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Multiplex real-time PCR assay for detection and differentiation of *Bordetella pertussis* and *Bordetella parapertussis*

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ABSTRACT

Background and Objective: Rapid diagnosis of pertussis is important for the timely isolation of the infection source and early prevention measures among the contact persons, especially among non-vaccinated infants for whom pertussis is life-threatening.

Materials and Methods: Targets *IS481*, *IS1001, BP0026* and human GAPDH gene were used to develop a multiplex real-time PCR assay based on the TaqMan technology for detection and identification of *Bordetella pertussis* and *Bordetella parapertussis* in clinical samples. A total of 121 human clinical specimens obtained within 2012-2013 were used to evaluate the multiplex real-time PCR assay. Clinical specimens were also tested for culture and conventional PCR. Sensitivity and specificity for culture, conventional PCR, and multiplex real-time PCR were measured in comparison with a clinical standard for *B. pertussis* infection.

Results: The lower limit of detection (LLOD) of the multiplex assay was similar to the LLOD of each target in an individual assay format, which was approximately 1 genomic equivalent per reaction for *IS481*, *IS1001* and 10 genomic equivalents per reaction for *BP0026* target. When the *B. pertussis* assays were compared with a clinical standard for *B. pertussis* infection, sensitivity was 5, 59 and 89% the specificity was 100, 100 and 100% for culture, conventional PCR, and multiplex real-time PCR, respectively.

Conclusions: Developed multiplex real-time PCR offers a fast tool with high sensitivity and specificity for the diagnosis of *B. pertussis* and *B. parapertussis* infections which is suitable for implementation in a routine laboratory diagnostics.

Keywords: Multiplex real-time PCR, *Bordetella pertussis*, *Bordetella parapertussis,* diagnosis

INTRODUCTION

Despite vaccination, pertussis remains endemic in many areas of the world (1-3). A reliable diagnosis of infection is required for the timely initiation of treatment and need for prevention of the disease in contact persons. In some cases, the disease occurs with typical symptoms of infection. At the same time as the majority of cases in adolescents and adults, who can be a major source of infection for children, occur atypically and require confirmation using laboratory methods for diagnosis. Rapid diagnosis of pertussis is important for the timely isolation of the infection source and early prevention measures among the contact persons, especially among non-vaccinated infants, for whom pertussis is life-threatening (4).

Bacteriological method has been considered as the "gold-standard" for diagnosis of pertussis due to its high specificity (100%) (5). However, its sensitivity varies from 7 to 60%, according to the sampling time and it takes seven days or longer to obtain a final result (5-7). Application of this method in the is-

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sue leads to low efficiency of diagnosis of infection (8). Serological tests help to diagnose pertussis and are more often used in practice. However, despite a higher sensitivity compared to the bacteriological method, they provide only a retrospective diagnosis of infection. They are based mainly on the increase in antibody titer in paired sera (9).

Usage of a single serum for the diagnosis is not widely used because it requires determination of diagnostic titer of the previous infection (10). Moreover, some of the commercial ELISA test systems are not standardized and do not allow differentiation between the *B. pertussis* and *B. parapertussis* (11). To overcome these limitations, a PCR method for diagnosis of pertussis, which is fast (2-24 hours) with specificity of 86-100% and sensitivity of 70-99% has been developed (5). Conventional PCR was introduced in 1989 to identify DNA of *B. pertussis* (12). It was substituted in clinical diagnostics by real-time PCR, but standardization of this method remains problematic (13,14).

A lot of genes were used as targets for identification and differentiation of human pathogens of genus *Bordetella* in PCR: promoter region of pertussis toxin gene (12, 15- 20), the region upstream of porin gene (21), porin gene (22), adenylate cyclases gene (*cyaA*) (13), pertactin gene (19, 23, 24), *recA* gene (22), repeating mobile elements *IS481* and *IS1001* (21, 25-28). The most common targets used for detection of *B. pertussis* are repetitive insertion sequence *IS481* and promoter region of pertussis toxin gene and repetitive insertion sequence *IS1001* for *B. parapertussis*. At present, however, no single target PCR assay is universally considered as a "gold standard" for PCR diagnosis of pertussis. In addition, the differentiation between members of the genus *Bordetella* is not possible using only one target (22, 29).

