Rapid and combined detection of *Mycoplasma pneumoniae*, Epstein–Barr virus and human cytomegalovirus using AllGlo quadruplex quantitative PCR

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Acute respiratory infections (ARIs) cause substantial morbidity and mortality worldwide. The causes of ARI are dynamic, and co-infections of *Mycoplasma pneumoniae*, Epstein–Barr virus and human cytomegalovirus are recently developed causes of ARI. Here, we established a quadruplex quantitative PCR (qPCR) method to rapidly identify and simultaneously detect a single infection or co-infection of these three pathogens and an internal control in a single tube using AllGlo probes. The analysis demonstrated a wide linear range of detection from $10^1$ to $10^8$ copies per test and a low coefficient of variation of less than 5%. The amplification efficiencies were all close to 1, and the correlation coefficients ($r^2$) were all greater than 0.99. We found no significant difference in a comparative reagent test ($P>0.05$). Moreover, the results of tests on clinical samples using AllGlo quadruplex qPCR and TaqMan uniplex qPCR were in near-perfect agreement ($\kappa = 0.97$). Clinically, the availability of this method will enable better differential diagnosis, disease surveillance and controlled outcomes.

INTRODUCTION

Acute respiratory infections (ARIs) are a common cause of morbidity and mortality among adults and children, and the aetiology of ARI is dynamically changing (Verani et al., 2013; Zumla et al., 2014). During the past decade, studies have demonstrated that mycoplasma and viral infections are increasing (Jain et al., 2015; Rasmussen et al., 2010; Wallihan & Ramilo, 2014). *Mycoplasma pneumoniae* (MP), Epstein–Barr virus (EBV) and human cytomegalovirus (HCMV) are common pathogens of respiratory infections (Li et al., 2014). EBV and HCMV frequently cause infectious mononucleosis (IM) accompanied by respiratory tract infections (Grilli et al., 2012; Restrepo-Gualteros et al., 2014; Wang et al., 2010; Xiong et al., 2014). In contrast, MP is characterized by acute onset, rapid progress and long course (Meyer Sauteur et al., 2014; Waites & Talkington, 2004). Studies have indicated that co-infections between MP, EBV or HCMV can lead to severe clinical consequences, so that there is a particular need to vigilantly monitor IM-like symptoms (Huijskens et al., 2014; Ito et al., 2009; Klein et al., 2013; Li et al., 2014; Meyer Sauteur et al., 2014; Narita, 2010; Schneider et al., 2013; Wang et al., 2010). Because co-infection often cannot be excluded, single-pathogen detection is likely to cause misdiagnoses and missed diagnoses. Therefore, a reliable, sensitive and rapid test that can simultaneously detect and differentiate among MP, EBV and HCMV should be established to aid in diagnosis.

Clinically, a correct aetiological diagnosis of MP, EBV or HCMV infection relies heavily on laboratory examination, including microbiological culture, serology, quantitative PCR (qPCR) and so on (Cannon et al., 2010; Daxboeck et al., 2003; Reddington et al., 2013). To our knowledge, the methods based on serology and qPCR are widely used to detect these pathogens at present. However, owing to their high sensitivity, specificity and throughput, qPCR-based assays are increasingly being adopted.

Advances in technology have allowed the development of real-time qPCR. Nevertheless, in current reports of qPCR methods three pathogens (MP, EBV and HCMV) and an internal
control in a single tube have not yet been simultaneously detected. In this study, novel AllGlo probes (Alle Logic Biosciences), which yield higher sensitivity and specificity than traditional TaqMan probes (Bai et al., 2014; Yu et al., 2012; Zhang et al., 2014), were designed and used to develop a multiplex qPCR method that rapidly, reliably and simultaneously detected and identified the three pathogens in a single tube in clinical samples. Importantly, we introduced a human housekeeping gene, GAPDH, as an internal control to strengthen the surveillance and control of detection quality. Based on these results, the AllGlo quadruplex qPCR assay is worth optimizing and improving for clinical application to meet the needs relating to the aetiological diagnosis of co-infection.

**METHODS**

**Clinical specimens and DNA template extraction.**

This study was approved by the Ethics Review Committee of Hangzhou First People’s Hospital. With permission from the outpatients and inpatients, 300 clinical specimens, including 130 sputum specimens and 170 nasopharyngeal swab specimens, were collected from Hangzhou First People’s Hospital, China. According to Yu et al. (2012), DNA was extracted from all samples (details are given in S1.1, available in the online Supplementary Material).

