Detection of respiratory bacterial pathogens causing atypical pneumonia by multiplex Lightmix® RT-PCR

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ABSTRACT

Pneumonia is a severe infectious disease. In addition to common viruses and bacterial pathogens (e.g. *Streptococcus pneumoniae*), fastidious respiratory pathogens like *Chlamydia pneumoniae*, *Mycoplasma pneumoniae* and *Legionella* spp. can cause severe atypical pneumonia. They do not respond to penicillin derivatives, which may cause failure of antibiotic empirical therapy. The same applies for infections with *B. pertussis* and *B. parapertussis*, the cause of pertussis disease, that may present atypically and need to be treated with macrolides. Moreover, these fastidious bacteria are difficult to identify by culture or serology, and therefore often remain undetected. Thus, rapid and accurate identification of bacterial pathogens causing atypical pneumonia is crucial. We performed a retrospective method evaluation study to evaluate the diagnostic performance of the new, commercially available Lightmix® multiplex RT-PCR assay that detects these fastidious bacterial pathogens causing atypical pneumonia. In this retrospective study, 368 clinical respiratory specimens, obtained from patients suffering from atypical pneumonia that have been tested negative for the presence of common agents of pneumonia by culture and viral PCR, were investigated. These clinical specimens have been previously characterized by singleplex RT-PCR assays in our diagnostic laboratory and were used to evaluate the diagnostic performance of the respiratory multiplex Lightmix® RT-PCR. The multiplex RT-PCR displayed a limit of detection between 5 and 10 DNA copies for different in-panel organisms and showed identical performance characteristics with respect to specificity and sensitivity as in-house singleplex RT-PCRs for pathogen detection. The Lightmix® multiplex RT-PCR assay represents a low-cost, time-saving and accurate diagnostic tool with high throughput potential. The time-to-result using an automated DNA extraction device for respiratory specimens followed by multiplex RT-PCR detection was below 4 h, which is expected to significantly improve diagnostics for atypical pneumonia-associated bacterial pathogens.

1. Introduction

Community-acquired pneumonia (CAP) is a severe infectious disease. The most common causative agents include viruses (like influenza) and bacterial pathogens (like *Streptococcus pneumoniae* and *Haemophilus influenzae*) (WHO, 2016). Besides these "common" causes of CAP, also fastidious bacterial pathogens (like *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Legionella pneumophila* and *Coxiella burnetii*) can cause so-called atypical pneumonia that accounts for up to 15% of all CAP cases (Cunha, 2006). Reliable detection of agents of atypical pneumonia is crucial as they do not respond to beta-lactams (Sharma et al., 2016; Lamoth and Greub, 2010). Therefore, empiric treatment

with penicillin derivatives will be ineffective for their eradication if no additional antibiotics (e.g. macrolides) are administered to the patient (Bennett et al., 2014). Still, the detection of atypical bacterial agents of CAP by culture or serology remains a challenge. Especially for *C. pneumoniae* detection, serology shows low sensitivity and insufficient specificity, making this test clinically useless (Wellinghausen et al., 2006). Rapid and accurate molecular methods are required for fast identification of bacterial pathogens causing atypical pneumonia and to subsequently adapt the antibiotic regimen of the patients.

In this study, we present the first evaluation of the multiplex Lightmix® RT-PCR for simultaneous detection of the most important bacterial pathogens causing atypical pneumonia, namely *Bordetella*

parapertussis, *Bordetella pertussis*, *Chlamydia pneumoniae*,[1] *Chlamydia psittaci*, *Legionella* spp., *Legionella pneumophila* and *Mycoplasma pneumoniae* in clinical respiratory specimens.

Among the bacterial pathogens, the majority of CAP in children and young adults is caused by *M. pneumoniae* resulting in mild to life-threatening infections (Lieberman et al., 1996). For specific multiplex RT-PCR detection, the repetitive element *repMp1* was chosen as target. Compared to single-copy targets, like the *P1* adhesin gene, *repMp1* enables more sensitive detection of *M. pneumoniae* (Dumke et al., 2007).

