Laboratory Performance and Clinical Correlation of rt-PCR as a Diagnostic Test for *Bordetella pertussis* Isolated from Patients in Oman

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Abstract

Objective: The prompt diagnosis of pertussis (whooping cough) is essential to limit its transmission. Compared with culture which has been for a long time considered the gold standard, rt-PCR has significantly increased the detection rate of pertussis. Limited studies, however, have incorporated clinical data when assessing the specificity of rt-PCR. This study aimed to evaluate rt-PCR for the diagnosis of pertussis, emphasising the importance of clinical correlation in determining its specificity.

Methods: Nasopharyngeal/ throat samples in charcoal media received from all over Oman at Central Public Health Laboratories (CPHL) from January 2014 to December 2016 were included in this study. These samples were tested using both culture and rt-PCR. rt-PCR was compared with culture to calculate its sensitivity and specificity. Further clinical correlation was conducted for the discrepant cases using different case definitions.

Results: A total of 590 nasopharyngeal/ throat samples were included in the study. Out of the 590 samples, 73 were positive by rt-PCR compared with 26 positive samples by culture (which were also positive by rt-PCR). The sensitivity and specificity of rt-PCR compared with those of culture were 100% [confidence interval (CI): 86.77%−100%] and 91.67% (CI: 89.07%−93.81%), respectively. To rule out false-positive results by rt-PCR, clinical correlation was performed. Out of 47 cases that were positive by rt-PCR but negative by culture, 44 cases were clinically evaluated by access to clinical details. Out of these 44 cases, 21 (48%) met the pertussis clinical criteria according to the CDC-2014 case definition, 41 (93%) according to Europe-2008 case definition, and 44 (100%) according to the Canada-2009 and Australia-2014 case definitions. With only positive rt-PCR, these cases were classified as confirmed according to these case definitions, and hence, this increases the specificity of rt-PCR to 95.7-100%. The mean turnaround time (TAT) was 3.46 days for rt-PCR compared to 6.20 days for culture.

Conclusions: rt-PCR is a highly sensitive and specific test for the diagnosis of *B. pertussis* infection. Based on our results, we recommend setting up a PCR diagnostic facility in regional hospitals in Oman as this will lead to the timely and accurate diagnosis of pertussis.

Keywords: *B. pertussis*, clinical correlation, culture, PCR, pertussis, test performance

Introduction

Pertussis (whooping cough) is a highly contagious respiratory infection caused by the bacterial species *Bordetella pertussis* (1). Pertussis accounts for significant morbidity and mortality among infants and children, particularly in low income countries where immunisation programs are not well established (2). In 2008, 195,000 children were estimated to die from the disease worldwide (3).

Pertussis immunisation has resulted in a significant drop in pertussis cases worldwide (2). However, the resurgence of pertussis cases was noticed during the last decades, even in countries with high vaccination coverage (4). In Oman, there has been a significant increase in the number of reported cases, rising from 56 cases in 2011 to 309 cases in 2013 (5). Possible reasons for this resurge may include incomplete effectiveness of the vaccine, waning vaccine-induced immunity, the adaptation of *B. pertussis* strains, increased awareness, and improved reporting (6). In addition, the atypical clinical presentation of pertussis seen in immunised individuals and adults makes the clinical diagnosis challenging, with the ongoing risk of transmission to vulnerable individuals (7). The resurge of the disease and the challenging clinical diagnosis of pertussis highlight the importance of using a rapid and accurate diagnostic method for the early diagnosis of pertussis cases, leading to early treatment and the interruption of its transmission.

Culture is considered the gold standard for diagnosing pertussis because of its high specificity (8,9). However, studies have shown that it has suboptimal sensitivity ranging from 15% to 60% (10). In addition, it is labour-intensive and may take up to six or seven days to finalise the results.

The most commonly used molecular method for the detection of *B. pertussis* is real-time PCR (rt-PCR). Although previous studies have shown that rt-PCR has excellent sensitivity compared with culture, such specificity was variable among these studies. In addition, limited studies used clinical data to assess the specificity of rt-PCR (11–13). This study aimed to evaluate rt-PCR for the diagnosis of pertussis, emphasising the importance of clinical correlation in determining its specificity.