Synonymous and non-synonymous mutations were identified in some pathogenicity genes: *ptxA*, *prn*, *fim2*, *fim3* and *tcfA* (30). Use of genes with confirmed mutation variability is not recommended for development of PCR assay for diagnostic pertussis infection (13). Most of described real-time PCR assays used several targets but in different tubes. Only few studies described the PCR using two or three targets in the same tube for detection and differentiation of *B. pertussis* and *B. parapertussis* (31,32,33).

The aim of this study was to develop a method for molecular diagnosis of pertussis infection based on real-time PCR with multiple targets in a single tube for the detection and differentiation of *B. pertussis* and *B. parapertussis*.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used to analyze the specificity of the real-time PCR have been presented in Table 1. All bacteria were cultured according to standard methods. Bacteria of the genus *Bordetella* were grown for 4 days at 37ºC under high humidity on charcoal agar (HiMedia, India) containing 10% defibrinated horse blood. Non-*Bordetella* strains were cultured following standard procedures.

Collection and processing of samples. A total of 121 paired nasopharyngeal swabs (as dry swabs and in charcoal-based Amies transport medium) was obtained from patients with alleged pertussis infection in 2012-2013 for research on *B. pertussis* infection. Nasopharyngeal swabs taken with a dry swab were placed in 0.3 ml of RNase and DNase water, swirled vigorously and wrung out, and the swab was removed from the specimen. An aliquot of the sample volume of 30 µl was heated at 95°C for 15 minutes to disrupt the cells and 5 µl used in conventional PCR. An aliquot volume of 100 µl was used for DNA extraction and the following analysis in real-time PCR. All specimens were stored at -20°C.

Nasopharyngeal swabs in Amies transport medium were cultured for *Bordetella* spp. on charcoal agar containing 10% defibrinated horse blood and 40 mg/l cephalexin. Primary isolation plates were incubated at 35–37ºC, in a moist atmosphere, and maintained for 7 days. Plates were examined at 4 and 7 days. Putative colonies consistent with the appearance of *B. pertussis* were tested by Gram's stain, oxidase, catalase, slide agglutination with polyvalent antisera (Difco Laboratories, Detroit, Mich.) (28).

Clinical data were collected for all patients. Available patient serum was assayed for the presence of IgG antibodies to pertussis toxin using the ELISA test-system SERION ELISA classis (Institute Virion/Serion, GmbH, Germany). IgG titer to pertussis toxin 100 UI/ml or more was considered diagnostic, which indicates an active or recent infection caused by *B. pertussis*.

DNA extraction. Nucleic acids were extracted from bacterial suspensions and clinical specimens with QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). DNA of all samples were extracted accord-

Table 1. Specificity of the triplex real-time PCR assay.

Bacterial strains	Code	PCR results based on target gene		
		IS481	BP0026	IS1001
*Bordetella pertussis**	Tohama I	+	+	-
*Bordetella parapertussis**	1560	-	-	+
*Bordetella parapertussis**	285	-	-	+
*Bordetella bronchiseptica**	22067	-	-	-
*Bordetella bronchiseptica***	clinical isolate	-	-	-
Corynebacterium diphtheriae	NCTC 10648	-	-	-
Corynebacterium ulcerans	NCTC 12077	-	-	-
Staphylococcus aureus	ATCC 259237	-	-	-
Pseudomonas aeruginosa	ATCC 15442	-	-	-
*Enterococcus faecalis***	clinical isolate	-	-	-
*Escherichia coli***	ATCC 11229	-	-	-
*Streptococcus pyogenes***	clinical isolate 9996	-	-	-
*Streptococcus pyogenes***	clinical isolate 1366	-	-	-
*Pseudomonas aeruginosa***	clinical isolate 3696	-	-	-
*Legionella longbeachae***	clinical isolate	-	-	-
*Legionella micdadei***	clinical isolate	-	-	-
*Klebsiella pneumoniae***	clinical isolate 2494	-	-	-

ATCC, American Type Culture Collection

NCTC, National Collection of Type Culture, Central Public Health Laboratory, London

* Typical strains provided by G.N.Gabrichevsky Research Institute of Epidemiology and Microbiology, Moscow

** Clinical isolates from the collection of the laboratory of Clinical and Experimental Microbiology, RRPCEM, Minsk

ing to the manufacturer's instructions with 100 µl of samples in each extraction. Concentrations of DNA extracted from bacterial isolates were determined with a NanoDrop ND-2000 spectrophotometer. Equivalent of bacterial genomic DNA was calculated based on the concentration of DNA.