L. pneumophila is an opportunistic bacterial pathogen that causes severe pneumonia, especially in elderly and immunocompromised patients (Yu, 2000). The multiplex RT-PCR targets the 16S rRNA gene and allows unambiguous differentiation of *L. pneumophila* from other *Legionella* spp. by melting curve analysis.

B. pertussis and a specific lineage of *B. parapertussis* infect humans, resulting in the typical symptoms of paroxysmal cough and whooping. Many countries recently reported an increase of pertussis cases, especially in infants and adolescents that are unimmunized or incompletely immunized with the pertussis vaccine (Heininger et al., 2014). Moreover, mixed outbreaks and co-infections with *B. parapertussis* and *B. pertussis* have been reported (Zouari et al., 2012). To maximize sensitivity, the multiplex RT-PCR targets the insertion sequences *IS481* (50–100 copies per cell) and *IS1001* (35–50 copies per cell) for specific detection of *B. pertussis* and *B. parapertussis*, respectively.

C. pneumoniae is an obligate intracellular pathogen that accounts for up to 10% of CAP cases and can be responsible for long-lasting disease (Kuo et al., 1995). Although less common in recent series, *C. pneumoniae* still regularly causes small outbreaks (Asner et al., 2014). Noteworthy, *C. pneumoniae* infection may sometimes mimic inaugural asthma (Asner et al., 2014; Webley and Hahn, 2017) and thus may remain undetected, since not tested. The multiplex RT-PCR targets the single-copy gene *ompA* that encodes the *C. pneumoniae* major outer membrane protein (MOMP) (Kuo et al., 1995).

Atypical pneumonia may also be caused by zoonotic transmission of respiratory pathogens (e.g. Schack et al., 2014). *C. psittaci* is mostly transmitted by domestic birds and causes psittacosis in humans leading to potentially life-threatening disease (Smith et al., 2011). In addition, poultry, wild bird species and even horses have also been reported to transmit *C. psittaci* to humans, leading to single cases of infections or even outbreaks (Smith et al., 2011; Rehn et al., 2013; Huijskens et al., 2016). Thus, in order to prevent underdiagnosis, the analysis of respiratory samples from patients with atypical pneumonia should include detection of *C. psittaci*. The multiplex RT-PCR targets the *rpoB* gene encoding the β-subunit of RNA polymerase that provides high taxonomic resolution and thus facilitates unambiguous *C. psittaci* detection (Adékambi et al., 2009).

In this study, the diagnostic performance of the new multiplex Lightmix® RT-PCR assay was evaluated for the detection of bacterial pathogens causing atypical pneumonia.

2. Materials and methods

2.1. Clinical specimens, DNA extraction and singleplex RT-PCR assays used in routine diagnostics

Respiratory clinical specimens (including sputum, *bronchoalveolar* lavage, respiratory swabs, *tracheal* and *nasopharyngeal secretions)* from patients with symptoms of atypical pneumonia were sent to our diagnostic laboratory for analysis (N = 355). These clinical specimens have been previously analysed by culture and viral PCR on the presence of

typical agents of CAP (data not shown) and were found to be negative. We received extracted DNA from respiratory specimens (N = 13) that were tested positive for *C. psittaci* and *C. pneumoniae*, respectively from the University Hospital of Lausanne, Switzerland.

One ml of the respiratory sample was centrifuged (10 min, 14,000 rpm) and the supernatant was removed. Subsequently, 195 μl digest buffer and 5 μl proteinase K were added to the pellet and incubated on a thermomixer (2 h, 56 °C, 1000 rpm). DNA from clinical specimens was extracted on the EZ1 Advanced XL (QIAGEN, Hombrechtikon, Switzerland) using the EZ1 DNA Tissue Kit (QIAGEN, Hombrechtikon, Switzerland), following the manufacturer's instructions. Respiratory pathogens were detected by singleplex in-house RT-PCR assays (SI Methods, Table S1). DNA from the samples was stored at −20 °C for further analysis.

