification (SDA) and rolling circle amplification (RCA). An LCR method is provided, for example, in International Publication No. WO89/09385, which is herein incorporated by reference. An SDA method can be found, for example, in U.S. Patent No. 5455166 which is herein incorporated by reference. An RCA method can be found, for example, in International Publication No. WO97/19193 which is herein incorporated by reference.

Preferably, the nucleic acid sequence amplification technique is PCR.

As used herein, an "*amplification product*" is a nucleic acid generated by a nucleic acid sequence amplification technique.

As used herein, "*hybridization*" refers to formation of a hybrid nucleic acid through base-pairing between complementary or at least partially complementary nucleotide sequences under defined conditions, as is well known in the art. Normal base-pairing occurs through formation of hydrogen bonds between complementary A and T or U bases, and between G and C bases. It will also be appreciated that base-pairing may occur between various derivatives of purines (G and A) and pyrimidines (C, T and U). Purine derivatives include inosine, methylinosine and methyladenosines. Pyrimidine derivatives include sulfur-containing pyrimidines such as thiouridine and methylated pyrimidines such as methylcytosine. For a detailed discussion of the factors that affect hybridization of PCR primers, the skilled addressee is directed to Chapter 15 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY Eds Ausubel et al. (John Wiley & Sons NY 1995-1999). For a detailed discussion of the factors that generally affect nucleic acid hybridization, the skilled addressee is directed to Chapter 2 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, *supra*. A preferred

probe hybridization method using commercially available reagents and methodology is provided hereinafter.

As used herein, "*-specific*" in the context of primers and probes refers to an ability to preferentially hybridize to a particular nucleotide sequence under defined hybridization conditions, without displaying cross-reactivity with other related sequences. Operationally, this means that the "*-specific*" primer or probe must hybridize to said particular nucleotide sequence but not to any other nucleotide sequence present in said sample, during amplification. Clearly, as used herein "*-specific*" does not imply absolute specificity. For example, the preferred *Bordetella parapertussis*-specific primer and probe used in the present invention does not distinguish between *Bordetella parapertussis* and *Bordetella bronchiseptica* porin gene sequences, but does distinguish between *Bordetella parapertussis* and *Bordetella pertussis* porin gene sequences.

As used herein, a "*genetic locus*" is any component of a genome which is heritable. A locus may be a "*gene*", and thereby include elements such as an amino acid coding region present in one or more cistrons, together with an operator, a promoter, a terminator and any other regulatory nucleotide sequence(s) common to prokaryotic or eukaryotic genes. Alternatively, a genetic locus may include non-coding sequence repeats, a ribosomal RNA gene, or a tRNA gene. A genetic locus may therefore comprise any, some or all of the above.

As used herein, "*conserved*" in relation to a genetic locus or region of that locus refers to a level of nucleotide sequence similarity exhibited by nucleic acids corresponding to said locus, whether obtained from organisms in separate taxonomic groups, or obtained from organisms representing isolates or strains of a single taxonomic group. Hence, the more conserved the locus or region of the locus, the greater the degree of sequence similarity.

Suitably, said sample includes nucleic acid(s) that may be in the form of DNA or RNA. DNA includes single-stranded and double-stranded genomic DNA and cDNA as are well understood in the art. RNA includes single-stranded and double-stranded unprocessed RNA, mRNA and tRNA.

Preferably, in order to minimize handling of said sample, genomic DNA is applicable to the method of the invention. However, in principle, cDNA

could be generated by reverse-transcribing isolated RNA as is well known in the art, as for example described in Chapter 15 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, *supra*.

Suitably, the sample is a nasopharyngeal aspirate (NPA), a throat swab or sputum.

Suitably, the sample is obtained from a mammal.

Preferably, the mammal is a human.

Preferably, the *Bordetella* nucleic acid corresponds to or is derived from a genetic locus present in a plurality of isolates of *Bordetella* species, so that the method and kit of the invention can be used to detect *Bordetella* in a wide variety of clinical samples.

Preferably, the genetic locus includes a gene which encodes a structural protein.

Preferably, the structural protein is porin (por), encoded by the porin (*por*) gene.

Suitably, the *Bordetella*-specific primer, the *Bordetella pertussis*-specific primer and the *B. parapertussis*-specific primer have respective nucleotide sequences which hybridize to the genetic locus, which locus is present in a genome of both *B. pertussis* and *B. parapertussis*. Furthermore, the locus has a region which is conserved between *B. pertussis* and *B. parapertussis* and a region which is non-conserved between these *Bordetella* species. Thus, the *Bordetella*-specific primer is hybridizable to the conserved region of the locus, and the *B. pertussis*- and *B. parapertussis*-specific primers is hybridizable to the non-conserved region of the locus particular to each species.

Preferably, the *Bordetella*-specific primer is capable of hybridizing to a 3' region of the porin gene (*por*), which region has a nucleotide sequence conserved in *Bordetella pertussis* and *Bordetella parapertussis*.

