Rapid Detection of *Bordetella pertussis* and *Bordetella parapertussis* DNA in Clinical Samples by Duplex Real Time PCR Assay Using Taqman Probes

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Abstract

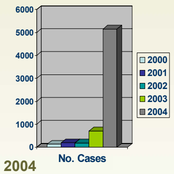
Recent outbreaks of *Bordetella pertussis*, the causative agent for whooping cough in Wisconsin have resulted in the development of rapid homebrew assays, since treatment of disease and control of outbreaks depends on rapid and accurate laboratory diagnosis. Here we report a rapid and sensitive assay for simultaneous detection of *B. pertussis* and *B. parapertussis* in clinical samples by duplex real time PCR using OmniMix HS beads in the SmartCycler®. The MHDL assay was designed to detect *B. pertussis* and/or *B. parapertussis* in single or duplex, two step, 45 cycle PCR using TET and CalRed-labeled Taqman probes with minimum template and low background fluorescence. Out of 154 nasopharyngeal swabs tested, a total of 20 (12.98 %) and 3 (1.94 %) samples were positive for *B. pertussis* and *B. parapertussis* respectively. The MHDL assay was capable of detecting 10 fg of genomic DNA using OmniMix HS beads. The specificity and sensitivity of our assay was compared with *B. pertussis* IS*481* sequence specific 'Analyte Specific Reagent' (ASR) based assay from Cepheid. A total of 30 samples were tested with these two assays using 2 µl of template and 10/30 and 8/30 samples were identified as positive for *B. pertussis* in the MHDL and Cepheid assays respectively. The results were similar when 5 µl or more DNA templates were used. The turnaround time for MHDL duplex assay was 29 min compared to 78 min with the Cepheid assay. The MHDL assay is a rapid, sensitive, specific, reproducible and cost effective real time PCR assay that can be used during any outbreak or routine diagnosis of *B. pertussis* and/or *B. parapertussis* in the clinical laboratory.

Introduction

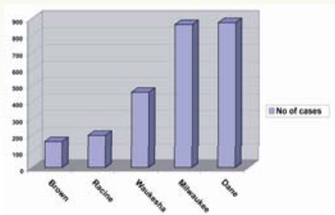
Bordetella pertussis affects unvaccinated children as well as previously vaccinated children and adults world wide, where immunity has waned (1, 2). The disease has significant morbidity and is one of the reportable diseases to the State of Wisconsin Department of Health. The cough is debilitating and the disease may be prolonged. Secondary complications may include bacterial pneumonia, encephalitis, and hemorrhages from the explosive cough. *B. pertussis* is transmitted by respiratory droplets and causes disease only in human. Two closely related species, *B. parapertussis* and *B. holmesii* may also cause 'Pertussis-like syndrome' (3). The disease is treatable with erythromycin or azithromycin. The recent 'Pertussis' outbreaks in the State of Wisconsin and increased occurrence of 'Atypical Pertussis' cases demand the need for rapid and accurate diagnostic methods to guide therapeutics and preventive interventions.

Pertussis Outbreaks in US: 2003

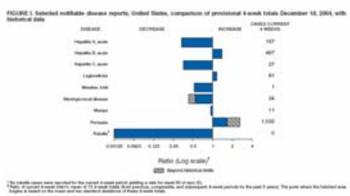
Increasing trends of Pertussis cases in WI



Pertussis Outbreaks in Wisconsin: 2004



MMWR December 2004 report on increase in Pertussis cases in US



Principle

PCR provides a unique mean for identifying *B. pertussis* and *B. parapertussis* from clinical samples, because the organisms are fastidious and culture is fraught with difficulty. The current protocol uses two-step Real time PCR that targets conserved insertion sequences; namely IS*481* (Accession # M28220) and IS*1001* (Accession # X66858; S51601) genes of *B. pertussis* and *B. parapertussis* respectively. The IS*481* and IS*1001* genes are internally amplified approximately 80 - 100 and 20 times within the bacterial chromosome, thus they are very sensitive areas in genomes to target for amplification (4).

Primers and Probes positions for the MHDL Assays

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                 1                   20
BPN(F):   ...............atccgaaccggatttgagaa...............
                         ||||||||||||||||||||
BpIS481:  ttgtatgcatggttcatccgaaccggatttgagaaactggaaatcgccaa
                 38                  57

                 1                   20
BPN(R)    ...............taggaaggtcaatcgggcat...............
                         ||||||||||||||||||||
                         taggaaggtcaatcgggcat
BpIS481:  tgaacacccataagcatgcccgattgaccttcctacgtcgactcgaaatg (Reverse)
                 138                 119
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BP Probe: 5'-TET-CCAGTTCACTCAAGGAGCCCGGC–BHQ-1-3'

Fragment size for the IS*481* gene = 103 base pairs
Fragment size for the IS*1001* gene = 193 base pairs

Materials

1. Primers and Probes: Primers and probes for the MHDL Single or Duplex assays were synthesized by Biosearch Technologies, Inc, CA.

2. OmniMix HS beads: OmniMix beads (2X) (Cepheid, Inc, CA) were used for master mix preparations during the MHDL PCR assays.

3. *B. pertussis* Analyte Specific Reagent (ASR): Cepheid ASR (2X) beads were used with OmniMix beads and excess $MgCl_2$ during *B. pertussis* assay. This contains an internal control for PCR.

4. Taqman β-Actin control: β-Actin controls (BACT) were used for inhibition assay during MHDL PCR using Taqman β-Actin control kit (Applied Biosystems, CA) and OmniMix beads.

Methods

1. DNA extraction from patient samples: DNA was extracted from NP swabs using QIAamp DNA Mini kit (Qiagen, Valencia, CA) with minor modifications for viscous samples. The swabs were washed in PBS and suspended in 200 µl AL buffer with 20 µl Qiagen Proteinase-K, followed by manufacturer's protocols and eluted with 50 µl AE buffer.

