Diagnostic Microbiology and Infectious Disease 76 (2013) 10–15

Contents lists available at SciVerse ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Molecular detection and quantification of pertussis and correlation with clinical outcomes in children

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ARTICLE INFO

Article history:
Received 3 October 2012
Received in revised form 14 December 2012
Accepted 19 December 2012
Available online 11 March 2013

Keywords:
Pertussis
PCR
Semi-quantitative PCR
Bacterial load

ABSTRACT

Pertussis is an under-recognized serious infection. Conventional cultures are insensitive and of limited utility after antibiotic exposure. We corroborated the utility of real-time polymerase chain reaction (PCR) as a diagnostic tool in pertussis and investigated its role as a prognostic tool by evaluating its benefit in the quantification of pertussis bacterial load. All pertussis-positive PCR tests ($n = 104$) submitted over 5 years were collected for retrospective study. PCR cycle threshold was compared to quantitative culture in 43. Compared to PCR, the sensitivity of culture was 41%. Our PCR assay reliably quantified bacterial load and was quantitatively reproducible. Higher bacterial load correlated with longer duration of hospitalization ($P = 0.0003$), and multivariate logistic regression models demonstrated this association to be independent. The study confirmed PCR as a superior diagnostic tool in pertussis. PCR quantification of bacterial load at initial diagnosis predicts later clinical disease severity, suggesting a potential benefit of PCR as a prognostic tool in pertussis.

1. Introduction

Each year, 20–40 million cases of pertussis occur globally and cause up to 400,000 fatalities, mostly in young infants (Tan et al., 2005). An increased incidence of pertussis has been reported in many countries, including the United States, over the past 15 years (Tan et al., 2005). Diagnosis is important because proper and early treatment can control and prevent community epidemics. Culture has traditionally been regarded as the gold standard for laboratory diagnosis of infection with *Bordetella pertussis*, but low sensitivity can limit its diagnostic utility. The sensitivity of culture-based diagnosis of pertussis decreases with disease progression (Cimolai et al., 1996), increase in age of the patient (Cimolai et al., 1996; Grimprel et al., 1993), and prior antibiotic exposure. These factors may be overcome by using polymerase chain reaction (PCR) as a diagnostic tool for pertussis. Additionally, the paroxysmal nature of the disease makes it challenging for clinicians to predict the disease severity, as the clinical picture can vary. Nucleic acid amplification (PCR) might be able to overcome these diagnostic and prognostic problems, as it theoretically allows both improved detection sensitivity and quantification of bacterial load. There has been increased interest in more rapid and accurate methods for *B. pertussis* diagnosis, and a large number of PCR protocols have been published (Dragsted et al., 2004). In most previous comparisons of culture and PCR, the diagnostic yield has been increased about 2- to 4-fold by the use of PCR, despite variations in sampling methods, sample preparation methods, primers, and detection methods (Heininger et al., 2000; Schlapfer et al., 1995; van der Zee et al., 1996). Overall, the diagnosis of *B. pertussis* infections by nucleic acid amplification–based methods has been shown to be both highly sensitive and specific (Muller et al., 1997). Nonetheless, little is known of the bacterial load of pertussis and its importance, if any, as a predictor of disease severity. To the best of our knowledge, the role of PCR as a prognostic tool in childhood pertussis has not been previously studied. In real-time PCR assays, the bacterial load can be measured by cycle threshold (CT) value, which is the point at which the amplification becomes exponential. Generally, the CT value is inversely proportional to the log of the starting concentration of the target DNA. The bacterial load of pertussis as a marker of disease severity has not been well described. We therefore developed a real-time semi-quantitative PCR assay diagnostic for pertussis,

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http://dx.doi.org/10.1016/j.diagmicrobio.2012.12.015

evaluated its performance relative to conventional culture assays, and studied the relationships between clinical disease severity and PCR-based quantification of bacterial load.

2. Methods

2.1. Study design

We implemented the pertussis PCR assay as a routine clinical diagnostic method on the date of 2 February 2004. The records of all pertussis-positive PCR tests submitted from this date through 5 May 2009 were retrospectively collected ($n = 104$). For purposes of statistical comparison, for every third consecutive positive PCR test we selected a negative PCR test, the specimen of which was collected nearest in time to the collection of the positive PCR specimen ($n = 35$).

2.2. Study population and data collection

The clinical data were extracted from medical records by a team of designated data extractors. Information captured included patient's age at admission, sex, ethnicity, history of prematurity (<37 weeks estimated gestational age), immunization status, presence of paroxysmal cough and cyanosis, and the length of hospital stay. Laboratory data included culture results, PCR results, white blood cell count (WBC), and absolute lymphocyte count (ALC). Patients were classified as "immunized," "unimmunized," or "unknown" based on their vaccination status as listed in the medical records. The patients with up-to-date vaccination records were defined as "immunized." The patients whose vaccination records were not found to be up-to-date for age were defined as "unimmunized." Finally, those patients whose vaccination status was not known or not listed in the medical records were defined as "unknown." Classical pertussis illness has 3 stages (catarrhal, paroxysmal, and convalescent) (Cherry and Heininger, 2009). The paroxysmal phase is characterized by episodes of coughing, and cyanosis can occur during these paroxysms. Previously, clinical studies have implicated cough and cyanosis as valuable markers in clinical pertussis (Lurie et al., 2009). Similarly, we elected to look at paroxysmal cough and cyanotic episodes because of the standardized manner in which these observations were recorded in the patient's chart.