Even more preferably, the *Bordetella*-specific primer has a nucleotide sequence according to SEQ ID NO:4.

Preferably, the *Bordetella pertussis*-specific primer has a nucleotide sequence capable of hybridizing to a non-conserved region of said porin gene.

More preferably, the non-conserved region of said genetic locus is a

5' region of the porin gene.

Even more preferably, the *Bordetella pertussis*-specific primer has a nucleotide sequence according to SEQ ID NO:1 or SEQ ID NO:2.

Advantageously, the *Bordetella pertussis*-specific primer has a nucleotide sequence according to SEQ ID NO:1.

Preferably, the *Bordetella parapertussis*-specific primer has a nucleotide sequence capable of hybridizing to a non-conserved region of said porin gene.

More preferably, the non-conserved region is a 5' region of the porin gene.

Even more preferably, the *Bordetella parapertussis*-specific primer has a nucleotide sequence according to SEQ ID NO:3.

Other primer sequences applicable to the method of the invention have respective nucleotide sequences as set forth in SEQ ID NOS:11-13 listed in Table 3.

Similar considerations apply to the *Bordetella pertussis*-specific probe and the *Bordetella parápertussis*-specific probe, in that each is capable of hybridizing to a respective non-conserved region of said genetic locus which has a nucleotide sequence particular to each species. Suitably, the genetic locus is that to which the primers of the invention are capable of hybridizing. However, it is preferred that each said probe has a nucleotide sequence distinct from each said primer.

Preferably, the *Bordetella pertussis*-specific probe has a nucleotide sequence according to SEQ ID NO: 5.

Preferably, the *Bordetella parapertussis*-specific probe has a nucleotide sequence according to SEQ ID NO: 6.

Preferably, a *Bordetella*-specific probe which is capable of hybridizing to a same or different conserved region of said locus is also provided.

More preferably, the *Bordetella*-specific probe is capable of hybridizing to a conserved region different to that which the *Bordetella*- specific primer hybridizes.

Even more preferably, the *Bordetella*-specific probe has a

nucleotide sequence according to SEQ ID NO:7.

Other probes applicable to the method of the invention have respective nucleotide sequences as set forth in SEQ ID NOS:14-16 listed in Table 3.

In light of the foregoing, it will be appreciated that a *Bordetella*-specific probe is utilized at step (ii) according to the method of the invention, in order to determine whether said sample includes said *Bordetella* nucleic acid, prior to distinguishing between *Bordetella* species. If said sample is found not to include said *Bordetella* nucleic acid, preferably a nucleic acid corresponding to a *por* gene or fragment thereof, then no further detection is required. However, if said sample is found to contain said *Bordetella* nucleic acid, then discrimination between a *B. pertussis* and *B. parapertussis* nucleic acid can be performed by hybridization with *B. pertussis*- and *B. parapertussis*-specific probes. This preferred method eliminates "negative" samples as a preliminary step, so that only positive samples are scrutinized further. This reduces the cost and increases the speed of detection, which are particularly important factors where a plurality of samples are analyzed such as during mass screening. Thus, samples which have been found to contain a *Bordetella* nucleic acid have undergone two rounds of detection, hence providing more confidence in the positive result so obtained.

Preferably, in cases where more than one amplification product is produced, the respective amplification products are of at least similar size. An advantage provided by similar-sized amplification products is that this reduces the likelihood of variation in the efficiency of amplification between products.

More preferably, the amplification products are within 10% of each other in terms of the predicted number of base pairs.

Even more preferably, the amplification products are within 5% of each other in terms of the predicted number of base pairs.

Advantageously, each said amplification product has an equal number of base pairs.

In one embodiment, the *Bordetella pertussis* amplification product and the *Bordetella parapertussis* amplification product each have approximately 112 base pairs.

Preferably, in order to monitor the efficiency of amplification, a reference nucleic acid is added to the sample at step (i).

Preferably, the *Bordetella pertussis*-specific primer is capable of hybridizing to a region of said reference nucleic acid, and the *Bordetella*-specific primer is capable of hybridizing to a region distinct from the *Bordetella pertussis*-specific primer.

In light of the foregoing, it will be understood that the reference nucleic acid when added to the sample at step (i) will subsequently undergo amplification to produce a reference amplification product. Accordingly, it is preferable that the reference amplification product is of at least similar size to the *Bordetella pertussis* amplification product. For this to occur, the reference nucleic acid suitably includes an intervening nucleotide sequence located intermediate the regions to which the primers are capable of hybridizing.

