South Asian Journal of Experimental Biology

ISSN: 2230-9799 Vol. 3, Issue 4, Page 166-171 http://www.sajeb.org

REGULAR ARTICLE

Assessment of a Loop-Mediated Isothermal Amplification Assay targeting *lytA* genes with conventional PCR for the direct detection of *Streptococcus pneumoniae* in clinical samples

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ARTICLE INFO

Article History:
Received: 5 Jul 2013
Revised: 21 Jul 2013
Accepted: 21 Jul 2013

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Keywords: S. pneumoniae, LAMP, PCR, *lyt A* , PCR

ABSTRACT

Streptococcus pneumoniae is one of the most encountered pathogens in developed and developing countries. It is a leading cause of invasive bacterial disease in adults as well as in children. This study focuses on the loop-mediated isothermal amplification (LAMP) assay to validate its suitability for directly detecting *lyt A* target genes of *S. pneumoniae* in clinical samples. We studied the clinical sensitivity and specificity of the LAMP assay targeting *lyt A* using 42 selected CSF specimens from children with suspected meningitis in the Kingdom of Saudi Arabia. Conventional polymerase chain reaction (PCR) and culture tests were also performed. The detection rate of the LAMP assay was significantly higher than the rates of PCR and culture tests and the detection limits (10 copies by LAMP) were considerably lower than those for PCR (10^3 copies). Our study suggests that LAMP reaction-based detection of target genes of suspected pathogens could be applied in a various clinical settings. In addition, the lower cost of LAMP assay than PCR makes it more economical, allowing its use in laboratories with limited resources.

1. Introduction

Streptococcus pneumoniae is one of the most frequently encountered pathogens in human adults and more in children, but its differentiation from closely related but less pathogenic streptococci remains a challenge (Alia et al., 2010). This pathogenic bacterium is involved in human disease causing bronchitis, pneumonia, as well as life-threatening meningitis and blood stream infections (Spanjaard et al., 2007). Culture-based methods are usually time consuming, and positivity depends upon so many factors such as the time taken for sample to reach the laboratory: the earlier it is brought for culture, the better it is as- it is well known that the cell wall is lysed by anautolysing enzyme (Fernebro et al., 2004). Differentiation is also important for resistance testing, since different antibiotic susceptibility breakpoints are applied for *S. pneumoniae* with regard to other viridans species (Rozkiewicz et al., 2006). Culture-based identification methods usually rely on optochin susceptibility, agglutination and bile-solubility, which need expertise with reference to quality testing,

especially when pseudo pneumoniae showing partial sensitivity to optochin are reported (Keith, 2013).

Molecular detection techniques have been promising for the detection of *S. pneumoniae* by using polymerase chain reaction (PCR) targeting *lyt A* (Alia et al., 2010). Real time PCR has shown sensitivity to amplify the *lyt A* gene with a DNA copy number as low as 10 (Carvlho et al., 2007). Finding specific pneumococcal genes for detection, however, continues to be challenging. *LytA* and *ply* genes are two 2 targets that are widely used in PCR for the identification of *S. pneumoniae* in clinical specimens. Pneumolysin was first used to identify *S. pneumoniae* in an agglutination assay, and its application in highly specific PCR-based assays for clinical specimens has been previously considered (Blaschke, 2011). Several studies have used the *ply* gene target for the detection of *S. pneumoniae* from patients of pneumococcal disease, and the relatively poor sensitivity and specificity for invasive diseases overall has been described (Carvlho et al., 2007). These studies make clear that *ply* can also be detected in non-pneumococcal Viridans-group streptococci, particularly *S. pseudopneumoniae* and *S. mitis* (Blaschke, 2011). Few other studies have identified the autolysin gene target *lyt A*. Carvalho et al., compared 3 gene targets; *ply*, *lyt A*, and *psaA*, and their studies have shown that the autolysin gene *lyt A* was the most specific and sensitive target gene among these 3 genes (Carvlho et al., 2007). Primers designed to target the autolysin gene showed no amplification from any of the non-pneumococcal bacteria tested.