Materials and Methods

Study Design

This study is a retrospective diagnostic test accuracy study. It was conducted at Central Public Health Laboratories (CPHL), which receives samples from clinically suspected pertussis cases from all over Oman to perform diagnostic tests for pertussis.

Study Sample

The study included nasopharyngeal/throat specimens sent in appropriate media (i.e. charcoal media) and tested by both culture and rt-PCR during the three-year study period: January 2014 to December 2016. The samples sent in viral transport media were tested only by rt-PCR and thus were excluded from this study.

Culture

Culture was performed from nasopharyngeal/throat swabs transported in appropriate media supporting the viability of organisms (e.g. charcoal media) sent in a cool box. In the lab, the swabs were pre-warmed and then streaked in Bordetella selective agar (charcoal agar). A quality control sample was run for each batch of samples. The specimens were incubated at 37°C in an ambient aerobic atmosphere for five days. The plates were inspected after 48 hours and onwards to check for growth. If any growth detected, it would be tested to identify Bordetella. *B. pertussis* was identified by characteristic pearl-like colonies, Gram stain, oxidase test, and antisera.

rt-PCR

The nasopharyngeal/throat samples were put in 2 ml of phosphate buffer saline and then underwent extraction according to the kit manufacturer's instructions (Qiagen® DNeasy Blood & Tissue Kit, Germany). Amplification was then run using an in-house multiplex assay targeting the IS 481, IS 1001, and hIS 1001 genes based on CDC protocol (14) in addition to internal control. These targets can detect different species of Bordetella, including *B. pertussis*, *B. parapertussis*, and *B. holmesii*, with a distinct positivity profile for each species. Positive IS 481 with negative IS 1001 and hIS 1001 indicates *B. pertussis*. Although uncommonly encountered, samples with positive IS 481 with high cycle threshold value (i.e. Ct $\geq$ 35) were further tested using commercial CE marked FTD kit (Fast Track Diagnostics, Luxembourg) to confirm *B. pertussis.*

Statistical Analysis

The data (lab number, date of sample collection, date of sample reception in CPHL, culture and rt-PCR results and date of results release) were collected from laboratory records and entered in Epidata software. The analysis of the data was performed through IBM SPSS version 22. Total turnaround time (TTAT; defined as the interval from sample collection to the reporting of results) and turnaround time (TAT; defined as the interval from sample receipt to the reporting of results) were calculated for both rt-PCR and culture. The transit time of the

samples (from sample collection to sample reception in CPHL) was also calculated. In addition, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for rt-PCR in comparison to culture.

Clinical Data Collection and Analysis

Cases that were pertussis-positive by rt-PCR but negative by culture were further assessed to rule out false-positive results. The clinical data were collected from the electronic health information system which connects all Ministry of Health hospitals. Demographic data, clinical data (including presence and duration of cough, presence of paroxysms of cough, inspiratory whoop, post-tussive vomiting, and apnoea), presence or absence of alternative diagnoses, and epidemiologic links were collected in data collection sheets and entered in Epidata software before being exported to IBM SPSS for statistical analysis. The clinical data were compared against the cinical criteria of four different pertussis case definitions (CDC-2014, Canada-2009, Europe-2008, and Australia-2014) (15).

Ethical approval

Ethical Approval was obtained from Research and Ethical Review & Approve committee, Ministry of Health, Sultanate of Oman, in August 2017, Proposal Code: MoH/CSR/17/5902. All work in relation to the patients reported anonymously. Hence, informed consent was not required.

Data Availability Statement: Data in this study is available on request.

Results

Out of the 907 nasopharyngeal/throat specimens received at CPHL during the three-year study period, 590 samples from 590 patients fulfilled the inclusion criteria (sent in charcoal media and kept in appropriate temperature) and so were included in the study (diagram1). The demographic data of the 590 patients are shown in Table 1. The majority of the included samples (90.7%) were from children less than 1 year of age.