Preferably, the intervening nucleotide sequence corresponds to a region of a mouse β-actin cDNA, as for example provided under GenBank Accession No. X03672. Preferred primers SEQ ID NO:8 and SEQ ID NO:9 anneal to regions corresponding to nucleotides 893-910 and 946-964, respectively, of the aforementioned mouse β actin cDNA. These hybrid primers thereby incorporate *Bordetella pertussis*-specific and *Bordetella*-specific primer annealing sequences into the reference nucleic acid when the reference nucleic acid is PCR-amplified from mouse β actin cDNA template. These primer sites thereby result in PCR amplification of the reference nucleic acid (if added to the PCR reaction mix) during the PCR method of detection according to the present invention.

It will be appreciated by persons skilled in the art that this principle is generally extendible to other β actin cDNA sequences and to a variety of other cDNAs routinely used in recombinant DNA technology.

Detection of a reference nucleic acid amplification product is preferably performed by hybridization of said amplification product to a reference nucleic acid-specific probe.

A preferred reference nucleic acid-specific probe nucleotide sequence is set forth in SEQ ID NO:10.

In light of the foregoing, it will be appreciated that in a preferred embodiment, there is provided a method of detecting a *Bordetella* nucleic acid corresponding to a porin gene or fragment thereof, said method including the steps of:

(i) subjecting a human nasopharyngeal aspirate sample to polymerase chain reaction (PCR) using a *Bordetella*-specific primer, a *Bordetella pertussis*-specific primer and a *Bordetella parapertussis*-specific primer, said sample comprising a reference nucleic acid to which the *Bordetella*-specific primer and the *Bordetella pertussis*-specific primer are respectively hybridizable;

(ii) determining whether or not said sample includes:

(a) a reference nucleic acid amplification product according to whether or not said reference nucleic acid amplification product hybridizes to a reference nucleic acid-specific probe; and

(b) a *Bordetella* nucleic acid according to whether or not one or more amplification products produced at step (i) hybridize to a *Bordetella*-specific probe; and

(iii) if a *Bordetella* nucleic acid is detected at step (b), determining whether said amplification products hybridize to a *Bordetella pertussis*-specific probe as an indication that said sample includes a *Bordetella pertussis* nucleic acid, and/or to a *Bordetella parapertussis*-specific probe, as an indication that said sample includes a *Bordetella parapertussis* nucleic acid.

The present invention will be now described in more detail by reference to the following non-limiting examples.

EXAMPLE 1

Materials and Methods

1.1 *Bacterial strains and genomic DNA*

Bacterial strains and genomic DNA for sensitivity and specificity studies were the same as described previously (Farrell *et al.*, 1999a, *supra*). Additional strains tested were: *Bordetella avium* strain TC9 and *Bordetella hinzii* strain TC58, both kindly donated by Patrick Blackall from the Queensland Department of Primary Industries Animal Research Institute, Brisbane, Australia; and four *Bordetella*

holmesii strains.

1.2 Patient specimens

Sample collection, initial treatment and storage were as previously described (Farrell *et al.*, 1999a, *supra*). A total of 705 samples (705 patients) were tested. Sample types were as follows: nasopharyngeal aspirates (NPA) 608, throat swabs (TS) 82, sputum 15. Six hundred and fifteen specimens were from patients with respiratory symptoms. Sixty-five (65) NPA samples were from healthy asymptomatic adults, initially thought to be contacts in a pertussis outbreak, which was later, confirmed as an influenza A outbreak. Twenty-five (25) throat swabs were collected from healthy adult volunteers. Samples were removed from the freezer (-70°C) and thawed at 37°C for 30 min. Twenty (20) µL was added to 80 µl of sterile DNA'se and RNA'se free water, vortexed for 30 sec, heated in a dry block heater for 20 min at 99.9°C and then centrifuged in a bench microfuge for 30 seconds. Positive and negative controls were treated identically to specimens. Specimens in which inhibitory substances were detected (see method below) underwent a DNA extraction procedure using the High Pure™ PCR template kit (Boehringer Mannheim) as per the manufacturers' instructions prior to retesting.

1.3 PCR methods

Nested IS481 PCR was performed as previously described (Farrell *et al.*, 1999a, *supra*). Pertussis toxin operon PCR (PTO) was also performed as described previously (Farrell *et al.*, 1999b, *supra*). Oligonucleotides for porin gene amplification (POR) used in this study were designed using previously published sequence data (Li *et al.*, 1994, *supra*) and are listed in Table 2. PCR oligonucleotides were synthesised commercially by Life Technologies (USA).

For POR, 10 µl of treated sample was added to 40 µL of a master mix (made immediately before use) containing 5µL of 10 x PCR buffer, PCR nucleotide mix (Boehringer Mannheim, Sydney) (200 µM dATP, dCTP, dGTP; 600 µM dUTP), 12.5 pmol of primers DFPOR1F (SEQ ID NO:1), DFPOR2F (SEQ ID NO:3) and DFPORRB (SEQ ID NO:4), 2.0 U Platinum *Taq* polymerase (Life Technologies) per reaction , 2.5 mM $MgCl_2$ (Life Technologies) and titrated internal control. One (1) µL of uracil-DNA glycosylase (heat labile) (Boehringer Mannheim) was added to each master mix and incubated at room temperature 10

minutes before amplification. Amplification was performed in a Perkin-Elmer 9600 GeneAmp 9600 thermal cycler (PE Applied Biosystems, Melbourne) with the following parameters: 2 min at 95°C followed by 44 cycles of 10 s at 94°C, 10 s at 56°C, 5 s at 72°C, with the last cycle concluding with a reaction for 5 min at 72°C.

