Comparison of loop-mediated isothermal amplification and real-time PCR for detecting *Bordetella pertussis*

Whooping cough is an acute human respiratory illness caused by *Bordetella pertussis*. This illness remains a serious infectious problem in developing countries and still affects vulnerable neonates. Even developed countries are struggling to cope due to the re-emergence of pertussis in the last two decades, which is generally unexplained (Chiappini *et al.*, 2013; Pai & Kangath, 2012).

Despite the existence of a conventional culture method and the development of PCR-based assays, the accurate detection of *B. pertussis* is a problem (Probert *et al.*, 2008). Real-time PCR is utilized routinely in clinical laboratories (Lanotte *et al.*, 2011; Vincart *et al.*, 2007), but the results obtained may be difficult to interpret, whilst the expensive materials and thermal cyclers required to perform this type of analysis have prompted laboratories to consider other diagnostic methods (Kösters *et al.*, 2001; Notomi *et al.*, 2000).

Loop-mediated isothermal amplification (LAMP) is an accurate, cost-effective and rapid novel amplification method that is highly sensitive and specific (Notomi *et al.*, 2000). In a previous study in Japan, a LAMP assay was developed to detect the PT promotor region of *B. pertussis* (PT-LAMP) (Kamachi *et al.*, 2006). In the present study, we developed a LAMP assay based on insertion sequence IS481 of *B. pertussis* (IS481-LAMP). We also evaluated the efficiency, speed, sensitivity and specificity of IS481-LAMP for analysing *B. pertussis* clinical specimens and we compared its performance with real-time PCR based on the same target. Finally, we compared the performance of IS481 LAMP with that of PT-LAMP as a single-copy target.

In total, 165 nasopharyngeal swabs were cultured on Regan–Lowe medium. All samples were obtained from Iranian patients with clinical symptoms, such as prolonged coughing, post-tussive vomiting and fever. The samples were incubated for 7 days at 35–37 °C with 100 % humidity to obtain visible colonies of fastidious *Bordetella* spp. A slide agglutination test (Difco) was performed using the samples that contained putative *Bordetella* spp. (Kösters *et al.*, 2001).

The nasopharyngeal swabs were immersed in PBS and extracted to obtain pure DNA using a commercially available High Pure PCR Template Preparation kit (Roche Applied Science), which was followed by real-time PCR. In addition, DNA samples were extracted from *B. pertussis* ATCC 9797, *Bordetella parapertussis* ATCC 15311, *Bordetella bronchiseptica* ATCC 4617, *Bordetella holmesii* ATCC 51541, *Klebsiella pneumoniae* ATCC 9997, *Streptococcus pneumoniae* ATCC 49619, *Haemophilus influenzae* ATCC 35056 and *Pseudomonas aeruginosa* ATCC 27853. The suspensions were stored at 4 °C.

The IS481-LAMP assay was developed using six primers (Table 1), which were designed using Primer Explorer version 4 (http://primerexplorer.jp/elnclamp4.0.0/index.html). The LAMP reaction was carried out in a volume of 25 μl, which contained 0.8 M betaine (Sigma), 1.2 mM each dNTPs (Genet Bio), 20 mM Tris/HCl (pH 8.8), 10 mM KCl, 10 mM (NH₄)₂SO₄, 6 mM MgSO₄, 0.1 % Triton X-100, 6 U Bst DNA polymerase (New England Biolabs) and template DNA (3 μl). The reaction was adjusted to 0.2 μM for the F3 and B3 primers, 1.6 μM for the FIP and BIP primers, and 0.4 μM for the loop primers.