Loop-mediated isothermal amplification (LAMP), a novel nucleic acid detection method was reported in early 2000 (Notomi et al., 2000). In this method, specific DNA products can be obtained in a shorter time than PCR by utilizing a unique priming mechanism. The availability of the LAMP method offers the opportunity to develop a novel assay for the detection of *S. pneumoniae*, which is more reliable and easier to perform than bacterial culture, antigen detection, or PCR based assays. However, until date, only a few articles have been published targeting the *lyt A*gene using the LAMP assay (Seki et al., 2005; Kim et al., 2010).

In our present study, we performed the LAMP assay and evaluated its detection limit by using *lyt A* gene targets. Using this assay, we also compared the sensitivity and specificity targets directly in clinical samples collected from patients with suspected meningitis.

2. Materials and Methods

2.1. Bacterial strains

In total, 51 *S.pneumoniae* clinical isolates were collected from King Khaleed Hospital at King Saud University, Riyadh, Saudi Arabia. Six other non-S.pneumoniae isolates included in this study were *Streptococcus mitis* (ATCC 9811), *S. oralis* (ATCC 10557), *S. gordonii* (ATCC 10558), *S. mutans* (ATCC 700610), *S. sanguis* (ATCC 10556), and *S. salivarius* (ATCC13419). We also included eight standard reference *S. pneumoniae* strains (serotype 1, 4, 5, 6B, 6A 14, 19F and 23 F). The serotype was confirmed by sequential multiplex PCR using the respective primers, as previously described (Pai et al., 2006).

2.2. Extraction of chromosomal DNA

Genomic DNA was extracted from all the isolates listed above using a QIAamp DNA minikit (Qiagen, Valencia, CA) according to manufacturer's protocol. To ascertain the detection limit of LAMP assay, genomic DNA of the control strain *S.pneumoniae* ATCC 49619 was serially diluted (10-fold) to obtain about 10^8 to at least 10 copies, and this was used in the amplification reactions. The results for the corresponding dilutions were compared with those obtained using conventional PCR. To confirm the results of detection limits, we performed all tests in triplicate in a single day, using 10-fold dilutions of genomic DNA. A spiking study was also performed with 10-fold dilutions of *S. pneumoniae*-negative samples, and the results were compared between LAMP and conventional PCR.

2.3. Conventional PCR targeting the lyt A gene

PCR was performed in a total volume of 25 ul, comprising a 1× reaction buffer, 3.5 mM MgCl2, 1.5 mM deoxynucleoside triphosphates, and 1 U of DNA Taq polymerase (Qiagen). The primer concentration in each reaction tube was 2.5 pmol, to which 2.5 ul DNA templates were added. The mixture was amplified for 1 cycle at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 54°C for 45 seconds, extension at 65°C for 2.5 min, and a final extension at 72°C for 1 min. The PCR products were run on a 2% agarose gel and visualized under UV light to identify the presence of the 160-bp DNA fragment.

2.4. LAMP primer design for S. pneumoniae lyt A

Five LAMP primers were designed for gene sequencing of the autolysin-encoding gene (*lytA*; GenBank accession numbers M13812, M55414, and M55415) by using the LAMP primer support software program (Net Laboratory, Kanagawa, Japan). LAMP primers consisted of 2 outer primers (F3 and B3), a forward inner primer (FIP), a backward inner primer (BIP), and a loop primer forward (Figure 1).

2.5. LAMP reactions for lyt A

LAMP reactions were performed in a total volume of 25 ul, containing 1.5 ul each of FIP and BIP, 0.2 BM each of F3 and B3, 0.4 BM LF, 6 U of Bst DNA polymerase large fragment (New England Biolabs, Ipswich, MA), 1.5 mM deoxynucleoside triphosphates, 0.8 M betaine (Sigma-Aldrich, St. Louis, MO), 25 mMTris-HCl (pH 8.8), 10 mMKCl, 10 mM (NH4)2SO4, 8 mM MgSO4, and 0.1% Tween 20. DNA templates (2.5 Bl) were added to the respective reaction mixture tubes. The mixture was incubated at 63°C for 35 min, and the reaction was terminated by heating the tubes at 80°C for 2 min.