Detection of amplification products was performed using a modification of the PCR DIG ELISA kit (Boehringer Mannheim). The oligonucleotide probes DFPORP1 (SEQ ID NO:5), DFPORP2 (SEQ ID NO:6), DFPORP3 (SEQ ID NO:7) and DFMBAP (SEQ ID NO:10) were labeled with digoxigenin-dd-UTP/dATP using the DIG oligonucleotide 3'-end labelling kit (Boehringer Mannheim) as per the manufacturer's instructions. Unless specified, the reagents described in the following methodology are those present in the PCR DIG ELISA kit. Twenty (20) µL of denaturation solution (reagent 1a) was added to each straptavidin-coated microwell. Five (5) µL of each amplified specimen was added to each of two wells. The microwell plate was gently mixed and allowed to sit at room temperature for 10 minutes. During this time, probes were prepared for addition to the microwells. DIG-labelled probes (DFPORP3 for *Bordetella* spp., DFMBAP for internal control) were diluted 1/200 in hybridisation buffer (reagent 2) and gently mixed by inversion. Two hundred µl of diluted probe was added to the appropriate microwells and the plate was covered with a plastic plate cover and incubated at 45°C on a heating block for 30 minutes. During hybridisation, the conjugate was prepared by diluting the anti-DIG-POD 1/100 with conjugate dilution buffer (reagent 4). At 30 minutes, the plate was washed 6 times on an automated EIA plate washer (Sorin, CSL Biosciences, Melbourne) using PCR ELISA buffer. One hundred µl of the prepared conjugate was added to each well and the plate was covered and incubated at 37°C for 30 minutes in a plate incubator (Sanofi Diagnostics, Sydney). The plate was then washed six times. One hundred µL of freshly combined TMB substrate (Roche Diagnostics, Sydney) was added to each well. The reaction was stopped exactly five minutes after the addition of the substrate by adding 100 µL of 10% H_2SO_4.

A cut-off for the positive result was obtained from the mean of five readings (from 5 separate runs) of a specimen diluted to approximately 1 viable cfu/reaction and an equivocal result was determined from a similar series using 1/5

to 1/2 dilutions of the positive cut-off specimen. A specimen was considered negative for *Bordetella spp.* if the A_{450} <= 0.2, positive if the A_{450} >= 0.8, equivocal if the A_{450} >0.2 and <0.8. Any run in which a negative control was positive or a positive control negative was repeated. Any specimen in which the internal control value was < 0.5 was considered to have PCR inhibition and a DNA purification step was performed using the High Pure PCR template kit (Boehringer Mannheim) before the test was repeated.

If a positive signal for *Bordetella* spp was obtained, the hybridization step was repeated using the probes DFPORP1 (for *B. pertussis*) and DFPORP2 (for *B. parapertussis/B. bronchiseptica*).

Modifications to the above method were as follows. POR PCR was performed using biotinylated primers. Amplification products were detected and interpreted as previously described (Loeffelholz *et al.*, 1999, J.Clin. Microbiol. **37** 2872), using a colorimetric microwell assay in which unlabelled POR capture probes were immobilized in plate wells. Wells that contained hybridized PCR product generated color after the addition of avidin horseradish peroxidase and tetramethylbenzidine substrate. Following washing and color development steps, plates were read in a microwell plate reader at A_{450}.

1.4 *Construction of an assay specific internal control*

Mouse cDNA was prepared from Balb/c mouse spleens. Mouse cDNA was used as it was readily available and a simple DNA extract of mouse DNA would suffice. Hybrid primers (DFPORFMB; SEQ ID NO:8 and DFPORRMB; SEQ ID NO:9) were designed using the sequence for mouse β-actin (Genbank accession No. X03672) and the nucleotide sequences are listed in Table 2. A master mix was prepared which had the following final concentration: 1 x PCR reaction buffer (Life Technologies); 200 μM of each of dATP, dCTP, dTTP & dGTP (Pharmacia Biotech); 10 pmol/reaction of specific oligonucleotide; Platinum Taq 2.0U/ reaction (Life Technologies); 1.5 mM $MgCl_2$ (Life Technologies). Two μl of mouse cDNA was added to 47.5 μL of master mix and amplification was performed on a Perkin Elmer 9600 GeneAmp thermal cycler (PE Applied Biosystems) using the following parameters: 94°C for 2 minutes; 29 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute; 72°C for 5 minutes.