Primers for conventional PCR. PCR amplification of *B. pertussis* was performed using primers which amplified a 408-bp region from 529 to 919 bp of *IS481* (accession no.BX470248) and a 637-bp region located within a putative thiolase gene tagged *BP0026* (accession no. BX470248). PCR amplification for *B. parapertussis* was performed using primers that amplified a 493-bp region from 735 to 1208 bp of the *IS1001* (accession no. BX470249). Primers and probe sequences are shown in Table 2.

Conventional PCR. Briefly, the PCR was performed in 25 µl of reaction mixture consisting of 10 µl AmpliSens PCR mixture, 10 pmol of each primer to amplify a fragment *IS481* and *IS1001*, 15 pmol of each primer to amplify a fragment *BP0026* gene, 5 µl of extracted DNA. The PCR thermal profile for *IS481* and *IS1001* consisted of an initial incubation of 5 min at 95°C; followed by 35 cycles of 30 sec at 94°C, 20 sec at 53°C (52°C for *IS1001*), and 30 sec at 72°C; and finally a 5-min hold at +72°C; *BP0026* consisted of 5 min at 95°C; followed by 40 cycles of 30 sec at 94°C, 20 sec at 65°C, and 40 sec at 72°C; and 5-min hold at 72°C. The PCR products were detected by agarose gel electrophoresis.

PRIMERS AND PROBES FOR REAL-TIME PCR

Gene IS481. Consensus sequence was obtained by alignment of three nucleotide sequences of *B. pertussis IS481* (accession no. AB473880, M22031 and M28220). The primers and probes were designed based on region of 292 bp with 100% homology. This region was aligned with nucleotide sequences of *IS481* from *B. bronchiseptica* (accession no. EF043395) and *B. holmesii* (accession no. DQ420073) to determine polymorphism in the region. The homology of this fragment with the nucleotide sequences of these strains was equal to 99%.

Table 2. Primers and probes for PCR.

Target	Primer and probes for sequence (5›- 3›)	Primer or probe	Amplicon size (bp)	Optimal concentration (nM)
Conventional PCR				
IS481	CATCAAGAAGCTGGGACG TCGGTGTTGGGAGTTCTG	Forward primer Reverse primer	408	400
BP0026	AACCCGATGACTCGTATGCT GTGACGATTACCAGCGAGATTA	Forward primer Reverse primer	637	600
IS1001	CCGCCTACGAGTTGGAGA CCGCTTGATGACCTTGATAG	Forward primer Reverse primer	493	400
Real-time PCR				
IS481	ATCAAGCACCGCTTTACCC TGAGCGTAAGCCCACTCAC FAM -ACCGCCCACAGACCAATGGC-BHQ1	Forward primer Reverse primer Probe	95	450
BP0026	AAACCCGATGACTCGTATGC ATCTGGGAGATCGCATGAAC FAM -TGCCGTATGGGTCAGATTGGGA-BHQ1	Forward primer Reverse primer Probe	118	300
IS1001	ACAGGCGGAGATCGTCTATG ATCCTGGCGTAGTTGATTGG Cy-5 -ACGAGAGGTCATTGATCGGGTGC-BHQ2	Forward primer Reverse primer Probe	103	150
Gene GAPDH	GGCTCCCTTGGGTATATGGT TTGATTTTGGAGGGATCTCG TAMRA-ACCTTGTGTCCCTCAATATGGTCCT- BHQ2	Forward primer Reverse primer Probe	120	200

A putative thiolase gene tagged BP0026: The unique genome sequence of approximately 3.8 kbp (28315-32100 bp of *B. pertussis* whole-genome sequencing, accession no. BX470248) was used for design primers and probes. Amplified fragment length of 118 bp located within the locus tagged *BP0026*.