2.6. Analysis of LAMP products

LAMP reaction generates turbidity proportional to the amount of amplified DNA. Amplified products can be visualized by electrophoresis on 2% agarose gels with ethidium bromide staining (13). To confirm DNA amplification, a 10 ul aliquot of the amplified products was digested using the restriction enzyme Hpy188I (New England Biolabs, Ipswich,MA), and their sizes were analyzed by electrophoresis on 3% agarose gels with ethidium bromide staining, as described earlier. Thereafter F2 and B2 primers for *lyt A* genes were used to sequence the target region (between F1 and B1) (Figure 1). The amplified products were sequenced using an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA) as per the manufacturer's instructions.

2.7. LAMP Assay for Clinical CSF specimens

To evaluate the sensitivity and specificity of designed LAMP reaction and its primers on clinical samples, 42 CSF samples were obtained from patients with suspected bacterial meningitis. Out of 42, 17 CSF samples were positive, and the remaining 25 samples were negative for *S. pneumoniae*, as detected by PCR targeting of the *lyt A* gene (capsular polysaccharide) and Binax test. Genomic DNA was extracted from all the CSF samples using QIAamp Blood DNA mini kit (Qiagen) as per the manufacturer's protocol.

3. Results

The target regions comprising of *lyt A* genes were successfully amplified at 63 °C for 35 min in the LAMP reaction, and the product was visualized on agarose gel. The products appear as ladder in the gel that is characteristic of the LAMP reaction (Figure 3).

3.1. Specificity of the LAMP reaction

In our study, we evaluated 51 *S. pneumoniae* of different serotypes and 8 *S. pneumoniae* serotype control strains together with 6 other non-streptococcus isolates to assess the specificity of the LAMP reaction targeting *lyt A* genes. For testing each isolate, we used template concentrations of approximately 10^8 copies of genomic DNA. Amplification of the *lyt A* genes was observed after 35 min (Table 1), whereas genomic DNA of the non-streptococcus strains was not amplified even after 60 min of incubation (Table 1). Amplification specificity was further confirmed by sequencing, and the sequences were compared to those of the targeted region between F1 and B1 (Figure 1). The sequences obtained were similar to the expected sequences.

3.2. Sensitivity of LAMP reaction

Genomic DNA was serially diluted 10-fold and consistently amplified with a minimum of 10 genome copies per reaction; the amplification was considered as positive only when at least 2of the 3 reactions showed an amplification of the target sequence, although the sample was diluted until only 1 copy of the gene was present per reaction (Table 2). No amplification was observed in the LAMP reaction when the sample lacked target DNA. Using the CSF spiking specimens, the detection limit of the LAMP reaction for both the target genes used in our study was 10 genomic copies, whereas PCR analysis using the *lyt A* genes established a detection limit of 10^3 genome copies (data not shown).

3.3. LAMP Assay for clinical isolates

Results of the LAMP assay for *lyt A* genes were concordant with PCR results for *S. pneumoniae* and non-streptococcal strains. All 51 clinical isolates were positive for *lyt A* by PCR and LAMP assay targeting both genes.

Assay	*S. pneumoniae* copy number									
		10^7	10^6	10^5	10^4	10^3	10^2	10^1	1	0
lyt A PCR[b]	+	+	+	+	+	-	-	-	-	-
lyt A Lamp Assay[b]	+	+	+	+	+	+	+	+[a]	-	-

Table 1: Detection limits of lyt A PCR, *lyt A* LAMP.
a= experiments performed in triplicate
b= electrophoresis analysis only

Results	*lyt A* Lamp Assay Results		Total number
	Positive sample (22)	Negative sample (20)	Total
lyt A PCR positive	17	0	17
lyt A PCR negative	5	20	25
Culture positive	15	0	15
Culture negative	7	20	27

Table 2: Comparison of lyt A PCR, *lyt A* LAMP.