Amplification products were visualized on a 2% Tris Borate EDTA (TBE) agarose gel using ethidium bromide.

The resulting amplification product was of the same size as the product for the *B. pertussis* assay and had the same sequences at 5' and 3' ends but included mouse β-actin DNA sequence intermediate the 5' and 3' ends. The internal control was then amplified using the diagnostic PCR method to produce a large amount of internal control. The internal control was then serial diluted, amplified in the standard *B. pertussis* assay and the cutoff for positivity was determined. One dilution higher than the cutoff concentration was the concentration used in the master mix. If PCR inhibitory substances were present in the specimen they would be detected by failure to amplify the internal control, which was detected in a separate microwell using an oligonucleotide probe (DFMBAP; SEQ ID NO:10) specific for mouse β-actin DNA.

EXAMPLE 2

Results

Analytical specificity was 100% using the strains previously described (Farrell *et al.*, 1999a, *supra*), *B. avium* strain TC9, *B. hinzii* strain TC58, and four *Bordetella holmesii* strains (see Table 5). No positive results were obtained in 65 NPA or 25 throat swabs collected from 90 asymptomatic healthy adults. The analytical sensitivity of the assay was between 1 and 10 cfu per reaction. Table 4 illustrates that analytical sensitivity was not adversely affected for *B. pertussis* or *B. parapertussis* if either organism was present in minimal numbers and the other in large numbers.

The results of the comparison between POR and IS481 nested PCR for 705 clinical specimens is shown in Table 6. The 2 POR positive and IS481 negative specimens came from 2 patients who fulfilled a clinical definition of pertussis (Nygren *et al.*, 2000, J. Clin. Microbiol. **38** 55) and were also positive by PTO PCR. PCR inhibition was detected in 21/705 specimens (2.98%). Inhibitors were detected more frequently in extremely mucoid specimens – 8 of which were sputum and 13 NPA. After DNA extraction no inhibitors were detected for any of the 21 specimens and all were positive by bacterial 16S rRNA PCR (method not shown but positive and negative controls were used in the method) showing that

loss of DNA did not occur during processing. One of the inhibitory specimens was positive for *B. pertussis* after DNA extraction.

EXAMPLE 3

Discussion

Disadvantages of the nested IS481/1001 method arise from the inherent problems of nested PCR assays, in particular the increased risk of carryover of amplified DNA. In addition, the carryover cannot be minimised by the use of the uracil-*N*-glycosylase carryover prevention system. Because of these problems, this method should only be performed in laboratories with staff experienced in nested PCR techniques and hence is not suitable for routine laboratory use. The next step was to develop a simpler assay using the IS481/1001 assay as a "gold-standard" to evaluate the analytical and clinical parameters of the assay.

Two of the candidates tested as an appropriate target were the repetitive insertion sequence IS481 and the regulatory region of the pertussis toxin gene. IS481 is present in all strains of pertussis tested so far at a rate of 50 – 100 copies per chromosome (McLafferty *et al.*, 1988, J. Gen. Microbiol. **134** 2297; McPheat & McNally, 1987, J. Gen. Microbiol. **133** 323; Farrell *et al.*, 1999a, *supra*). IS481 would seem like an appropriate target due to its high copy number yet many have questioned its use. Grimprel *et al.*, 1993, J. Clin. Microbiol. **31** 2745 argued that the copy number might vary between strains and that insertion sequences may also be present in other *Bordetella* species. To assess the specificity of the insertion sequence, Glare *et al.*, 1990, J. Clin. Microbiol. **28** 1982 performed hybridisation studies with 45 different species of bacteria (from 24 genera) using a *Cla*I fragment as a probe. Strong hybridisation occurred for *B. pertussis*, a weak hybridisation was observed for *B. bronchiseptica* (after prolonged autoradiography), and no hybridisation was observed for any other species. In all studies except the study of Glare *et al.*, 1990, *supra*, the specificity of IS481 based PCR has been 100% when tested against a multitude of non-pertussis *Bordetellae* and a high correlation has been observed with clinical pertussis and culture. On the other hand, Glare *et al.*, 1990, supra reported a 12 base pair difference when compared to the sequence published by McLafferty *et al.* (McLafferty *et al.*, 1988, *supra*), suggesting that the IS is not absolutely conserved. Also, a recent study

(Loeffelholz *et al.*, 2000, *supra*) showed that an IS481 PCR cross-reacted strongly with six strains of *B. holmseii*. Unfortunately, the carriage rate and clinical significance of *B. holmseii* is unknown, confounding assays that target IS481.

