Addition of a Homologous Internal Control to a Real-Time PCR Assay for Detection of Bordetella pertussis, Christoph Koidl,1 Michael Bozic,1 Jörg Berg,2 Markus Stöcher,2 Gerhard Mühlbauer,3 Egon Marth,4 and Harald H. Kessler5

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Pertussis, also called whooping cough, is caused by Bordetella pertussis. The disease may show an atypical course, particularly in neonates and elderly patients. A rapid and safe diagnostic method is thus essential for appropriate treatment and prophylaxis. Culture has been considered the gold standard for detection of B. pertussis, but this method often lacks sensitivity, and a minimum of 4 days may be required to obtain results (1, 2). PCR is a rapid, sensitive, and specific method for the diagnosis of pertussis (3–5).

In this study, a new molecular assay was established based on real-time PCR and including a homologous internal control (IC). We evaluated the performance of this assay with a commercially available genomic DNA isolate and with clinical samples.

The new molecular assay consisted of a protocol for manual extraction of DNA followed by generation of the amplification product by real-time PCR. The assay was based on the amplification of a 181-bp fragment of the repetitive insertion sequence IS481, which has been described in B. pertussis and Bordetella holmesii and may be present in Bordetella bronchiseptica (6–10) (see Table 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue12). We determined assay linearity and detection limit by analyzing 10-fold dilutions of the ATCC genomic DNA isolate 9797D from B. pertussis. Interassay variation was determined with 7 dilutions of the genomic DNA isolate (5 determinations on 5 different days), whereas intraassay variation was determined with 3 samples (5 determinations within a single assay). All assays for determination of inter- and intraassay variation included negative controls.

A total of 219 nasopharyngeal swabs were tested in this study. All specimens were collected with the Copan Venturi Transystem® culture swab transport system (CO-PAN Italia Spa) according to the manufacturer’s instructions. Samples were obtained from patients (99 females, 120 males; mean age, 9.1 years; range, 0–86 years) with a clinical presentation compatible with B. pertussis infection.

We devised the B. pertussis–specific IC as a PCR competitor that contained B. pertussis–specific forward and reverse primer sequences bordering a stretch of the neomycin phosphotransferase gene (neo) as heterologous DNA. The IC was constructed as described recently (11). Briefly, a stretch of neo (bp 471 to 603; GenBank accession no. V00618) was amplified in preparative PCR with composite primers that consisted of the B. pertussis–specific primer sequences in 5′ and the neospecific primer sequences in 3′. The obtained product (201 bp) was cloned into the plasmid vector pCR2.1-TOPO by the TOPO TA Cloning Kit according to the manufacturer’s instructions (Invitrogen). Plasmid DNA was purified from transformed bacteria by use of the Quantum Plasmid Miniprep Kit (Bio-Rad) linearized with HindIII restriction enzyme (New England Biolabs, Inc.), and purified with the High Pure Viral Nucleic acid reagent set (Roche Applied Science), according to the manufacturer’s instructions. The obtained DNA amounts were assessed by ultraviolet spectrophotometry at 260 nm. To guarantee sufficient
stability, the IC was introduced into lambda phage particles, as described recently (12).

Preparation of bacterial DNA started with dipping the swab into a mixture of 200 μL of tissue lysis buffer and 40 μL of proteinase K solution (20 g/L). The tip of the swab was cut, and the suspension (including the swab tip) was incubated at 55 °C for 60 min. After removal of the swab, 5 μL of the IC, equivalent to 20 copies of IC DNA, was added, and the High Pure PCR Template Preparation Kit (Roche) was used for DNA extraction according to manufacturer’s instructions.

Real-time PCR was performed on the LightCycler 2.0 (LC) instrument (Roche). All samples were assayed with the LC Fast Start DNA Master Hybridization Probes Kit (Roche). The PCR master mixture contained 2 μL of Fast Start Master DNA Hybridization Probes reaction mixture, 1.6 μL of MgCl₂ (final concentration, 3 mM), 1.0 μL each (final concentration, 0.5 μM) of the forward and reverse primers, 2.0 μL each (final concentration, 0.2 μM) of BP FL and BP LC Red 640 hybridization probes, 0.2 μL each (final concentration, 0.2 μM) of the Neo FL and Neo-LC Red 705 neo-hybridization probes, and PCR-grade sterile water (7.0 μL) to a final volume of 17 μL. A 3-μL aliquot of extracted sample was added to 17 μL of PCR master mixture in each LC glass capillary. The LC capillaries were then sealed, inserted into the specially designed LC Carousel (Roche), and centrifuged at 735 g for 15 s. Finally, the LC Carousel was placed into the LC instrument.