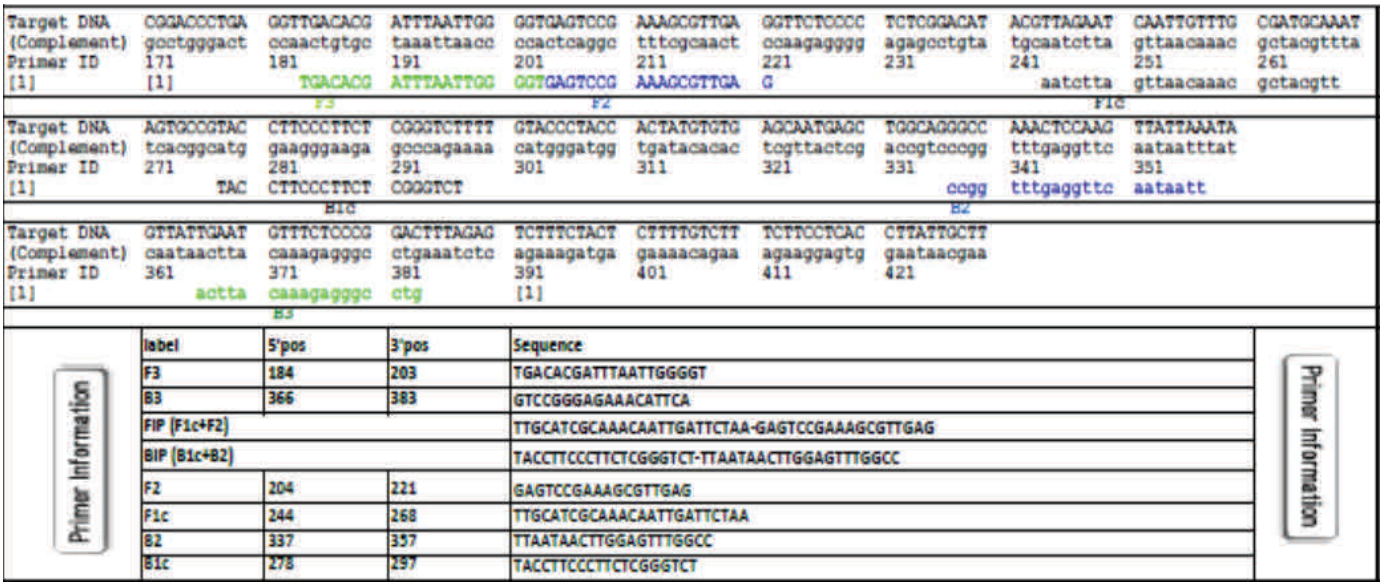

Figure 1: Sequence of primers used in the *lyt A* LAMP assay.

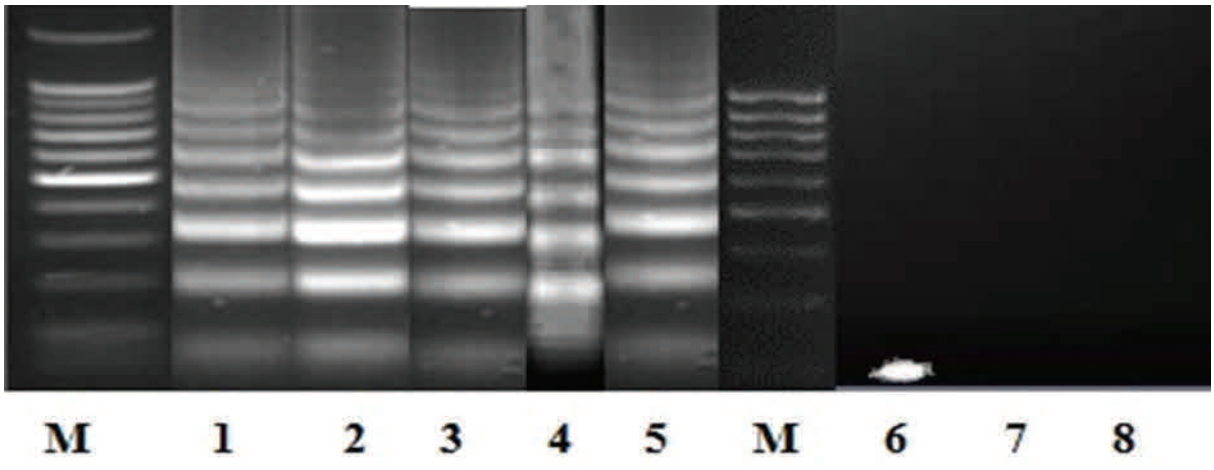

Figure 2: Electrophoretic analysis of the *lyt A* LAMP-amplified products. Lane M, 100-bp ladder; lanes 1–5, LAMP products from individual samples with different genomicDNA concentrations; lane 6, product after digestion; lanes 7 and 8 controls without a template.