2.2. Respiratory multiplex Lightmix® RT-PCR assay and melting curve analysis

We used DNA from respiratory specimens (N = 368) that has been previously tested positive for at least one in-panel organism by singleplex RT-PCR and retrospectively analysed it by multiplex Lightmix® RT-PCR. All Lightmix® primers and probes (TIBMolbiol, Berlin Germany) used in this study are commercially available and are currently labelled "for research use only". For multiplex RT-PCR, extracted DNA was added to a mixture consisting of PCR grade water (Roche, Rotkreuz, Switzerland), a LightCycler® DNA multiplex master mix (Roche) and the Lightmix® primers and probes (TIBMolbiol). RT-PCR mastermix composition and the LightCycler® (LC) amplification protocol were in accordance with the guidelines provided by TIBMolbiol. The RT-PCR was performed using a LightCycler480-II® (for more information see SI Methods).

2.3. Analytical performance of the respiratory multiplex Lightmix® RT-PCR

Bronchoalveolar lavage (BAL) specimens that were tested negative in the singleplex RT-PCR assays were pooled and extracted on the EZ1 Advanced XL (QIAGEN), according to the manufacturer's instructions using the EZ1 DNA Tissue Kit (QIAGEN). Analytical sensitivity for each bacterial pathogen was determined by serial dilution of the respective positive control plasmid (5–1000 DNA copies per RT-PCR reaction; Fig. S1) in the BAL extracts. This allowed the generation of a standard curve and the quantification of positive samples (Fig. S1). The intra-run reproducibility was assessed in duplicates using the respective positive control plasmid diluted in the BAL extracts (5–1000 DNA copies per RT-PCR reaction; Fig. S2 and Table S2). To determine the inter-assay variability, 6 clinical respiratory samples were analysed by 3 different technicians on 3 consecutive days (Table S3).

2.4. Performance characteristics of the respiratory multiplex RT-PCR in comparison to in-house RT-PCRs

To assess the performance of the multiplex RT-PCR, bacterial strains that are commonly found in respiratory samples or that are part of the human microbial flora and reference strains of 8 *Legionella* spp. were analysed, respectively (Table 2 and Table S4). The 8 *Legionella* spp. were chosen based on 16S rDNA diversity and expert opinion. Furthermore, we investigated the performance of the multiplex RT-PCR with clinical respiratory specimens that have been previously characterized by singleplex in-house RT-PCR assays (N = 368) (Fig. 1 and Table 1).

2.5. Statistical methods

To determine differences in diagnostic performance of the singleplex and multiplex RT-PCR, K-statistics (Cohen, 1960) and the Bland and Altmann plot (Bland and Altman, 1995) were used. The inter-assay