**Primer and probe design.**

Conserved gene sequences of MP, EBV, HCMV and GAPDH were downloaded from GenBank. We designed the primers and probes using Primer Premiers 5.0 software. Primers and probes with the best specificity and similar melting temperatures ($T_m$) were selected based on a blast sequence comparison (Table 1). The primers used in the multiplex qPCR were synthesized by Life Technology, and the AllGlo probes were synthesized by Huirui Bio.

**Construction of standard plasmids.**

To confirm the anticipated sizes of the MP, EBV, HCMV and GAPDH target DNA, which were extracted from sputum specimens, the PCR products were detected in a 2.0% agarose gel after electrophoresis. Next, the products were purified and cloned into the plasmid pMD19-T (the plasmid and products were ligated at 16°C for 30 min). The recombinant plasmids were transformed into E. coli DH5α competent cells, and plated on Luria–Bertani agar plates including 100 µg ml$^{-1}$ ampicillin and 60 µg ml$^{-1}$ X-Gal. Plasmid DNA was extracted and purified using a mini purification kit (TaKaRa Bio) according to the manufacturer’s instructions. The recombinant plasmid inserts were then submitted for sequencing by Life Technology. The quality and concentration of the plasmid DNA samples were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Finally, 10-fold serial dilutions of the four plasmid DNA templates and a mixed plasmid template containing MP, EBV, HCMV and an internal control (concentration ratio 1:1:1:1) were prepared for use as DNA quantification standards at concentrations from $10^1$ copies per test to $10^8$ copies per test. These standards were divided into aliquots and stored at ~80°C for subsequent use.

**AllGlo multiplex real-time qPCR.**

**Optimization of the quadruplex qPCR conditions.** The annealing temperature for quadruplex qPCR detection was set to 60°C based on the results of the uniplex qPCR optimization experiment (S1.2). We also selected two primer/probe combinations and concentrations (200/200 and 200/100 nM) that had performed better than the other tested combination (S1.2) for use in the quadruplex qPCR. Single-plasmid templates (MP, EBV, HCMV or GAPDH) and a mixed-plasmid template (containing MP, EBV, HCMV and GAPDH) were used to further determine the optimal quadruplex qPCR conditions and the specificity and sensitivity of the primers and probes. Other conditions of quadruplex

<table>
<thead>
<tr>
<th>Table 1. Primers and probes for the AllGlo qPCR</th>
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<tbody>
<tr>
<td><strong>Gene ID</strong></td>
</tr>
<tr>
<td>M10593.1</td>
</tr>
<tr>
<td> </td>
</tr>
<tr>
<td> </td>
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<td> </td>
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<tr>
<td>Z33395.1</td>
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<td> </td>
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<td>NG_009329.4</td>
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<td> </td>
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<td> </td>
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<td> </td>
</tr>
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</table>

*GenBank.
†MAR, URA, NEP and JUP are four kinds of different AllGlo probe fluorochromes, and correspond to the currently used FAM, ROX, CY5 and VIC fluorescence, respectively.
qPCR were set equal to those used in the uniplex qPCR. Following these experiments, an orthogonal experiment was designed to explore the optimal parameters (dNTPs, Mg\(^{2+}\) and Hot Start Taq DNA polymerase) of a homemade quadruplex qPCR Mix (S1.3).

**Indicator Analysis for the quadruplex qPCR.** The indicators of the quadruplex qPCR noted by Yu et al. (2012), including sensitivity, specificity, repeatability and standard curves, were analysed (details in S1.4).

**Comparison of TaKaRa and Vazyme reagents.** Mixed plasmid templates containing MP, EBV, HCMV and an internal control (concentration ratio 1:1:1:1) at final concentrations (in copies per test) of \(10^8\), \(10^5\) and \(10^3\) were divided into six aliquots for each sample concentration. The quadruplex qPCR was performed in triplicate for each reagent pair at different concentrations with 4 µl DNA template to compare the TaKaRa and Vazyme (Vazyme Biotech) reagents under the same conditions. We analysed the data by comparing the \(C_t\) (cycle threshold) values obtained using the two reagents to obtain experimental results that were more reliable.

**Detection of clinical samples.** DNA templates obtained from 300 clinical specimens that had been tested previously were each divided into five aliquots. One aliquot was used for the detection of MP, EBV and HCMV using our AllGlo quadruplex qPCR method, three aliquots were tested using TaqMan uniplex qPCR (Da’an Reagent; Guangzhou Da’an Diagnostic) for MP, EBV or HCMV, and the last aliquot was stored at \(-80\) °C for review. For both methods, every test was performed in triplicate using 4 µl DNA template, and standard curves were constructed at the same time. The clinical sample test results obtained using AllGlo quadruplex qPCR and TaqMan uniplex qPCR were then compared with each other (Table 2), and statistical methods were used to assess the consistency of the results of the two methods. Finally, a retrospective analysis of clinical information was made to evaluate the diagnostic value of AllGlo quadruplex qPCR.