3.4. Comparison of the LAMP assay and traditional PCR using CSF samples

lyt A PCR was applied to 42 CSF specimens, and the PCR results obtained were identical to that of the Binax test. Among the 22 samples that were LAMP-positive for *lyt A* specimens, 17 (77.3%) were lyt A-PCR positive, whereas 5 specimens were negative for *lyt A* PCR (Table 3). Twenty *lyt A* LAMP-negative specimens were also determined to be negative by PCR assays (Table 3). Fifteen CSF specimens were culture-positive for *S. pneumoniae*. Among the culture-positive specimens, all specimens were positive for both LAMP and PCR, whereas among the 27 culture-negative samples, 20 were negative for both LAMP and PCR. Rest seven specimens were found to be positive for *lyt A* LAMP and 2 for lyt A PCR.

4. Discussion

In our study, the LAMP assay gave a detection limit higher than that for conventional PCR, and the assay was found to be accurate enough to differentiate *S. pneumoniae* strains from non-*S. pneumoniae* strains of known identity (Bi et al., 2012). The detection limit of the LAMP assay was uniform from 10 copies for up to 10^8 copies of DNA templates; the same results were observed even with spiked samples, which confirmed the reliability of the LAMP assay. The comparatively lower detection limit of the LAMP assay captured 5 additional positive samples using *lyt A* LAMP thus contributing to its higher detection rate (22 positive samples) compared with that of PCR (17 positive samples) out of the 42 tested clinical CSF specimens from children with suspected meningitis. LAMP assays for the detection of meningitis bacteria have been previously established, but the efficacy for their diagnosis has not been tested so far (Kim et al., 2012).

In the present study, we compared the culture methods (15 positive samples) and lyt A PCR (17 positive samples), and we found that PCR showed relatively higher sensitivity compared to that of culture, as described in previous studies (Bartlett et al., 2011). Two more samples, which were culture-negative, were detected as positive by PCR; moreover, no culture-positive sample was detected as negative by PCR, showing the high specificity of PCR. Indeed, *lyt A* LAMP detected 5 more positive samples than PCR and 7 more positive samples than the culture tests; on the other hand, no culture- or PCR-positive samples were detected as negative by the LAMP assay. Furthermore, the LAMP assay did not show any negative reaction for culture- or PCR positive samples. The LAMP assay used in our study showed promisingly high analytical specificity and low detection limit (of up to 10 DNA copies), and this is in agreement with previously reported findings for *S. pneumonia* LAMP assays (Seki et al., 2005; Kim et al., 2011).

In the present study, 100% (n =15) of the culture-positive specimens were both PCR- and LAMP positive. The *lyt A* LAMP assay was more sensitive than the previously described PCR method. The discrepancy in the results obtained in our study (5 CSF specimens were negative by PCR and positive by *lyt A* LAMP assay) is likely due to the lower detection limit of DNA achieved by the *lyt A* LAMP assay.

Our study results suggest that LAMP reaction-based detection of target genes of suspected pathogens could be more feasible in a wide variety of clinical settings. Among molecular based techniques, where the detection limit is up to 10^3 genome copies for PCR and 10 genome copies for LAMP, the LAMP assay is relatively more sensitive. In addition, compared to PCR, LAMP reactions are not repressed due to enzymatic factors, and previous studies have shown that the LAMP reaction is more tolerant to the presence of potentially inhibitory biological substances, as compared to PCR (Nijiru et al., 2008). In addition, the LAMP reaction does not require a thermal cycler; this key feature makes the LAMP assay appropriate for resource-limited settings that are frequently faced by many laboratories in developing countries. Our experience in using LAMP assays suggests that the cost of using LAMP assays is remarkably lower than that for PCR, However, further evaluation of the LAMP assay in a prospective manner is required to confirm assay characteristics, compared to bacterial culture, antigen detection, as well as PCR, in order to judge its clinical applications and specificity.

5. Conclusion

In summary, we conclude that the LAMP-based assay can be successfully established as a highly analytical, sensitive, and specific assay compared to conventional PCR assays, antigen detection tests, and other culture techniques. Furthermore, the cost of LAMP assay is lower than that of PCR assays, and this makes it economical and applicable in laboratories with limited resources.

Acknowledgements

The authors would like to extend their sincere ap-

preciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group Project no. RGP-VPP-314.

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