The PT-LAMP assay was performed according to a method described in a previous study conducted in Japan (Kamachi *et al.*, 2006) and the primers were the same. Briefly, the 25 μl LAMP reaction was optimized for clinical specimens, which contained 0.8 M betaine (Sigma), 1.4 mM each dNTPs (Genet Bio), 20 mM Tris/HCl (pH 8.8), 10 mM KCl, 10 mM (NH₄)₂SO₄, 8 mM MgSO₄, 0.1 % Triton X-100, 6 U Bst DNA polymerase (New England Biolabs) and template DNA (3 μl). The primer combinations were changed slightly to ensure that efficient amplification was achieved: 0.2 μM for the F3 and B3 primers, 1.8 μM for the FIP and BIP primers, and 0.4 μM for the loop primers. For both of the LAMP reactions, amplifications were performed at 64 °C for 60 min and the enzyme was inactivated by heating at 80 °C for 10 min.

To confirm the LAMP products, 3 μl of the amplified reaction product was analysed by electrophoresis on a 2 % agarose gel. White turbidity was also visual evidence of LAMP amplification. The amplified LAMP products were also detected by adding 1 μl of 1 : 10-diluted SYBR Safe DNA Gel Stain (Invitrogen). The amplified LAMP products were then illuminated under UV light and the green colour of the positive samples was visible to the naked eye (Fig. 1). To compare the sensitivity and specificity of the two LAMP assays, a conventional IS481-TaqMan-based real-time PCR was performed (Kösters *et al.*, 2001) using an internal process control (Ding *et al.*, 2009).

All of the statistical analyses were conducted with MedCalc biostatistical software.

Clinical samples obtained between January and December 2012 were subjected to the entire process because they had already been referred to the Pasteur Institute of Iran. The positivity and negativity of samples were confirmed by IS481 real-time PCR and culture. The culture of samples was rarely positive, confirming the difficulties of this procedure. The conventional IS481 real-time PCR obtained 80 positive and 85 negative samples compared with the 76 positive samples by IS481-LAMP. The sensitivity and specificity of the IS481-LAMP and PT-LAMP methods were calculated relative to real-time PCR as the reference test (Table 2). For the PT-LAMP assay, the positive predictive value (PPV) and negative predictive value (NPV) were 92.4 % [95 % confidence interval (CI): 83.2–97.4 %] and
80.8 % (95 % CI: 71.6–88.0 %), respectively, and for the IS481-LAMP assay, PPV and NPV were 92.1 % (95 % CI: 83.6–97.0 %) and 88.7 % (95 % CI: 80.3–94.4 %), respectively. Over a range of six serial dilutions (from 100 pg to 1 fg), amplification using 10 fg by IS481-LAMP and 100 fg by PT-LAMP obtained a sensitivity of 2.26 and 22 genomic copies of B. pertussis, respectively. However, the limitation threshold of 5 fg with real-time PCR was more sensitive than that with both of the LAMP-based methods.

There were no cross-reactions among any of the Bordetella spp. tested (i.e. only true positivity for B. pertussis) or the other tested organisms when PT-LAMP was used for B. pertussis detection. However, B. holmesii harbours an IS481 element and had a positive reaction in the IS481-LAMP assay. For the other organisms, no cross-reactions interfered with the results.

According to previous studies, the sensitivity and specificity of the LAMP assay are reasonable compared with real-time PCR (Lin et al., 2012). Theoretically, the IS481-LAMP test should be more sensitive than PT-LAMP, which we confirmed in our study (Table 2). However, as expected, the specificity of PT-LAMP was higher than that of IS481-LAMP (94.1 vs 92.9 %). In addition, the presence of insertion sequence IS481 in some Bordetella spp. (Lin et al., 2012) can lead to false positives in the IS481-LAMP assay, which is why the specificity of PT-LAMP is higher than that of IS481-LAMP. However, the detection of B. holmesii from clinical samples is rare, so it can be overlooked (Fishbain et al., 2014).