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[1] In this study, the genus name *Chlamydia* and not *Chlamydophila* is used throughout for *C. pneumoniae* and *C. psittaci* according to the most recent taxonomic classification and expert opinion (Gilbert Greub; Bavoil P, Kaltenboeck B, Greub G. In *Chlamydia* veritas. Pathog Dis 2013;67:89-90. doi: 10.1111/2049-632X.12026).
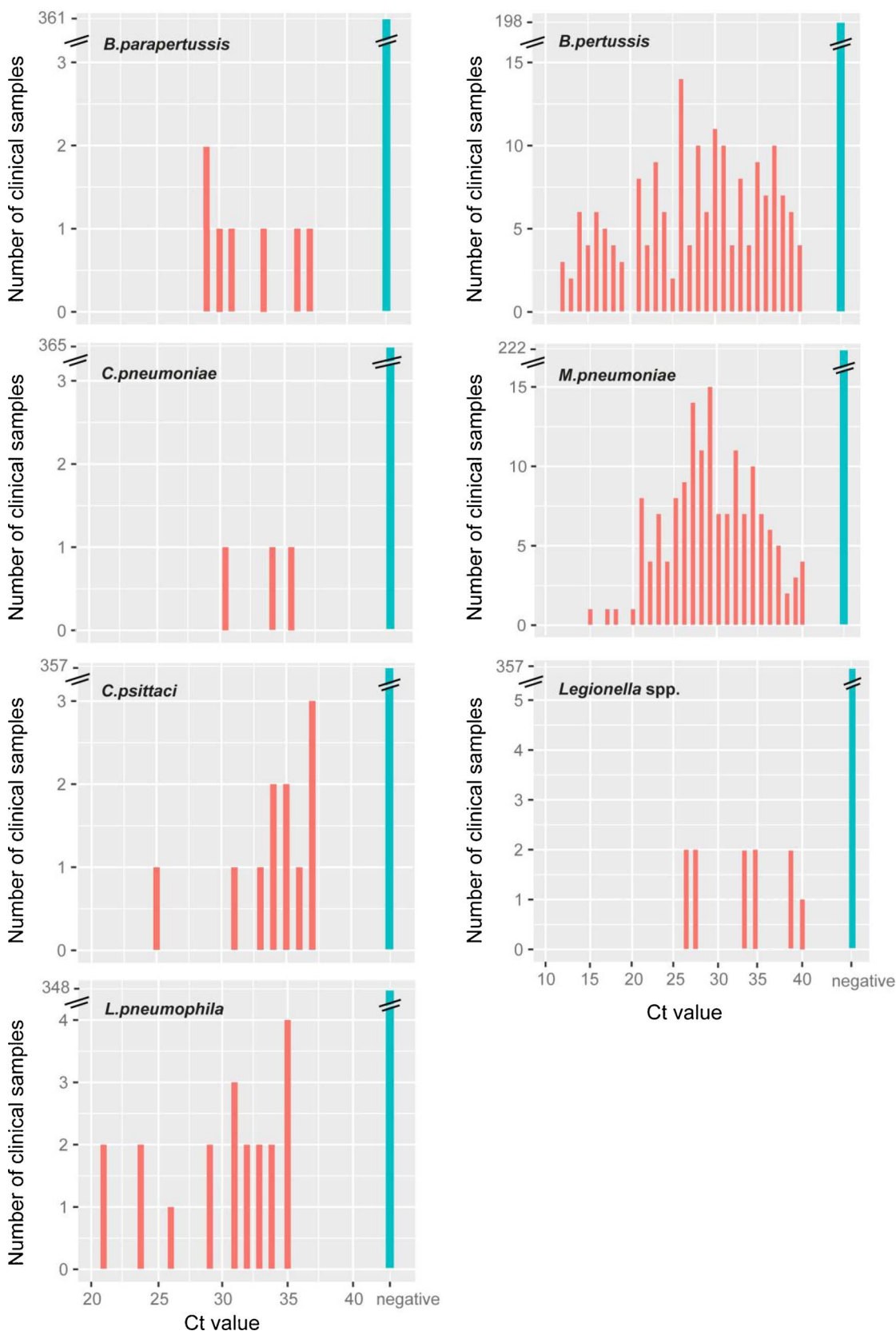
Fig. 1. Detection of respiratory pathogens by multiplex Lightmix® RT-PCR in clinical specimens (N = 368) and the corresponding cycling threshold (Ct) values.

and intra-assay variability (i.e. variation coefficients) were calculated by dividing the standard deviation of the cycling threshold (Ct) values from the replicates in the multiplex RT-PCR by the mean of the Ct values from the replicates. All data analysis and data visualization was done in R (Team R, 2011).

Table 1
Detection of atypical respiratory pathogens in clinical specimens by multiplex Lightmix® RT-PCR and agreement of singleplex RT-PCR vs. multiplex RT-PCR results (ƙ statistics).