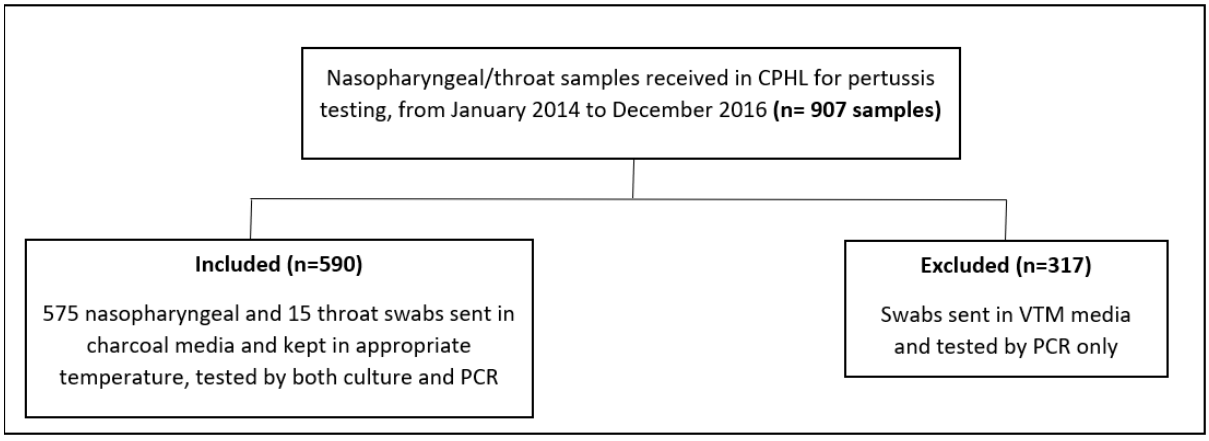

Diagram 1: A flow-chart showing the included samples in the study.

Table 1: Demographic data of the included patients.

		Frequency (n)	Percentage (%)
sex	Male	326	55.3
	Female	264	44.7
	Total	590	100
Nationality	Omani	586	99.3
	Non-Omani	4	0.7
	Total	590	100
Age (years)	<1	535	90.7
	1–5	36	6.1
	6–15	9	1.5
	16–50	8	1.4
	>50	2	0.3
	Total	590	100
Sample type	Nasopharyngeal	575	97.5
	Throat	15	2.5
	Total	590	100

rt-PCR was positive for 73 out of the 590 samples (0.12%), while culture was positive for 26 samples (0.04%). All the 26 positive samples by culture were also positive by rt-PCR, giving rt-PCR a sensitivity of 100% (Table 2). Out of 564 negative samples by culture, 47 of them tested positive by rt-PCR. The calculated specificity, PPV, and NPV for rt-PCR compared with those for culture were 91.67%, 35.62%, and 100%, respectively.

Table 2: 2 × 2 table showing the number of positive and negative samples tested by rt-PCR compared with those via culture.

		Culture Result		Total
		Positive	Negative	
PCR Result	Positive	26	47	73
	Negative	0	517	517
Total		26	564	590

The mean TAT was 3.46 days for rt-PCR compared with 6.20 days for culture. The transit time ranged between <24 hours to 23 days, with an overall mean transit time of 2.26 days. The calculated mean of TTAT was 5.72 days for rt-PCR compared to 8.46 days for culture.

To overcome the issue of a less sensitive gold standard (culture) with the potential of overcalling false positive results for the evaluated test (rt-PCR) – which may, in turn, affect the calculated specificity and PPV – further analysis was done for the rt-PCR-positive culture-negative cases. A total of 44 out of the 47 rt-PCR-positive culture-negative cases were further analysed using four different published case definitions. Table 3 summarises the clinical presentations of these 44 cases. The numbers of cases that met the clinical criteria according to -the four case definitions -are shown in Table 4. Adding rt-PCR-positive results to the clinical

data, 44 (100%) cases were classified as confirmed pertussis cases using the Canada-2009 and Australia-2014 case definitions. Applying the CDC-2014 and European-2008 case definitions, the numbers of confirmed cases were 21 (48%) cases and 41 (93%) cases, respectively. The calculation of specificity after the addition of these confirmed cases and the exclusion of three cases with unavailable clinical data yielded a higher specificity for rt-PCR that ranges between 95.7% and 100% considering the different pertussis case definitions. The calculation considering the CDC-2014 definition is illustrated in Table 5.