In a first attempt to develop a simpler assay, the pertussis toxin operon was used as a target. The PTO assay performed well, both analytically and clinically, against nested IS481/1001 (Farrell *et al.*, 1999b, *supra*). Although *ptx* is present as a single copy gene, a comparison of PTO and IS481 as targets showed equivalent sensitivity (Houard *et al.*, 1989, Res. Microbiol. **140** 477). On the other hand an identical PCR protocol was used for both IS481 and PTO yet the published T_m of the primers differed by 14°C and 16°C between the two methods (Houard *et al.*, 1989, *supra*). One could argue that with better optimisation the IS481 PCR might have been a great deal more sensitive than PTO PCR. Arguments for not using PTO as a target are 1) the pertussis toxin gene is also present but not expressed in *B. parapertussis* and *B. bronchiseptica*. This concern is most probably ungrounded as the reason these organisms do not express the toxin is because multiple mutations have been located in the *ptx* promoter region (Locht & Keith, 1986, Science **232** 1258). On the other hand, an IgG response to pertussis toxin in patients with parapertussis infections has been described, indicating that expression of the toxin genes may occur (Li *et al.*, 1994, *supra*;, Farrell *et al.*, 1999b, *supra*) mutations in the pertussis toxin S1 subunit have been recently described and are thought to be due to selective evolutionary pressure on the organism caused by vaccination (Mooi *et al.*, 1998, Infect. Immun. **66** 670). These authors also provide evidence that contemporary strains of *B. pertussis* differ in the genetic sequence of the S1 subunit possibly through vaccine-driven evolution. For diagnostic methods it may be wise not to use genes that code for antigens present in commercial vaccines. In addition, polymorphisms in the pertussis toxin promoter region have also been described (Nygren *et al.*, 2000, *supra*).

The outer membrane porin gene as a target for diagnostic PCR has only been used in one previous study (Li *et al.*, 1994, *supra*). The assay described in that study had a sensitivity of 79% against culture when gel detection was used and a sensitivity of 95% when digoxigenin immunoblot was used. In the present

study, new primers and probes were designed using the outer membrane porin sequence data described in Li *et al.*, 1994, *supra*. A rapid cycle protocol was designed, a carryover prevention system (uracil-N-glycosylase) was included, probe detection allowed differentiation between *Bordetella* spp., an assay specific internal control was developed allowing specimen preparation to be minimal, and the assay was evaluated against a "gold-standard" method targeting a different gene. This method is the first to fulfil the international consensus requirements for the use of PCR in the detection of *B. pertussis* as proposed by Meade and Bollen (Meade & Bollen, 1994, *supra*) and outlined in Table 1. The asssay does not distinguish between *B. parapertussis* and *B. bronchiseptica* but this should only cause concern in interpreting results from specimens obtained from immunocompromised patients as *B. bronchiseptica* is only rarely part of the normal flora of humans. Nevertheless, interpretation of a positive *B. parapertussis/B.bronchiseptica* POR result should be considered with caution. The POR PCR assay allows a rapid, accurate, standardized, simplified approach to the laboratory diagnosis of pertussis.

It will be appreciated that the present invention is not limited to the particular embodiments described in detail herein, and that various modifications and variations are contemplated which nevertheless fall within the broad spirit and scope of the invention.

All scientific and patent literature, computer programs and algorithms referred to in this specification are incorporated herein by reference.

TABLE 1

• Sample processing should be kept to a minimum
• Nasopharyngeal aspirate (NPA) is the preferred specimen
• Differentiation between *Bordetella pertussis* & *B. parapertussis* is necessary
• Use of carry-over control (eg.UNG) to minimize contamination
• Appropriate use of controls (Positive and Negative)
• Detection systems - use of probes for added specificity and sensitivity
• Confirmation of questionable results with an alternative target
• 100% of culture positive should be NAA positive
•Other commensals and pathogens should test negative
•Testing of samples from a healthy population assumed to be negative for the pathogen
• Use of internal control to check for inhibition of the reaction

TABLE 2

Primer/probe	Nucleotide Sequence
DFPOR1F	5'-GGCTCCTTGAGTGAACTGG-3'
DFPOR1'F	5'-GGCTCCTTGACTGAACTGG-3'
DFPOR2F	5'-TGAGGTCGGGCGAATCGTC-3'
DFPORRB	5'Biotin-GGTAAGTTGCAACATCCTGTC-3'
DFPORP1	5'-GAACCATGCATACAACCTATT-3'
DFPORP2	5'-GAGCTTGTTGCATTGCGATGC-3'
DFPORP3	5'-CGCGCGCGATTCCGGATTAAG-3'
DFPORFMB	5'-GGCTCCTTGACTGAACTGGCTGTGGCATC CATGAAAC-3'
DFPORRMB	5'-GGTAAGTTGCAACATCCTGACGCATAGAG GTCTTTACGGA-3'
DFMBAP	5'-TCAATTCCATCATGAAGTGTGACGTTGA-3'