The gene IS1001. Consensus sequence was obtained by alignment of two nucleotide sequences of *B. parapertussis IS1001* (accession no. X66858, BX640436). The primers and probes were designed based on region of 686 bp with 100% homology.

*Human GAPDH gene****.*** Designed primers and probes for human gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase, accession no. AY340484.1) were used for an internal control in real-time PCR.

The ptxS1 gene. For amplification of *ptxS1* were used primers and probe for proposed by Tatti et al (34). All primers and probes were designed using Primer Express software v. 3.0 (Applied Biosystems). Vector NTI v.10.0.1 program was used for alignment and estimation probability of formation of dimmers or secondary structures by primers, probes and amplified fragments. A BLAST search was performed to check specificity of DNA sequences of the primers and probes. Designed primers and probes are shown in Table 2.

Real-time PCR. Multiplex TaqMan real-time PCR was performed in the format of two duplex reactions using ABI 7500 system (Applied Biosystems) in a volume of 25 µl in an optical 96-well plate. According to this scheme, amplification proceeded in two parallel tubes. The amplification mixture contained in a final concentrations of 1x PCR buffer 0.2 mM of each dNTP, 4 mM $MgCl_2$, 2.5% dimethylsulfoxide, and 5 µl aliquot of extracted sample DNA. In first tube PCR reaction mixture contained primers and probes for *IS481* and *IS1001.* In second tube reaction mixture contained primers and probes for *BP0026* and GAPDH. Optimal concentrations for primers and probes are shown in Table 2. The simplex real-time PCR assay was performed in the same conditions of reaction mixture. The PCR protocol used was as follows: hold for 5 min at 95ºC; amplification for 15 sec at 95ºC and 1 min at 60ºC, repeat for 45

cycles. The protocols were similar for simplex and multiplex PCR.

The background fluorescence was considered to obtain the correct cycle threshold (Ct) value. Thus, the threshold was drawn above the background fluorescence for each run in the exponential phase of the amplification curve.

The analytical sensitivity and specificity. The sensitivity of the real-time PCR assay was measured using serial tenfold dilutions of purified *B. pertussis* and *B. parapertussis* DNA. A stock concentration of DNA from *B. pertussis* strain Tohama1 and *B. parapertussis* 285 was determined based on the absorption of A260 and 10-fold serial dilutions (10^0–10^7 genomic equivalents per reaction), tested in triplicate by both the multiplex and simplex real-time PCR assays.

Five strains of the genus *Bordetella* and 12 pathogenic bacteria of non-*Bordetella* species were used to evaluate the specificity of the multiplex real-time PCR (Table 1). DNA from these strains was used in the individual evaluation of each real-time PCR target assay and in the multiplex assay for cross-reactivity at a 10 ng/ µl concentration.

Real-time PCR of clinical samples. Totally, 121 clinical specimens were tested with the multiplex real-time PCR in duplicate. Forty-six of them were evaluated with both the simplex and multiplex assays on the same 96-well reaction plate. An average Ct value of the duplicate real-time PCR was calculated to give a final value. A specimen was considered positive for DNA *B. pertussis,* when it produced signals in the two channels for the *IS481* and *BP0026* targets with a Ct value <40 or the signal was produced in the channel for *IS481* and the result could be confirmed in repeat research with new aliquot. When specimen produced a signal in the channel for *IS1001*, the result was reported as positive for *B. parapertussis*. The result was reported as 'negative', when amplification was not observed for any of the three targets or Ct value was 40 or more.

Clinical specimens were also tested for the human GAPDH gene using the real-time PCR assay to monitor the quality of DNA in the specimen and to check inhibition. To be considered positive for GAPDH, a specimen had to have a Ct value of <40. If a specimen was negative for all targets, including human gene GAPDH, it was considered as low-quality and was not taken into account in the analysis.

A positive control sample contained DNA of *B. pertussis*, *B. parapertussis* (100 copies of genomic DNA per reaction) and human DNA (50 pg per reaction). PCR grade water was used as a negative control sample.