**Statistical analysis.**

SPSS software (v19.0) was used for the statistical analysis. ANOVA was applied to the orthogonal design experiment. The Student–Newman–Keuls (SNK) test was used to determine the optimal combinations and concentrations of dNTPs, Mg\(^{2+}\) and Taq DNA polymerase. The reagents were compared using a paired-samples \(t\)-test combined with a Bland–Altman analysis. The kappa (κ) statistic and McNemar’s test were analysed to compare the two methods for clinical sample detection. The mean of the \(C_t\) values of the uniplex qPCR and quadruplex qPCR using different plasmid templates at each concentration were compared using one-way ANOVA and the SNK test. Differences were considered statistically significant when the \(P\)-value was <0.05 (two-sided).

**RESULTS**

**Optimum parameters for AllGlo qPCR**

An annealing temperature of 60 °C was found to be suitable for multiplex detection in this study. The 200/100 nM (primer/probe) combination was considered the best combination in both the uniplex qPCR and the quadruplex qPCR because it presented the lowest \(C_t\) value and the highest gain in fluorescence. Based on the lowest \(C_t\) value, the optimum combination of dNTPs, Mg\(^{2+}\) and Hot Start Taq DNA polymerase for the quadruplex qPCR was obtained

**Table 2. Clinical sample detection using AllGlo quadruplex qPCR and TaqMan uniplex qPCR**

<table>
<thead>
<tr>
<th>Method</th>
<th>Total</th>
<th>MP*</th>
<th>EBV*</th>
<th>HCMV*</th>
<th>MP(\dagger), EBV</th>
<th>MP(\dagger), HCMV</th>
<th>EBV(\dagger), HCMV</th>
<th>No. negative samples</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan</td>
<td>130</td>
<td>43</td>
<td>41</td>
<td>20</td>
<td>18</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>170</td>
</tr>
<tr>
<td>AllGlo</td>
<td>133</td>
<td>42</td>
<td>43</td>
<td>20</td>
<td>19</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>167</td>
</tr>
</tbody>
</table>

*Single infection.

†Co-infection.

**Fig. 1. Bland–Altman analysis for comparing the detection results (\(C_t\) values) between the TaKaRa and Vazyme reagents.** The \(x\)-axis shows the detection results (\(C_t\) values) of the two reagents (TaKaRa and Vazyme) for a paired test at the same concentration. The black dashed line representing the mean of the differences is at zero, and the brown solid line shows the mean of the differences of the \(C_t\) values in this experiment (the two lines overlap). The upper and lower horizontal solid lines (black) indicate the acceptable limits of agreement that were strictly established for this experiment. Mixed-plasmid template final concentrations of \(10^5\), \(10^8\) and \(10^9\) copies per test were chosen for this experiment. Each paired test was performed in triplicate using AllGlo quadruplex qPCR.
Table 3. Comparison of the clinical sample detection results

<table>
<thead>
<tr>
<th></th>
<th>TaqMan uniplex qPCR</th>
<th>AllGlo quadruplex qPCR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Co-infection</td>
<td>Single infection</td>
<td>Negative</td>
</tr>
<tr>
<td>Co-infection</td>
<td>26</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Single infection</td>
<td>2</td>
<td>102</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>3</td>
<td>167</td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
<td>105</td>
<td>167</td>
</tr>
</tbody>
</table>

McNemar’s test: \( P=0.082 \). Kappa statistic: \( \kappa = 0.97, P=0.000 \).

Sensitivity, specificity and repeatability of AllGlo quadruplex qPCR assay

The sensitivity of this method was \( 10^7 \) copies per test for all detections of MP, EBV, HCMV and the internal control (Fig. S1). To verify the specificity of the test, the genomic DNAs of other human herpesviruses, including herpes simplex virus, varicella zoster virus and human herpesvirus-6, as well as a human adenovirus, that might cause similar clinical symptoms, were used as templates for amplification in this assay. As expected, MP, EBV, HCMV and the internal control were positively detected (Fig. S2), whereas the other samples yielded negative results (\( C_t > 40 \)). No apparent cross-reaction was observed in the AllGlo quadruplex qPCR. The amplifications of MP, EBV, HCMV and the internal control were obviously distinct in the single-tube multiplex qPCR assay. The coefficients of variation of the \( C_t \) values between each aliquot for MP, EBV, HCMV and the internal control were all less than 5 % (Table S2).