B. parapertussis			Singleplex RT-PCR		
			Negative	Positive	
Multiplex Lightmix® RT-PCR		Negative	361	0	K = 1
		Positive	0	7	
B. pertussis			Singleplex RT-PCR		
			Negative	Positive	
Multiplex Lightmix® RT-PCR		Negative	198	0	K = 1
		Positive	0	170	
C. pneumoniae			Singleplex RT-PCR		
			Negative	Positive	
Multiplex Lightmix® RT-PCR		Negative	365	0	K = 1
		Positive	0	3	
C. psittaci			Singleplex RT-PCR		
			Negative	Positive	
Multiplex Lightmix® RT-PCR		Negative	357	0	K = 1
		Positive	0	11	
L. pneumophila			Singleplex RT-PCR		
			Negative	Positive	
Multiplex Lightmix® RT-PCR		Negative	348	0	K = 1
		Positive	0	20	
Legionella spp.			Singleplex RT-PCR		
			Negative	Positive	
Multiplex Lightmix® RT-PCR		Negative	357	0	K = 1
		Positive	0	11	
M. pneumoniae			Singleplex RT-PCR		
			Negative	Positive	
Multiplex Lightmix® RT-PCR		Negative	222	0	K = 1
		Positive	0	146	

Table 2
Bacterial strains used for determining the specificity of the multiplex Lightmix˙ RT-PCR.

Species		RT-PCR Result
Corynebacterium spp.	*C. amycolatum*	−
	C. propinquum	−
	C. imitans	−
	C. pseudodiphtheriticum	−
Escherichia spp.	*E. coli*	−
Staphylococcus spp.	*S. aureus*	−
	S. caprae	−
	S. epidermidis	−
	S. gallinarum	−
	S. haemolyticus	−
	S. hominis	−
	S. lugdunensis	−
	S. pettenkoferi	−
	S. warneri	−
Streptococcus spp.	*S. anginosus*	−
	S. constellatus	−
	S. bovis	−
	S. gallolyticus	−
	S. gordonii	−
	S. intermedius	−
	S. mitis	−
	S. mutans	−
	S. oralis	−
	S. salivarius	−
Bordetella spp.	*B. holmesii*	+
	B. bronchiseptica	−
Chlamydia spp.	*C. trachomatis*	−
	C. abortus	−

2.6. Ethics

The study was conducted according to good laboratory practice and in accordance with the Declaration of Helsinki and national and institutional standards.

3. Results

3.1. Analytical performance of the multiplex Lightmix® RT-PCR

The analytical sensitivity of the multiplex RT-PCR was evaluated using serial dilutions (5–1000 DNA copies per RT-PCR reaction) of the positive control plasmids in negative BAL specimens. For all respiratory pathogens analysed, the limit of detection (LOD) was determined to be between 5 and 10 DNA copies per RT-PCR reaction (Fig. S1). The intra-run variability was assessed by plotting the Ct values of sample replicates analysed in the same RT-PCR run. The R^2 was excellent for each respiratory pathogen (i.e. $R^2 \geq 0.95$) (Fig. S2), and the variation coefficient of the intra-run variability was < 3% (Table S2). The inter-run variability was < 5% indicating high reproducibility of the multiplex RT-PCR results independent of the analyst performing the assay (Table S3).

3.2. Performance characteristics of the respiratory multiplex RT-PCR in comparison to in-house RT-PCRs

In total, 368 clinical respiratory specimens were analysed by multiplex RT-PCR. Seven samples tested positive for *B. parapertussis*, 170 for *B. pertussis*, 3 for *C. pneumoniae*, 11 for *C. psittaci*, 20 for *L. pneumophila*, 11 for *Legionella* spp. and 146 for *M. pneumoniae*, respectively (Table 1 and Fig. 1). Moreover, in two patients a co-infection of *B. pertussis* and *B. parapertussis* was observed (Fig. S3). This underlines the good diagnostic performance of the multiplex RT-PCR, also for clinical samples with more than one bacterial pathogen present (Fig. S3). In all 368 clinical specimens, the multiplex RT-PCR detected the same pathogen as the corresponding singleplex RT-PCR that is used in our ISO accredited diagnostic laboratory. K-statistics (Cohen, 1960) showed perfect agreement between the singleplex and the multiplex RT-PCR for the detection of all respiratory pathogens (Table 1). The Bland and Altman plot (Bland and Altman, 1995) showed that Ct values of the singleplex and the multiplex RT-PCR were evenly scattered above and below zero, revealing no bias of one approach versus the other (Fig. 2). In both, the singleplex and the multiplex RT-PCR, 5 Ct values were outside the 1.96 SD of the mean (Fig. 2).