Clinical criteria of pertussis case. A pertussis case was defined as cough lasting for at least 2 weeks, paroxysms of coughing or vomiting (35, 36) and one or more of the following symptoms or characteristics: apnea or cyanosis, subconjunctival bleeding, lymphocytosis, or a recent contact (up to 3 weeks) with a whooping cough patient. In addition to these clinical criteria, whooping cough was also considered in the case of positive culture of *B. pertussis* or four-fold increase in anti-PT antibody level was detected in paired sera or anti-PT levels ≥ 100 ME/ml in a single serum sample, only if the serum is collected after over a three-week cough and 3 years after a vaccine booster.

The clinical sensitivities and specificities and predictive values of the results were determined in two frequency tables with the clinical criteria for pertussis as the gold standard.

RESULTS

The analytical sensitivity and specificity of the multiplex TaqMan real-time PCR. The lower limit of detection (LLOD) for *IS481* and *IS1001* is one genome equivalent per reaction for the simplex and multiplex assays and 10 genome equivalents per reaction for *BP0026*. The amplification curves obtained from the same dilution series by the simplex reaction showed similar efficiency with multiplex real-time PCR assay (Table 3).

No amplification signal was found for the non-*Bordetella* species used for examination the specificity of the multiplex real-time PCR (Table 1).

Comparison of real-time PCR methods for the detection of *B. pertussis* and *B. parapertussis*. Forty-six clinical samples were tested simultaneously in multiplex and simplex real-time PCR. Comparative analysis of two assays showed that in both reactions 22 of 46 samples (47.8%) were considered positive for *B. pertussis* and 24 of 46 samples (52.2%) were negative. Among the 22 positive samples, 13 were positive for *IS481* and *BP0026* in both reactions; one sample was positive for two targets in simplex assay but only for IS481 in multiplex; eight samples were positive only for one target *IS481* in both reactions.

Table 3. Efficiency of simplex and multiplex TaqMan real-time PCR assays.

RT-PCR assay	IS481			BP0026			IS1001		
	Effic.(%)	Slope	R^2	Effic.(%)	Slope	R^2	Effic.(%)	Slope	R^2
Singleplex	90.858	-3.562	0.999	99.565	-3.332	0.998	100.513	-3.31	0.998
Multiplex	92.071	-3.528	0.998	97.078	- 3.394	0.997	101.021	-3.298	0.996

Five of eight samples positive only for *IS481* had high Ct value (between 37 and 40), indicating low concentration of DNA in starting material.

Clinical evaluation of real-time PCRs. One hundred twenty-one patients with alleged pertussis infection were tested. Nasopharyngeal swabs of all patients were investigated by bacteriological method, conventional PCR assay and multiplex real-time PCR. Two clinical samples were negative for all the targets in the real-time PCR, including the control human GAPDH gene. Therefore, they were not considered in the analysis. Sixty-five of the 119 (54.6%) patients had a pertussis infection according to clinical criteria as described in materials and methods. Three of the 119 (2.5%) were found positive by culture, 37 of 119 were found positive by conventional PCR (31.1%), and 57 of 119 (47.9%) were found positive by multiplex real-time PCR (Table 4). One swab was found positive for *B. parapertussis* by both PCR assays. All specimens from patients without symptoms, matching the clinical criteria of pertussis, were negative in PCR.

When *B. pertussis* assays were compared with the clinical standard for *B. pertussis* infection, the sensitivity was 5, 59 and 89%; the specificity was 100, 100 and 100%; the positive predictive value was 100, 100 and 100%; and negative predictive value was 47, 67 and 89% for culture, conventional PCR, and real-time PCR, respectively.

As it is shown, there were 20 samples positive by multiplex real-time PCR only, and all had clinical criteria for disease (Fig.1). Nine of them were positive by serological test and two were epidemiologically linked with laboratory-confirmed case of pertussis. One sample was positive in conventional PCR and negative in real-time assay. Thus, number of positive results increased by 52.6% in comparison with conventional PCR.