The cycling protocol was as follows: 1 cycle of 95 °C for 7 min followed by 50 cycles consisting of denaturation for 10 s at 95 °C, annealing for 10 s at 50 °C, and elongation for 20 s at 72 °C. After the final cycle, the melting curve analysis was started with the initial temperature set at 95 °C, followed by cooling to 45 °C for 2 min; the thermal chamber temperature was then slowly (0.2 °C/s) increased to 82 °C, and the fluorescence was measured continuously. Fluorescence curves were analyzed with the LC software (Ver. 4.0). Crossing point calculation was done by the automated second-derivative maximum method. Channel 640/530 was selected for the target sequence, with channel 705 selected for the IC.

The results of the linearity testing are shown in Fig. 1. When 10-fold dilutions of the ATCC genomic DNA isolate were analyzed, positive results were consistently obtained with dilutions 10⁻² through 10⁻⁸ (corresponding to ~1 genome-equivalent). The calibration curve was quasi-

![Amplification Curves](https://via.placeholder.com/150)

### Fig. 1. Detection of *B. pertussis* DNA.

Results obtained from a dilution series of the ATCC genomic DNA isolate 9797D. (A), plots for the target DNA; (B), calibration curve; (C), plots for the corresponding IC. neg, negative; GE, genome-equivalent.
linear over 6 logs (dilutions $10^{-2}$ through $10^{-7}$). The IC was consistently detected in dilutions $10^{-6}$ through $10^{-9}$.

To determine interassay variation, we assayed 7 clinical samples positive for *B. pertussis* DNA within the linearity range of the assay 5 times on 5 different days (including reextraction each day). The SD calculated from the crossing points obtained was 0.08–1.09. We determined intraassay variation by assaying 3 *B. pertussis*-positive clinical samples within the linearity range of the assay 5 times within a single assay (including different extractions). Mean crossing points were calculated, and the SD was 0.14–1.04. The mean melting peak was at 62°C (range, 61.5–62.5°C).

When clinical samples were tested, 46 of 219 nasopharyngeal swabs were found to be positive for *B. pertussis* DNA, with melting points at the expected temperature. Sera were obtained 4 weeks later from all patients with positive results and tested with the Serion ELISA classic for *B. pertussis* IgA (Institut Virion\Serion GmbH). A positive IgA result was obtained for all sera from patients with a positive PCR result. The new homologous IC was consistently detected in all negative and in 10 (22%) of 46 positive clinical samples. Extraction of samples was completed within 2 h. After the centrifugation step, real-time PCR took another 55 min. No contamination was observed during the entire study.

PCR amplification may fail because of interference from PCR inhibitors; therefore, ICs have been incorporated in molecular assays for detection of *B. pertussis* (10, 13). The homologous IC used in this study was coextracted with the clinical samples and amplified with the same primers used for the target DNA. This procedure ensures accurate control of the entire molecular assay and represents the state of the art for ICs. The *B. pertussis*-specific IC gave positive results for all negative samples throughout the whole study, indicating successful removal of potential inhibitors by the extraction method. Contrary to a recent study using an identical amplification protocol (13), the sensitivity of the assay in this study was not affected by introduction of the homologous IC. Sensitivity to strains other than *B. pertussis* may also not be affected by introduction of this IC. In 78% of positive samples, competitive inhibition prevented detection of the IC.

In conclusion, the newly established assay includes all of the features required for molecular detection of *B. pertussis* in the routine diagnostic laboratory. This molecular assay is suitable for the routine diagnostic laboratory and allows rapid and safe diagnosis of *B. pertussis*.

**References**


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**Process Improvement and Operational Efficiency through Test Result Autoverification, Narayan Torke, Leonard Boral, Tracy Nguyen, Angelo Perri, and Alan Chakrin**

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Through autoverification, a customized expert system within the Laboratory Information System (LIS), a computer performs the initial review and verification of test results based on a predetermined set of boundaries or rules, as established by the laboratory (1). A carefully designed system can be an important tool in addressing such crucial issues as medical errors, test turnaround time (TAT), shortages in personnel and funding, and operational efficiency. As part of an ongoing modular laboratory automation plan at John H. Stroger, Jr. Hospital of Cook County (JSHHCC, formerly Cook County Hospital, Chicago), autoverification was implemented in our clinical chemistry and urinalysis laboratories.

JSHHCC is a large, urban, tertiary acute-care public hospital and trauma center that serves as the centerpiece of a countywide integrated healthcare delivery system. As part of the hospital’s clinical pathology service, the clinical chemistry laboratory provides more than 150 different test procedures in general chemistry, toxicology, drug screening, endocrinology, urinalysis, and blood gas analysis. The laboratory has modern, automated instrumenta-