Standard curve preparation

The mixed standard plasmids at concentrations from \( 10^7 \) to \( 10^9 \) copies per test were used to achieve reliable standard curves using the AllGlo quadruplex qPCR assay. The amplification efficiencies for MP, EBV, HCMV and the internal control were 1.04, 1.06, 1.03 and 0.96, respectively, and the correlation coefficients \( (r^2) \) obtained were all greater than 0.99 (Fig. S3).

Comparison of reagents

No significant difference was observed between the use of TaKaRa and Vazyme reagents (mean of paired differences \( 0.00, 95 \% CI = -0.07 \) to 0.07, \( P=0.982 \)). The \( C_t \) values of detection using the two reagents are shown in Table S3. Detection results obtained using both reagents were consistent, and the maximum difference in the \( C_t \) values was less than 0.5, conforming to the acceptable limits of agreement that were strictly established for this experiment (Fig. 1).

Evaluation of clinical sample detection

The clinical sample detection results obtained using AllGlo quadruplex qPCR and using TaqMan uniplex qPCR are summarized in Table 2. The percentages of positive samples obtained from two sample types (sputum and nasopharyngeal swabs) were 55.4 and 35.9 %, respectively. Five different test results were obtained among the clinical specimens. Satisfactorily, the results obtained using the two methods were almost identical (Table 3) (\( P>0.05 \) McNemar’s test; \( P<0.05 \), kappa statistic \( \kappa = 0.97 \)). In addition, the clinical diagnostic value of AllGlo quadruplex qPCR is shown in Table S4.

DISCUSSION

In this study, we found that the positive rate of co-infections of MP, EBV or HCMV detected among clinical specimens was as high as 9.3 %, representing 21 % of all positive samples. Thus, it is essential to improve both our understanding of the impact of multiple infections of MP, EBV or HCMV in ARI and our capacity to identify non-specific clinical manifestations. These issues require the use of powerful laboratory examination tools.

AllGlo-probe-based quadruplex qPCR proved efficient in this study and offers some advantages for the rapid (2–4 h for one sample test) and simultaneous detection of MP, EBV and HCMV. In contrast, viral culture might overlook some possible pathogens, especially in multiple infections (Chen et al., 2013). Our technique not only overcomes the limitations of the serology assay (such as sensitivity) but is also quantitative and significantly simpler. In addition, optimization of the qPCR assay parameters, comparison of the reagents and evaluation of the clinical samples provide further evidence of the reliability of this method.

No cross-reactivity with other viruses was apparent in our experiment, revealing the high specificity and selectivity of the designed primers and probes. By comparing and analyzing the mean of the \( C_t \) values obtained under the optimum combination and concentration of these primers and probes, we found that the results of the uniplex qPCR and quadruplex qPCR with the single plasmid template were close (\( P>0.05 \)) and were about one \( C_t \) value less than those obtained using quadruplex qPCR with a mixed plasmid.
template at the same concentration (P<0.05) (Fig. 2). This difference was probably due to competitive effects of increasing the amount of nucleic acid templates, whether capable of participating in qPCR or not, which indicated the quadruplex qPCR reaction system with a mixed plasmid template might exhibit mutual interference among the primers and probes. However, the difference of one Ct value did not affect the sensitivity of AllGlo quadruplex qPCR and it was considered acceptable in daily clinical practice.

AllGlo technology, the latest generation of quantitative fluorescent probes, enables the creation of simple and efficient high-throughput diagnostics with good reproducibility and high sensitivity and specificity (Yu et al., 2012). This technology has been widely used for agricultural and medical studies, e.g. it was developed to evaluate predation on three rice planthoppers and detect single-nucleotide polymorphism associated with lung cancer (Cheng et al., 2012; Wang et al., 2013). Consequently, single-tube AllGlo quadruplex qPCR rather than TaqMan uniplex qPCR is expected to become widely used in early aetiological diagnosis and will play an important role in the rapid identification and simultaneous detection of MP, EBV and HCMV. Given that the test quality can be monitored using an internal control, this new method is suitable for both qualitative and quantitative detection in daily clinical practice. In this study, the clinical sensitivity of our method was 88.89% with a specificity of 96.39%. However, false-negative and -positive results did exist in our study. The clinical sensitivity of AllGlo quadruplex qPCR method was up to 95%.

In conclusion, the diagnosis of a single infection or co-infection with these three pathogens (MP, EBV and HCMV) in a single tube by multiplex qPCR is possible. This assay will improve clinical differential diagnosis capability, and allow better disease surveillance and controlled outcomes. Application of this promising technique as a screening or confirmation tool might enhance clinical decision-making during the early stage of pathogen infection, thereby reducing the overuse of antibiotics and improving patient prognosis.

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