To determine the specificity of the multiplex RT-PCR, different bacterial isolates were analysed that all tested negative, except *B. holmesii* that showed a positive amplification signal with the *B. pertussis* Lightmix® primer/probe set (Table 2). This is based on the fact that the *IS481* insertion sequence is also present in some *B. holmesii* isolates (Pittet et al., 2014).

3.3. Discrimination of L. pneumophila from other Legionella spp.

All *Legionella* spp. analysed showed a positive amplification signal in the multiplex RT-PCR and could thereby be clearly identified as positive. In melting curve analysis, all *L. pneumophila* serogroups showed a peak at 62 °C and enabled their unambiguous differentiation from other *Legionella* spp. (Fig. 3 and Table S4). Different *Legionella* spp. displayed either no peak or a peak between 48 °C and 55 °C (Table S4), respectively.

4. Discussion

We investigated the diagnostic performance of a commercial multiplex RT-PCR that targets therapeutically relevant bacterial agents of atypical pneumonia. In general, accuracy and reproducibility of the multiplex RT-PCR results were very good. Moreover, analytical sensitivity and specificity of the multiplex RT-PCR was equal to the singleplex in-house RT-PCRs that are used in our accredited diagnostics laboratory. Despite excellent specificity of the multiplex RT-PCR, we obtained one false-positive result with a sample containing *B. holmesii*. This owes to the fact that most PCR assays used in routine diagnostics
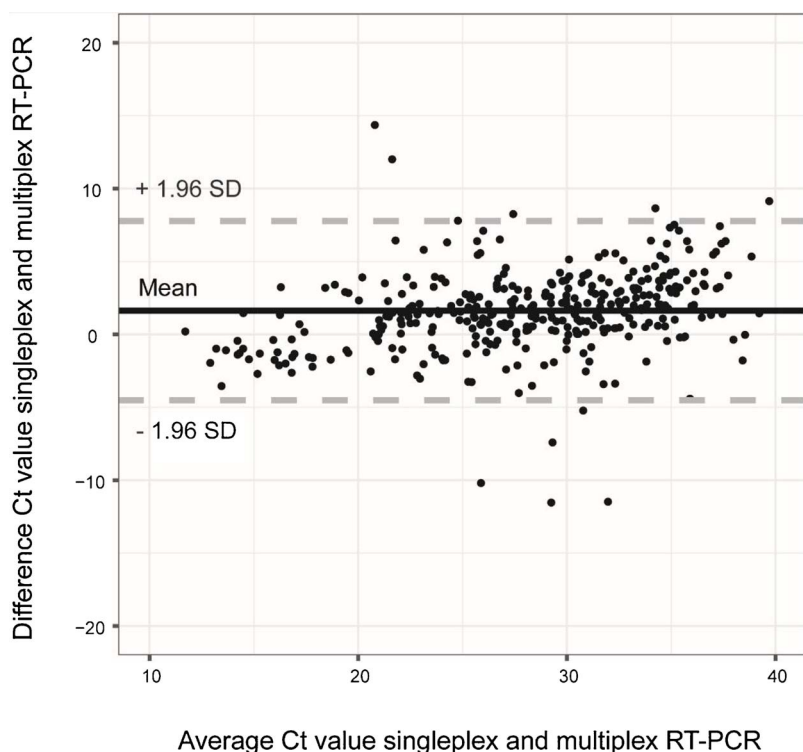
Fig. 2. Bland Altman Plot comparing the cycling threshold (Ct) values from the singleplex and multiplex RT-PCRs.
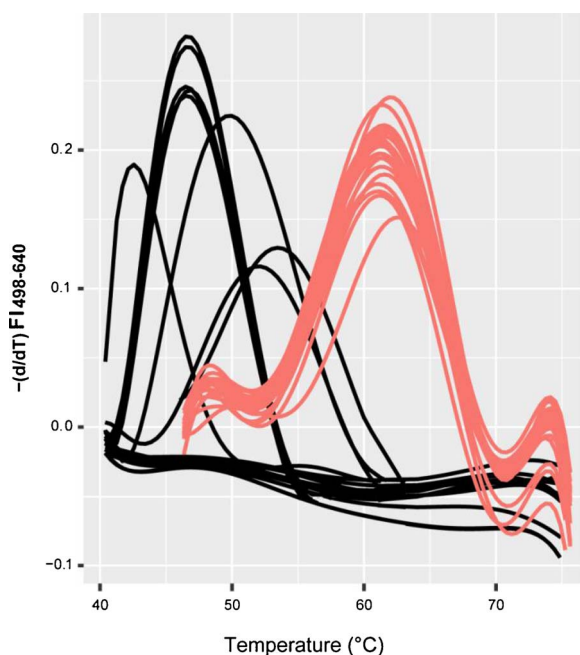