Table 3: The clinical presentation of the 44 rt-PCR–positive culture-negative cases of suspected pertussis.

		Number (total = 44)
Paroxysms of cough	Present	43
	Absent	0
	Not mentioned	1
Inspiratory whoop	Present	8
	Absent	0
	Not mentioned	36
Post-tussive vomiting	Present	21
	Absent	9
	Not mentioned	14
Apnea (if age is <1 year)	Present	17
	Absent	13
	Not mentioned	14
Cough duration	<2 week	22
	≥2 weeks	22
Epidemiologic link	Present	0
	Absent	2
	Not mentioned	42

Table 4: Classification of the 44 Positive-PCR negative- culture cases according to different pertussis case definitions.

	CDC 2014	**Canada 2009**	**European 2008**	**Australia 2014**
Cases that meet pertussis clinical criteria/evidence	**21**	**44***	**41**	**44**
Confirmed cases (consideringpositive PCR)	**21**	**44**	**41**	**44**

*Canada-2009 case definition has different clinical criteria for suspect and probable cases. 44 cases met the suspect case criteria, while 21 cases met the probable case criteria. With positive rt-PCR all cases that met the suspect criteria are classified as confirmed according to this case definition.

	Confirmed by culture or meeting CDC 2014 confirmed case definition			
PCR		Positive	Negative	
	Positive	47 **	23 ***	70
	Negative	0	517	517
		47	540	587*

Table 5: Modified 2 × 2 table comparing PCR cases with confirmed cases via either culture or the CDC 2014 case definition.

* Excluding the three cases (rt-PCR positive/culture negative) with no access to clinical data. ** Forty-seven is the sum of the 26 cases positive via both culture and PCR as well as the 21 cases classified as confirmed using the CDC 2014 case definition. *** Twenty-three cases resulted from the subtraction of 21 confirmed cases per the CDC 2014 case definition and the three cases with no access to clinical data from the total 47 cases (initially considered possible false-positive PCR results).

An analysis per age for all the 73 rt-PCR-positive samples, found that the majority of cases (67/73) were for children less than 1 year of age, the majority of whom (55/73) were less than 3 months of age.

Discussion

In this study, we evaluated the rt-PCR assay for the detection of *B. pertussis* in two steps (1) the evaluation of performance compared with the culture which is the gold standard; and (2) the evaluation of test specificity by clinical correlation of the discrepant results. The study included nasopharyngeal and throat samples. It is well-established that nasopharyngeal specimens have the best yield for *B. pertussis*, while throat swabs have unacceptable low rates of recovery (16). In our study, only 15 throat swabs were tested, and since all the samples underwent testing by both rt-PCR and culture, they were included in our study as their results affected both arms of the study equally. However, 14 of the 15 samples were negative by both tests, while only one throat was rt-PCR-positive/culture-negative case that met the pertussis clinical case definition.

It has been reported that the environmental contamination of clinical specimens in clinics and cross-contamination within laboratories has been associated with false positive PCR results and several pseudo-outbreaks of pertussis in recent years (8). However, the use of multiple targets in rt-PCR seems to improve specificity, as shown in the study published by Kathleen M. Tatti (14). The same targets and protocol were followed in our laboratory.

Our results showed that rt-PCR has high sensitivity, reaching 100% and a specificity of 91.67% compared with culture, which are in the same line with previous studies (9–16). However, the low sensitivity of the gold standard (i.e. culture) could have affected the calculated specificity and PPV of rt-PCR since 47 samples were culture negative and rt-PCR positive.