TABLE 3

Primer/probe	Nucleotide Sequence
DFPOR3F	5'-TATGGGTGTTCATCCGGCCG-3'
DFPOR4F	5'-GCGGGCACGGGCACACTTGG-3'
DFPOR2R	5'-CCCGCCCCATTGTTGGTAAG-3'
DFPORP4	5'-TTTCTCAAATCCGGTTCGGAT-3'
DFPORP5	5'-CGGCGCGGGGCAGGCGGGCAG-3'
DFPORP6	5'-CCGGATTAAGGGGACAGGAT-3'

TABLE 4

B. pertussis	*B. parapertussis*	DFPOR1	DFPOR2	DFPOR3
1	10^7	0.03	2.2	2.25
10	10^7	1.2	2.3	2.1
100	10^5	2.25	2.35	2.4
10^3	10^3	2.3	2.15	2.25
10^5	100	2.15	1.95	2.1
10^7	10	2.4	1.6	2.35
10^7	1	2.25	0.25	2.05

TABLE 5

Species	DFPORP1	DFPORP2	DFPORP3
B. pertussis (21)	+ (21)	- (21)	+ (21)
B. parapertussis (6)	- (6)	+ (6)	+ (6)
B. bronchiseptica (3)	- (3)	+ (3)	+ (3)
B. avium (2)	- (2)	- (2)	- (2)
B. hinzii (2)	- (2)	- (2)	- (2)
B. holmesii (4)*	- (4)	- (4)	- (4)
Non-*Bordetellae* (30)§	-(30)	-(30)	-(30)

TABLE 6

	IS481 Positive	IS481 Negative
POR Positive	51	2
POR Negative	0	652

CLAIMS

1. A method of detecting a *Bordetella* nucleic acid, said method including the steps of:

(i) subjecting a sample to a nucleic acid sequence amplification technique using a *Bordetella*-specific primer, a *Bordetella pertussis*-specific primer and a *Bordetella parapertussis*-specific primer; and

(ii) determining whether or not said sample includes a *Bordetella* nucleic acid according to whether or not one or more amplification products produced at step (i) hybridizes to a *Bordetella*-specific probe.

2. The method of Claim 2, wherein if a *Bordetella* nucleic acid is detected at step (ii), a subsequent hybridization step is performed to determine whether said one or more amplification products hybridize to a *Bordetella pertussis*-specific probe as an indication that said sample includes a *Bordetella pertussis* nucleic acid and/or to a *Bordetella parapertussis*-specific probe as an indication that said sample includes a *Bordetella parapertussis* nucleic acid.

3. The method of Claim 1 wherein a reference nucleic acid is added to said sample before step (ii).

4. The method of Claim 3, wherein the reference nucleic acid comprises regions respectively hybridizable to said *Bordetella pertussis*-specific primer and to said *Bordetella*-specific primer.

5. The method of Claim 4, wherein the reference nucleic acid further comprises nucleotides 893-964 of a mouse β-actin cDNA sequence intermediate said regions respectively hybridizable to said *Bordetella pertussis*-specific primer and to said *Bordetella*-specific primer.

6. The method of Claim 1, wherein the nucleic acid sequence amplification technique is PCR.

7. The method of Claim 1, wherein the *Bordetella*-specific primer, the *Bordetella pertussis*-specific primer and the *Bordetella parapertussis*-specific primer are each capable of hybridizing to a genetic locus present in a genome of both *B. pertussis* and *B. parapertussis*.

8. The method of Claim 9, wherein said locus has a region which is conserved between *B. pertussis and B. parapertussis* and a region which is non-conserved

between *B. pertussis and B. parapertussis*.

9. The method of Claim 8, wherein the *Bordetella*-specific primer is hybridizable to said conserved region of the locus, the *Bordetella pertussis*-specific primer and the *B. parapertussis*-specific primer being hybridizable to the non-conserved region present in *Bordetella pertussis* and *B. parapertussis* respectively.

10. The method of Claim 9, wherein said genetic locus corresponds to a porin (*por*) gene.

11. The method of Claim 1, wherein the *Bordetella*-specific primer, the *Bordetella pertussis*-specific primer and the *Bordetella parapertussis*-specific primer are designed so that said one or more amplification products each have a similar number of base pairs.

12. The method of Claim 11, wherein said one or more amplification products each have an equal number of base pairs.

13. The method of Claim 1, wherein the *Bordetella*-specific primer has a nucleotide sequence according to SEQ ID NO:4 or SEQ ID NO:13.

14. The method of Claim 1, wherein the *Bordetella pertussis*-specific primer has a nucleotide sequence according to SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:11.

15. The method of Claim 1, wherein the *Bordetella parapertussis*-specific primer has a nucleotide sequence according to SEQ ID NO:3 or SEQ ID NO: 12.

16. The method of Claim 1, wherein the *Bordetella*-specific probe has a nucleotide sequence according to SEQ ID NO:7 or SEQ ID NO:16.

17. The method of Claim 2, wherein the *Bordetella pertussis*-specific probe has a nucleotide sequence according to SEQ ID NO:5 or SEQ ID NO:14.