Fig. 3. *L. pneumophila* (red) showed a distinct melting temperature of 62 °C, whereas *Legionella* spp. (black) showed either no melting curve or a melting temperature lower 55 °C in melting curve analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

for *B. pertussis* detection target the insertion sequence *IS481* (Dalby et al., 2013), which is also present in some *B. holmesii* isolates. To circumvent this problem, respiratory specimens with a positive *B. pertussis* amplification signal in the multiplex RT-PCR could be subjected to a second RT-PCR assay that targets genes exclusively found in *B. pertussis*, like the pertussis toxin gene (Sloan et al., 2002). However, since these genes, unlike *IS481*, are single-copy targets, RT-PCR is less sensitive, and therefore may lead to false-negative results (Sloan et al., 2002). The 16S rRNA gene is also not an appropriate discriminator since *B. holmesii*

shares more than 99% sequence similarity in the 16S rRNA sequence with *B. pertussis* (Gross et al., 2010). In summary, we consider the utilization of *IS481* as an appropriate target for the detection of *B. pertussis* as its high sensitivity outweighs the limitations in specificity.

The multiplex RT-PCR allowed a very accurate differentiation between *L. pneumophila* and other, potentially apathogenic *Legionella* spp. Most cases of Legionnaires' disease reported are associated with *L. pneumophila*, especially serotype 1 (Fields et al., 2002). This may reflect a diagnosis bias, because most commercially available kits exclusively detect *L. pneumophila* serotype 1 antigen in urine specimens. The Lightmix® multiplex RT-PCR allowed identification of different *L. pneumophila* serotypes and *Legionella* spp. Some *Legionella* spp. showed no T_m peak in melting curve analysis. This may be explained by the fact that some *Legionella* spp. show basepair mismatches with the Lightmix® genus probe used in the assay. This does not pose a problem for the analysis, as *L. pneumophila* can readily be discriminated from other *Legionella* spp. by showing a peak at 62 °C. If identification of *Legionella* spp. other than *L. pneumophila* is required, 16S rDNA sequencing may be used for species identification. However, when 16S rDNA sequencing from specimens with Ct values higher 30 in the multiplex RT-PCR was performed, interpretation of the Sanger electropherograms was often not possible (i.e. weak peaks or multiple overlaying peaks in the electropherograms).