For this reason, the specificity of rt-PCR was further evaluated for those cases with discrepant PCR/culture results by carrying out clinical correlation using different case definitions. The majority of cases met the pertussis clinical criteria/evidence according to the Canada-2009 (100%), Australia-2014 (100%), and Europe-2008 (93%) case definitions. By adding positive PCR to the clinical criteria, these cases were considered confirmed. The CDC-2014 clinical case definition was an exception since only 21 of the 44 (47.7%) cases met its clinical criteria . A cough duration of at least two weeks was the only clinical criterion not met by most of the remaining cases(22/44). Although only 47.7% of the assessed cases met the CDC-2014 case definition, considering these cases as confirmed after adding positive rt-PCR, the specificity of rt-PCR improved to 95.7%. Thus, considering the different mentioned case definitions, specificity improved to 95.7% to 100%. Considering that PCR is more sensitive in the first three weeks of illness (16), it is not unexpected to find that the cases that presented early and did not meet the clinical criterion of a minimum cough duration of two weeks were PCR positive.

Of note, CDC has updated its case definition which was approved by the Council of State and Territorial Epidemiologists (CSTE) in June 2019 and went into effect January 1, 2020 (17) . The current CDC-2020 case definition classifies PCR-positive cases with acute cough illness as confirmed regardless of cough duration and presence of other pertussis symptoms. The updated CDC definition has resulted from increased confidence in the accuracy of PCR testing as a consequence of significant improvement in the quality of PCR testing, introduction of multiple target PCR and improved laboratory practice that led to minimal contamination and false-positive results, all of which have improved the specificity of PCR (18).

Previous studies that included clinical data when assessing the specificity of PCR showed improved specificity with clinical correlation, with specificity of 97-98% (12,13), which are in line with our results.

Our results emphasise the importance of clinical correlation in determining the rt-PCR specificity, particularly in the absence of sensitive gold standard, and clearly show combination of both laboratory and clinical data greatly increases the chance of early diagnosis, prompting early management and public health measures.

Bacterial load and viability are affected by several factors leading to culture suboptimal sensitivity. These factors include the stage of the disease when the sample was taken, the vaccination status of the patient, the age of the patient, and the specimen quality (19). In addition, culture methods, transit time, and delays in sample processing all affect the viability of the organisms leading to suboptimal culture sensitivity. rt-PCR is less likely to be affected by these factors since organism does not need to be viable to detect target genes. In addition,

culture is labour-intensive and may take up to five to seven days to finalise the results. Although rt-PCR can be completed within one day, the mean TAT for rt-PCR was longer (3.46 days) in our study due to capacity issues. However, it was clearly shorter than the culture TAT (6.2 days). Introducing rt-PCR in regional hospitals in Oman will likely improve the TAT, and result in timely accurate diagnosis of pertussis.

Our results showed that rt-PCR is a sensitive, fairly specific, and rapid test for diagnosing *B. pertussis*. Although our results did not show additional benefits for culture in pertussis diagnosis compared with rt-PCR, culture might still be helpful for the surveillance of circulating strains and for performing antimicrobial susceptibility testing when clinically indicated.

Although Oman has a high vaccination coverage (5), a high number of pertussis cases are still reported, particularly among infants. In fact, adolescents and adults who have not received tetanus-diphtheria-pertussis (Tdap) booster vaccinations can become infected or re-infected as immunity from childhood vaccination and natural disease wanes with time. Atypical presentation might be seen in this population as well as a high potential of missing the diagnosis (20). These individuals might be the source of infection for infants too young to be vaccinated and who are at the highest risk of severe complications and death. This makes an accurate and fast test to diagnose pertussis essential.

The strengths of our study include the large number of samples included, the use of a three-target multiplex PCR rather than a singleplex PCR, and the clinical correlation done for the discrepant results. However, it has some limitations including the retrospective design of the study, which led to the unavailability of some clinical information required in the clinical correlation.

Conclusion and Recommendations

rt-PCR is a highly sensitive and specific test for the diagnosis of *B. pertussis*. We recommend it to be part of the diagnostic tests for all suspected pertussis cases. Based on these results, we recommend setting up a PCR diagnostic facility in regional hospitals in Oman as this will lead to the timely and accurate diagnosis of pertussis.

Conflicts of Interest

The authors declare no conflict of interest.

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Disclosure

The abstract was presented as a poster in Oman Medial Specialty Board (OMSB) research and career day 2017/2018, and the abstract was also published in OMJ (2018), Vol.33, No. 1 under OMSB career and research forum 2018: Abstracts.

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