2.3. Laboratory methods: PCR and culture

2.3.1. Polymerase chain reaction

The real-time PCR assay targeted the insertion sequence IS*481* of *Bordetella*. The primers used were as follows: forward primer, 5′ GGT GTG AAG ATT CAA TAG GTT GT; reverse primer, 5′ GCC GCT TCA GGC ACA CAA AC. The probes used were 5′ TCG CCA ACC CCC CAG TTC ACT CA-FAM and 5′ LC-Red 640-AGC CCG GCC GGA TGA ACA CCC. Reaction conditions for the assay were chosen according to a standard LightCycler® protocol (Roche Diagnostics, Indianapolis, IN, USA). Fluorescence increase was measured during the annealing step at 65 °C. Readout of LC-Red 640 values (*B. pertussis*) was performed in channel F2/Back-F1, and readout of LC-Red 705 values (human albumin) was performed in channel F3/Back-F2. Oligonucleotides (TIB MOLBIOL, New Jersey, USA) from IS*481* for *B. pertussis* were as above. The reaction mixture was prepared in a controlled access reagent preparation room and consisted of 15 μL of PCR master mix plus 5 μL of DNA extract per reaction. The PCR master mix consisted of 4 mmol/L $MgCl_2$, 50 mmol/L KCl, 20 mmol/L Tris–HCl (pH 8.4), 0.2 mmol/L PCR Nucleotide Mix^{PLUS} (1 × dATP, dCTP, and dGTP, and 3 × dUTP; Roche Diagnostics, Indianapolis, IN, USA), 0.75 μmol/L (each) primers, 0.025% bovine serum albumin (Sigma, St. Louis, MO, USA), 0.2% HK-UNG thermolabile uracil-*N*-glycosylase (Epicentre Technologies, Madison, WI, USA), 0.2 μmol/L (each) fluorescein probe, 0.3 μmol/L (each) LC Red-640 probe, 0.5% dimethyl sulfoxide (Sigma), and 0.03 U/μL of Platinum *Taq* DNA polymerase (Life Technologies, Rockville, MD, USA). Each specimen was run in duplicate; samples were considered positive if both replicates had Ct values ≤50 and negative if there was no amplification.

2.3.2. Culture

The nasopharyngeal swabs submitted for *B. pertussis* culture were inoculated onto Regan-Lowe plates. The plates were incubated at 35 °C in the presence of CO_2 for as long as 6 days and were examined for the presence of typical colonies.

2.4. Laboratory methods: in vitro quantification of bacterial load by PCR and culture

To test the ability of our semi-quantitative real-time PCR to correlate with numbers of live *B. pertussis* bacteria, cultures of the *B. pertussis* ATTC strain 9340 were grown in vitro. A known concentration, measured in colony forming units (CFU), was prepared, serially diluted in saline, and aliquoted. The identical *B. pertussis* extraction and PCR procedure used for the series of clinical specimens were then performed on these aliquots of cultured *B. pertussis*. The quantity of bacteria as measured by CT value was correlated with that aliquot's CFU (log CFU/L) as determined by culture.

2.5. Statistical analyses

Conventional descriptive statistics were used to characterize the study population. To identify independent predictors of disease severity, predictive models were generated using multiple logistic regression analyses. The variable length of hospitalization, measured continuously or dichotomized by a median split (<4 days; ≥4 days), was identified as the outcome measure of pertussis-related disease severity for its availability in the medical record, ease of characterization, and clinical relevance. The independent variables (regressors) selected pre-analyses and tested for inclusion in the predictive models were age, sex, prematurity, immunization status, bacterial load (CT value of PCR), total WBC, and ALC. First, univariate analyses and simple association studies were used to test the associations of the listed regressors with the outcome variable. Next, independence of covariates was confirmed by correlation diagnostics testing for potential inter-regressor collinearity. Statistical models were then built by using multivariate logistic regression analyses, including all independent variables found to be associated ($P < 0.1$) with the outcome variable in the univariate analyses. Goodness-of-fit diagnostics were applied to the final models to demonstrate the adequacy of fit. All analyses were performed using the outcome variable length of hospitalization, first as a continuous measurement, and then repeated with the length of hospitalization dichotomized as described above. Predictor variables were considered significant when the *P* values associated with their respective beta coefficients in the final models were less than 0.05. All statistical analyses were performed using the SAS software (SAS Institute, Cary, NC, USA). Figures were generated using the GraphPad Prism Software (GraphPad Software, La Jolla, CA, USA).

3. Results

A total of 305 upper respiratory secretion samples were submitted to the clinical diagnostic laboratory during the 5-year period from the date of availability of the first PCR test (2 February 2004) through 26 May 2009. Of these, all patients with PCR positive for *B. pertussis* ($n = 104$) were included in this retrospective study. For purposes of comparison, for every third positive PCR test, the patient with the nearest ordered negative test was also included ($n = 35$). The characteristics of the patient population ($n = 139$) are outlined in Table 1. Of the 139 samples evaluated for pertussis by PCR, 43 were