2. PCR controls: Laboratory strains of *B. pertussis and B. parapertussis* were used as positive extraction controls. Human β-Actin was used as inhibition control.

3. Duplex PCR Assay in SmartCycler II: Duplex Taqman PCR assay was performed with OmniMix HS beads, 400 nM primers and 200 nM TET, CalRed-labeled probes and 2 µl template DNA in 25 µl volume. Cepheid ASR beads were used for identifying *B. pertussis* and Internal Control (IC) in separate duplex assay. The Taqman PCR Cycling conditions were as followings.

Table 1: PCR Cycle conditions

Stage 1			Stage 2		
Single Cycle			Repeat: 45 Cycles		
Temp (ºC)	Sec	Optics	Temp (ºC)	Sec	Optics
95	30	OFF	95	10	OFF
-	-	-	60	10	ON

References

1. Stojanov, S *et al*. 2000. Infection, 28: 106- 110
2. Khetsuriani N *et al*. 2001. Ped. Infect. Dis. J. 20: 1108- 1112
3. Mazengia, E *et al*. 2000. J. Clin. Microbiol. 38: 2330- 2333
4. van der Zee, A *et al*. 1993. J. Clin. Microbiol. 31: 2134-2140

Results

Fig. 1. Sensitivity of the MHDL PCR for *B. pertussis*

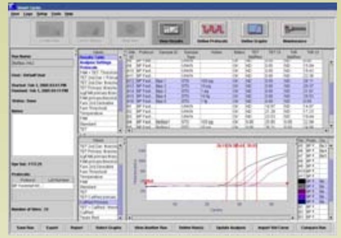


Fig. 2. Sensitivity of the MHDL PCR assay for *B. parapertussis*

Fig. 3. Sensitivity of the MHDL Duplex PCR Assay

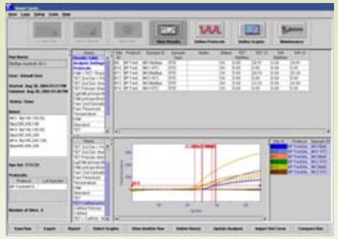


Fig. 4. MHDL Duplex assay with different primer and probe concentrations

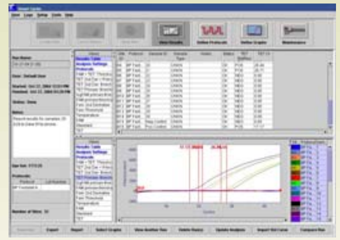


Fig. 5. Amplification of the clinical samples using MHDL PCR Assay

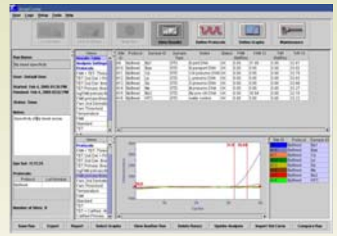


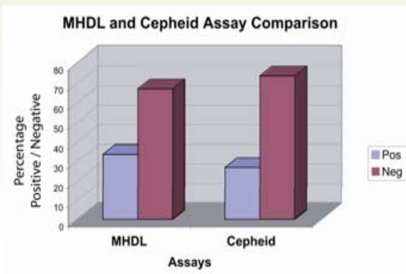
Fig. 6. Specificity of the Cepheid *B. pertussis* ASR bead-based PCR

Table. 2. Real Time PCR: A Comparison between Cepheid *B. pertussis* ASR and MHDL Duplex Assay

Sample ID	Expected results	Assay comparison and Final Results			
		Cepheid Duplex Assay[1] (40 Cycles)		MHDL Duplex Assay[2] (45 Cycles)	
		C_T value	Assay time (min)	C_T value	Assay time (min)
#4*	Positive	28.37	78	29.23	29
#6	Positive	34.73	78	36.40	29
#7*	Positive	27.13	78	27.64	29
#11**	Positive	-	78	39.20	29
#12	Positive	37.73	78	39.74	29
#13*	Positive	25.21	78	25.37	29
#17	Positive	37.65	78	39.34	29
#18	Positive	35.82	78	36.21	29
#23	Positive	36.49	78	38.51	29
#24*/**	Positive	-	78	36.66	29

1 Duplex ASR Assay for *Bordetella pertussis* and Internal Control (IC)
2 Duplex PCR with *Bordetella pertussis* and *B. parapertussis*
* No reaction for IC (2 or 5 µl DNA template for 25 µl reaction)
** No reaction for *B. pertussis* with 2 µl template. In repeat run with 5 µl DNA, PCR was positive for *Bp*

Fig. 7. A comparison of the MHDL and Cepheid Duplex PCR Assays



Limitations of the MHDL Assay

1. Unable to differentiate viable and non-viable organisms.
2. Possible cross-reaction with *B. holmesii* in clinical sample.
3. Depends on quality and integrity of the clinical sample.
4. Need to add Internal control

Limitations of the Cepheid ASR

1. Unable to differentiate viable and non-viable organisms.
2. Possible cross-reaction with *B. holmesii* in clinical sample.
3. Unable to detect *B. parapertussis.*
4. Depends on quality and integrity of the clinical sample.

Conclusions

1. The MHDL Taqman PCR demonstrated very high sensitivity and specificity for detecting *B. pertussis* and *B. parapertussis* in single reaction.
2. The turnaround time for MHDL Duplex PCR assay in SmartCycler II is 29 min.
3. Cepheid *B. pertussis* ASR bead assay is very specific for *B. pertussis* and equally sensitive when template concentration is increased.
4. Amplification using OmniMix HS beads provide high performance, reproducibility and ease during the assays.

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