DISCUSSION

The multiplex real-time PCR assay described provides an effective way to detect and differentiate *B. pertussis* and *B. parapertussis* infection. Only few studies describe the use of more than one target in a single tube for detection and differentiation of *B. pertussis* and *B. parapertussis* (33,34). The attempt to develop a triplex real-time PCR with three targets in a single tube showed significant decrease of efficiency of reaction in comparison with simplex real-time PCR (data are not shown). Comparative analysis of multiplex real-time PCR in the format of two duplex reactions with single real-time PCR showed similar result. The two duplex real-time PCR assay also allowed using the fourth target as internal control. Application of the internal control in reaction enabled

Table 4. Results of culture, conventional PCR, and real-time two duplex PCR from 119 patient samples with clinically alleged pertussis infection. Sixty five of these 119 fulfilled the clinical definition for pertussis infection.

Method	*No. (%) positive*				*No. with positive result in clinical pertussis test*	
	B. pertussis			*B.parapertussis*		
	All	*including*				
		IS 481	*BP0026*	*IS 1001*	*Positive*	*Negative*
Culture	3 (2.5)			0	3	62
Conventional PCR	37 (31.1)	37	14	1 (0.8)	38	27
Multiplex RT-PCR	57 (47.9)	57	38	1 (0.8)	58	7
Total	119			119	65	

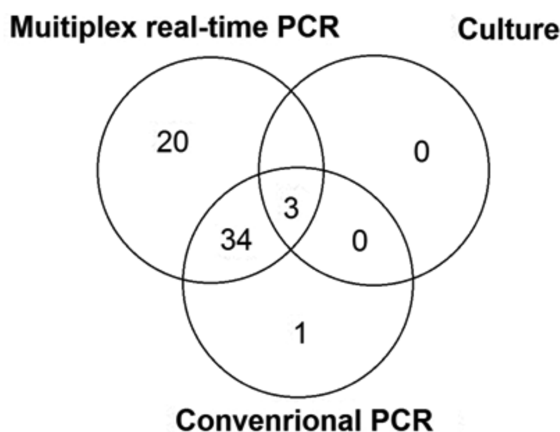

Fig. 1. Venn diagram showing assays demonstrating *B. pertussis* and *B. parapertussis* positivity.

monitoring quality of extracted DNA and inhibition of reaction.

The regions of *IS481* and *BP0026*, targeted in our assay are sensitive with LLOD of 1 and 10 *B. pertussis* genomic equivalent per reaction, respectively. The region of *IS1001* targeted in our assay is sensitive with LLOD of 1 *B. parapertussis* genomic equivalent per reaction. All regions are specific with no cross-reactivity with non-*Bordetella* spp. or human DNA. It is known that a small amount of *IS481* copies are found in the genomes closely related with pathogens *B. bronchiseptica* and *B. holmesii* (27,37). In order to identify DNA of these pathogens in our real-time PCR design of primers and probe for selection IS481 was performed on the fragment with 99% homology to the nucleotide sequences *IS481* from *B. bronchiseptica* (accession no. EF043395) and *B. holmesii* (accession no. DQ420073). Unfortunately two available *B. bronchiseptica* strains were negative in our real-time PCR and there were no strains of *B. holmesii* to analyze (Table 1).

A total of 119 specimens from patients with clinical symptoms of pertussis, 57 samples were positive for *IS481* by multiplex real-time PCR assay. Among 57 samples only 38 were positive for *BP0026*. Among nineteen samples positive for *IS481* and negative for *BP0026* 14 samples had high Ct value (between 37 and 40). According to our data analytical sensitivity of the multiplex real-time PCR amount one genomic equivalent for *IS481* target (mean Ct value 38.8). Thus, samples with so low concentration DNA couldn't be detected by *BP0026* target, because the LLOD for it was equal to 10 genomic equivalents (Table 5).

A similar situation has been observed when using other single copy genes as the second target. Negative results for *ptxS1* gene were observed, when positive results for *IS481* had high Ct value (34). We have compared analytical sensitivity of real-time PCR with primers and probe for *ptxS1*, proposed by Tatti et al. (34) and primers and probe for *BP0026* target. LLOD for both reactions was similar and equal to 10 genomic equivalents per reaction (Fig. 2). Besides, specimens positive for *IS481* and negative for *BP0026* were tested in real-time PCR with primers for *ptxS1* and all had a negative result.