18. The method of Claim 2, wherein the *Bordetella parapertussis*-specific probe has a nucleotide sequence according to SEQ ID NO:6 or SEQ ID NO:15.

19. The method of Claim 1, wherein the sample is a nasopharyngeal aspirate (NPA), a throat swab or sputum.

20. The method of Claim 19, wherein the sample is obtained from a human.

21. A method of detecting a *Bordetella* nucleic acid corresponding to a porin gene or fragment thereof, said method including the steps of:

(i) subjecting a human nasopharyngeal aspirate sample to polymerase

chain reaction (PCR) using a *Bordetella*-specific primer, a *Bordetella pertussis*-specific primer and a *Bordetella parapertussis*-specific primer, said sample comprising a reference nucleic acid to which the *Bordetella*-specific primer and the *Bordetella pertussis*-specific primer are respectively hybridizable;

(ii) determining whether or not said sample includes:

(a) a reference nucleic acid amplification product according to whether or not said reference nucleic acid amplification product hybridizes to a reference nucleic acid-specific probe;

(b) a *Bordetella* nucleic acid according to whether or not one or more amplification products produced at step (i) hybridize to a *Bordetella*-specific probe; and

(iii) if a *Bordetella* nucleic acid is detected at step (b), determining whether said amplification products hybridize to a *Bordetella pertussis*-specific probe as an indication that said sample includes a *Bordetella pertussis* nucleic acid, and/or to a *Bordetella parapertussis*-specific probe, as an indication that said sample includes a *Bordetella parapertussis* nucleic acid.

22. A kit for detecting a *Bordetella* nucleic acid comprising:

(i) a *Bordetella*-specific primer, a *Bordetella pertussis*- specific primer and a *Bordetella parapertussis*-specific primer; and

(ii) a *Bordetella*-specific probe.

23. The kit of Claim 22, further comprising a *Bordetella pertussis*-specific probe and a *Bordetella parapertussis*-specific probe.

24. The kit of Claim 23, further comprising a reference nucleic acid.

25. The kit of Claim 24, further comprising a reference nucleic acid-specific probe.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU01/00557

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12Q 1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

CA, WPIDS (C12Q), SEE ELECTRONIC DATABASES

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SEE ELECTRONIC DATABASES

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIDS (C12Q and keywords), Medline, CA (Bordetella pertussis, Bordetella parapertussis, Amplif?, PCR, Polymerase Chain Reaction)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P/X	Farrell, D. J. *et al.* 2000. "Rapid cycle PCR method to detect *Bordetella pertussis* that fulfils all consensus recommendations for use of PCR in diagnosis of Pertussis". Journal of Clinical Microbiology. **38**(12) pp.4499-4502. See whole document.	1-25
X/Y	Müller, F-M. C. *et al.* 1998. "Discrimination of *Bordetella parapertussis* and *Bordetella pertussis* organisms from clinical isolates by PCR using biotin labelled oligonucleotide probes". Molecular and Cellular Probes. **12** pp.213-217. See whole document, in particular, Abstract and Materials and Methods.	1-25

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 June 2001	2 July 2001
Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	**Terry Moore** Telephone No : (02) 6283 2632

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU01/00557

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Li, Z. *et al.* 1994. "Identification of *Bordetella pertussis* infection by shared primer PCR". Journal of Clinical Microbiology. **32**(3) pp.783-789. See whole document, in particular, Abstract, Materials and Methods, Results and Figure 2.	1,2, 6-11, 16, 19, 20
Y	In combination with Müller, F-M. C. *et al.* 1998. Molecular and Cellular Probes. **12** pp.213-217.	3-5, 12-15, 17, 18, 21-25
	Müller, F-M. C. *et al.* 1998. "The rationale and method for constructing internal control DNA used in pertussis polymerase chain reaction". Diagnostic Microbiology and Infectious Disease. **31**(4) pp.517-523. See Materials and Methods.	
Y	In combination with Müller, F-M. C. *et al.* 1998. Molecular and Cellular Probes. **12** pp.213-217.	1-25
A	WO00/17391 A (INNOGENETICS N.V.) 30 March 2000 See p.3 Lines 18-21, p.4 Lines 16-28, Example 2 part 4 and Table 4.	1-25
A	Hozbor, D. *et al.* 1999. "Detection of *Bordetella bronchiseptica* by the polymerase chain reaction". Research in Microbiology. **150**(5) pp.333-341. See Abstract and Materials and Methods.	1-25
A	Lind-Brandberg, L. *et al.* 1998. "Evaluation of PCR for diagnosis of *Bordetella pertussis* and *Bordetella parapertussis* infections". Journal of Clinical Microbiology. **36**(3) pp.679-683. See Abstract and Materials and Methods.	1-25

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU01/00557

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member	
WO	200017391	AU	61949/99

END OF ANNEX