Various multiplex panels for simultaneous detection of diverse pathogens causing pneumonia have been developed like the Unyvero® P55 (Curetis AG, Holzgerlingen, Germany), NxTAG® Respiratory Pathogen Panel (Luminex, Hertogenbosch, The Netherlands), RespiFinder® 22 (PathoFinder, Maastricht, The Netherlands) or Seeplex® PneumoBacter ACE Detection (Seegene Inc., Seoul, South Korea) (e.g. Babady, 2013; Beckmann and Hirsch, 2016; Ozongwu et al., 2017). However, these are mostly syndromic assays and costly, often needing test-specific equipment and consumables that are not aimed for high-throughput screening in molecular diagnostic laboratories. In addition, such test panels often include viral agents without specific therapeutic consequences. Moreover, the application of highly multiplexed assays as first-line tests in diagnostics of CAP is subject of controversial discussion (Schreckenberger and McAdam, 2015) since the mere presence of

an organism does not necessarily indicate its contribution to disease. Many bacterial and viral agents with pathogenic potential in the lung may also be present in the upper respiratory tract of healthy individuals in a carriage state or may be detected after resolution of infection due to prolonged shedding (e.g. Skevaki et al., 2015; Kumar et al., 2008). Therefore, asymptomatic carriage of viruses and bacterial pathogens (e.g. *S. pneumoniae*) in the upper respiratory tract poses a diagnostic problem. In contrast, no carriage of bacterial pathogens causing atypical pneumonia was found in respiratory secretions (Kumar et al., 2008), indicating that positive test results correlate with clinical disease. However, there are also contradictory reports stating that *M. pneumoniae* may be carried asymptomatically in the upper respiratory tract of children (Sauteur et al., 2016). Therefore, it is crucial to adhere to clinical case definitions and follow clinical decision rules as RT-PCR testing is unable to differentiate *M. pneumoniae* carriage from infection (Sauteur et al., 2016).

Our study has several limitations: It was designed as single centre laboratory-based, retrospective method evaluation study with preselected clinical specimens. The total number of samples tested positive for individual in-panel organisms like *C. psittaci* and *C. pneumoniae* is low at our institute (one positive *C. pneumoniae* detection in the past 5 years). In order to correct for low positive sample counts for *C. psittaci* and *C. pneumoniae*, additional samples that were tested positive at another University Hospital (CHUV, Lausanne, Switzerland) were added to the sample set. Since our study does not reflect a true epidemiology and since positive predictive values of molecular assays depend on the prevalence of in-panel organisms, clinical specimens with a positive test result for *Chlamydia* spp. should be confirmed by an alternative assay or send to a reference laboratory for confirmatory testing.

5. Conclusion

In conclusion, the multiplex Lightmix® RT-PCR provides a reliable tool to overcome underdiagnosis of atypical pneumonia, allowing improved, targeted therapy and application of appropriate infection control measures. The multiplex Lightmix® RT-PCR showed a good diagnostic performance that is comparable to singleplex in-house RT-PCR assays with respect to specificity and sensitivity. Moreover, the multiplex Lightmix® RT-PCR enabled accurate detection of *B. pertussis* and *B. parapertussis* in co-infected patients. We propose a diagnostic workflow with low per sample costs using an automated DNA extraction device (e.g. QIASymphony) and multiplex Lightmix® RT-PCR detection. Up to 24 samples can be analysed in parallel in this workflow within less than 4 h. Therefore, it is suitable for high-throughput routine screening of multiple important respiratory pathogens causing atypical pneumonia.

Conflicts of interest

Lightmix® kits for the analysis of 100 samples were donated by TIBMolbiol, Berlin, Germany. For the analysis of the remaining samples (N = 268), Lightmix® kits were bought from TIBMolbiol by the Institute of Medical Microbiology, University of Zurich.

This is an investigator-initiated project and TIBMolbiol had no involvement in the design and conduct of the study, collection, management, analysis, and interpretation of the data, nor in the preparation, review, and approval of the manuscript or decision to submit the manuscript. All authors declare no conflict of interests.

Acknowledgement

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijmm.2018.01.010.

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