At the same time the combined use of targets *IS481* and *ptxS1* does not allow differentiation between *B. pertussis* and *B. bronchiseptica* if samples are positive for both targets. Although strains of *B. bronchiseptica* primarily affect animals, but occasionally cause a disease in humans (38, 39). Using a fragment of putative thiolase gene tagged *BP0026* as a second target in our diagnostic PCR allows to confirm presence of DNA of *B. pertussis*. The specificity of this fragment to *B. pertussis* genome and possibility of its use in PCR to improve detection of the causative

Table 5. The minimum amount of DNA detectable in the multiplex TaqMan real-time PCR.

Genomic equivalents per reaction	mean Ct (95% CI)		
	***B. pertussis* IS481**	***B. pertussis* BP0026**	***B. parapertussis* IS1001**
10^7	13.2 (12.7-13.7)	19.8 (19.5-20.1)	15.0 (14.8-15.2)
10^6	16.6 (15.5-17.7)	23.3 (22.8-23.8)	18.0 (17.4-18.6)
10^5	20.3 (19.3-21.3)	26.6 (26.0-27.2)	21.1 (20.5-21.7)
10^4	23.8 (22.8-24.8)	30.1 (29.6-30.6)	24.5 (23.8-25.2)
10^3	27.4 (26.4-28.4)	33.4 (32.3-34.5)	27.5 (27.0-28.0)
10^2	31.3 (29.2-33.4)	36.9 (35.9-37.9)	31.1 (30.2-32.0)
10^1	34.7 (33.1-36.3)	38.9 (37.8-40.0)	34.8 (33.7-35.9)
10^0	38.8 (37.9-39.7)	-	37.9 (36.7-39.1)

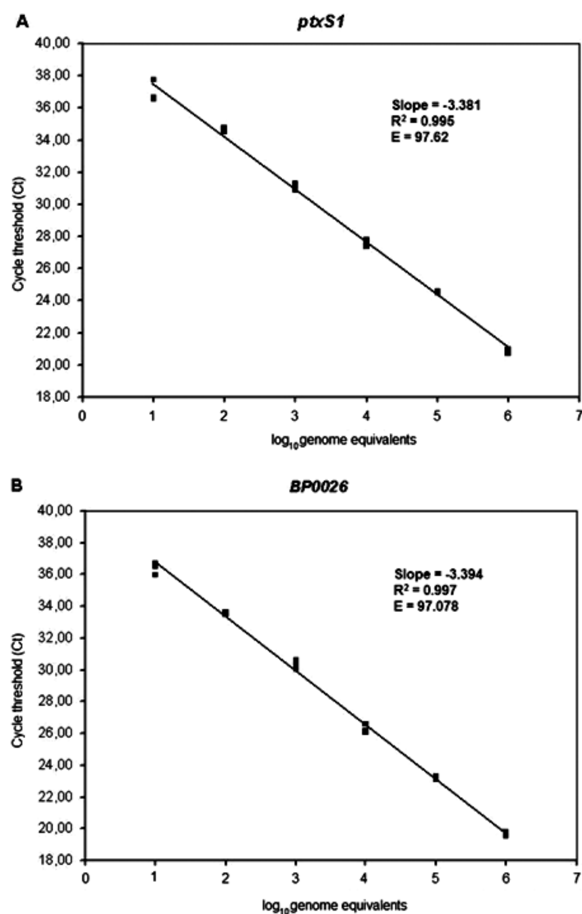

Fig. 2. Linear dynamic range of the real-time PCR assays using *B. pertussis* DNA extracts Dynamic range analysis of the real-time PCR assays *ptxS1* target (A) and *BP0026* target (B) The slope, correlation coefficient (R^2) and PCR efficiency (E) are displayed.

agent of whooping cough were described by Probert *et al.* (37). However, samples with high Ct value for *IS481* and with negative result for *BP0026* require comparison of PCR data with other laboratory tests such as bacteriological, serological, and with clinical and epidemiological data. Such specimens could be retested in PCR for correct interpretation of results.

In conclusion, developed multiplex real-time PCR in a format of two duplex reactions offers fast and suitable tools for implementation in a routine laboratory diagnostics. Targets used in this PCR assay provide high sensitivity and specificity for the diagnosis of *B. pertussis* and *B. parapertussis* infections.

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