Despite many advantages of real-time PCR, it has some limitations such as the requirements for thermal cyclers and expensive substrates (Reddy et al., 2010). Real-time PCR is used widely in diagnostic laboratories (Roorda et al., 2011; Kösters et al., 2001), but the LAMP assay has advantages compared with PCR for the following reasons. First, the LAMP amplification results can be visualized with a UV light and an appropriate fluorescent dye. Secondly, there are no requirements for complex interpretations or advanced operator skills. Finally, this method is less expensive than real-time PCR. Indeed, the short amplification time and cheaper substrates are major advantages compared with other methods (Notomi et al., 2000; Mori & Notomi, 2009).

LAMP can be used as a diagnostic method, but it has some disadvantages, including non-specific amplification, no control of inhibition and the risk of contamination. In this study, the use of six primers in the LAMP reaction reduced the risk of non-specific amplification compared with PCR, which used two primers, and preparation in a separate room can avoid the contamination problem. In addition, the LAMP method has several advantages for diagnostic laboratories, i.e. it is user-friendly and does not require skilled staff or sophisticated equipment. Therefore, this method is cost-effective for many clinical laboratories.

In conclusion, we developed a new LAMP assay based on IS481 to facilitate the rapid

| LAMP assay | Primer type | Sequence (5’→3’)
<table>
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<tbody>
<tr>
<td>IS481-LAMP</td>
<td>IS481-F3</td>
<td>GCCTACTACCAGCGCCTG</td>
</tr>
<tr>
<td></td>
<td>IS481-B3</td>
<td>GGGAGTTCTGTTAGGTGTA</td>
</tr>
<tr>
<td></td>
<td>IS481-FIP</td>
<td>AGCTCATGGCCACAGCGCGGACCATCCACGCGCTG</td>
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<tr>
<td></td>
<td>IS481-BIP</td>
<td>CGCTTTACCGCACCTTACCGCGTAAGGCCACTCACGCA</td>
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<tr>
<td></td>
<td>IS481-LF</td>
<td>GCCGAGCCATTGTGCGT</td>
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<tr>
<td></td>
<td>IS481-LB</td>
<td>AATGGCAAGGCGGACGC</td>
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Fig. 1. (a) Visual appearance after reaction. Positive (tubes 1 and 3) and negative (tubes 2 and 4) reactions are yellow and orange, respectively. (b) Visual appearance of LAMP reactions after the addition of 1 μl of 1:10-diluted SYBR Safe DNA Gel Stain (Invitrogen) under UV light. Positive samples (tubes 1 and 3) show a green colour whilst negatives (tubes 2 and 4) remain orange.
and sensitive detection of B. pertussis that is cheaper than conventional real-time PCR. This method is a possible alternative for the diagnosis of B. pertussis in clinical laboratories, particularly where it is not possible to diagnose pertussis infections with the reference molecular real-time PCR.

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**Abbreviation:** LAMP, loop-mediated isothermal amplification.

<table>
<thead>
<tr>
<th>Method (sample number)</th>
<th>PT-LAMP</th>
<th>IS481-LAMP</th>
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<tbody>
<tr>
<td>Positive (n=66)</td>
<td></td>
<td></td>
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<tr>
<td>Real-time PCR positive (80)</td>
<td>61</td>
<td>70</td>
</tr>
<tr>
<td>Real-time PCR negative (85)</td>
<td>5</td>
<td>6</td>
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<tr>
<td>Culture (165)</td>
<td>16</td>
<td>16</td>
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<tr>
<td>Positive (n=76)</td>
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<tr>
<td>Sensitivity</td>
<td>76.2 % (95 % CI: 65.4–85.0 %)</td>
<td>87.5 % (95 % CI: 78.2–93.8 %)</td>
</tr>
<tr>
<td>Specificity</td>
<td>94.1 % (95 % CI: 86.8–98.0 %)</td>
<td>92.9 % (95 % CI: 85.2–97.3 %)</td>
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<tr>
<td>Negative (n=99)</td>
<td>19</td>
<td>10</td>
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<tr>
<td>Real-time PCR negative (85)</td>
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<td>79</td>
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<tr>
<td>Culture (165)</td>
<td>149</td>
<td>149</td>
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