1   Research Article

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3   **Assessment of a loop-mediated isothermal amplification (LAMP) assay for the rapid detection of**

4   **pathogenic bacteria from respiratory samples in patients with hospital-acquired pneumonia**

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24   **Running Head:** LAMP to detect bacteria in respiratory samples

25

26   **Keywords:** loop-mediated isothermal amplification (LAMP); hospital-acquired pneumonia (HAP);

27   diagnostic techniques, respiratory system, critical care.

Abstract

Introduction. Hospital-acquired pneumonia (HAP) is the one that presents clinically two or more days after admission into the hospital. Rapid identification of the causative agent of HAP will allow an earlier administration of a more appropriate antibiotic therapy and could lead to an improved outcome of patients with HAP.

Methods. First of all, a rapid procedure (< 30 min) to extract the DNA from bronchoalveolar lavage (BAL), endotracheal aspirate (EA) or bronchoaspirate (BAS) was set up. A loop-mediated isothermal amplification reaction (LAMP) specific for *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae, Pseudomonas aeruginosa, Stenotrophomonas maltophilia* and *Acinetobacter baumannii* was carried out with the extracted solution. The reaction was performed at 65ºC for 30-40 min. LAMP was compared with bacterial culture method.

Results. Overall, 58 positive BAL and 83 EA/BAS samples were tested. The limits of detection varied according to the microorganism detected and to the respiratory sample analyzed. Validation of the LAMP assay with BAL samples showed that the assay was 100% specific and 86.3% sensitive (positive predictive value of 100% and a negative predictive value of 50%). Meanwhile for BAS/EA samples, the assay rendered the following statistical parameters: 100% specificity, 94.6% sensitivity, 100% positive predictive value and 69.2% negative predictive value. These scores were obtained including minor errors as correct. The turnaround time including preparation of the sample and LAMP was circa 1 hour.

Conclusions. LAMP method may be used to detect the most frequent bacteria causing HAP. It is a simple, cheap, sensitive, specific and rapid assay.

Introduction

Hospital-acquired pneumonia (HAP) is the one that presents clinically two or more days after hospitalization and includes ventilator-associated pneumonia (VAP), which is defined as pneumonia that presents after 48 hours with endotracheal intubation (1). Patients with VAP present longer periods with mechanical ventilation, as well as longer stay in the ICU and in the hospital (2). It is estimated that approximately 10-40% of the patients undergoing mechanical ventilation for more than two days will develop VAP (2), with great differences among countries, type of patient, and type of intensive care unit (ICU). The implementation of different preventive strategies enabled a decrease in VAP cases, but it is still a very important problem among ventilated patients (3).

VAP is frequently caused by Gram-negative aerobic bacteria (4–6), such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Acinetobacter* spp; while *Staphylococcus aureus* is the most frequent Gram-positive pathogen (7). In addition, the VAP can be polymicrobial, which complicates diagnosis and treatment. Regarding viruses, virus herpes simplex (HSV) (8) and cytomegalovirus (CMV) (9) can be reactivated in critical care patients and cause VAP. It should be noted that many of the pathogens causing VAP may present high levels of antibiotic resistance (2, 7).

Unfortunately, the diagnosis of VAP is complicated and there is no a reliable reference test (10). The signs and symptoms of pneumonia, such as fever, tachycardia, leukocytosis and purulent secretions, are frequent in patients with mechanical ventilation and are not specific to VAP. Moreover, radiographic changes are difficult to interpret in this group of patients. Therefore, it is necessary to confirm the clinical suspicion of VAP through the microbiological culture of pulmonary secretions. However, the microbiological diagnosis also has several limitations: 1) difficulty to differentiate between colonization and infection; 2) non-homogeneous distribution of

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73 the infection in the lung; and 3) negative cultures due to previous empirical antibiotic treatment.

74 Empirical treatment is usually initiated in patients with suspected VAP before having the definitive

75 diagnosis (11), since early and adequate treatment decreases the mortality (12). However, the

76 indiscriminate administration of antibiotics exposes patients to unnecessary side effects, increases

77 health care costs and favors the appearance of antibiotic resistance. Hence, the importance of

78 knowing if there is an infection or not and the identification of the etiological agent (2).

79   The application of rapid diagnostic techniques to identify microbial pathogens seems to

80 have a huge impact in the treatment of VAP, reducing inappropriate or unnecessary antimicrobial

81 treatments and mortality in these patients (13, 14). Molecular biology techniques have allowed a

82 faster diagnosis of VAP, especially in viral infections. They have the advantage of being faster than

83 culture, allowing the detection of the causative agents even though the patient is receiving

84 antibiotics and being able to quantify the bacterial concentration in the sample. On the other

85 hand, in general they are relatively expensive techniques and detect a limited number of

86 microorganisms.

87   The reaction based on the loop-mediated isothermal amplification (LAMP), developed in

88 2000 by Notomi *et al.* (15), is a rapid, simple, cheap and constant temperature nucleic acid

89 amplification method. This method is very sensitive and specific, and there are numerous

90 publications regarding its use in a wide range of applications (16). LAMP has been used to identify

91 respiratory pathogens, with promising results (17, 18). The purpose of this study was to develop

92 and evaluate a rapid protocol to identify the main microorganisms involved in HAP by LAMP

93 directly from respiratory samples.

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94   **Material and Methods**

95   *Study design*

96   We developed a rapid protocol to identify by LAMP six different bacteria (*P. aeruginosa*,

97   *Acinetobacter baumannii*, *K. pneumoniae*, *Escherichia coli*, *S. aureus* and *Stenotrophomonas*

98   *maltophilia*) and evaluated its performance compared to culture. We used three different types of

99   samples: bronchoalveolar lavage (BAL), endotracheal aspirate (EA) and bronchoaspirate (BAS).

100   Two different protocols were optimized and used to deal with the different consistencies of the

101   samples processed (aspirates being more difficult to work with due to sample thickness).

102   *Collection of samples*

103   Positive and negative BAL, EA and BAS samples were collected from the Clinical

104   Microbiology Laboratory at the Hospital Clinic of Barcelona (Spain), after being processed for

105   routine techniques. The microbiological result was collected, including Gram stain and culture

106   result. BAL collected during two-year period (2016/17) and EA/BAS during six months (January to

107   June 2018) were stored at -80ºC until use. To standardize the protocol, negative samples were

108   mixed to obtain a homogeneous matrix. Aliquots of this homogeneous negative sample was

109   spiked with different microorganisms and used to determine the limits of detection for each

110   microorganism.

111   *Routine microbiological methods*

112   Respiratory samples were collected in sterile containers and transported to the laboratory

113   in less than two hours. Gram staining of the samples in the area of maximal purulence was

114   examined for leukocytes and epithelial cells. Only respiratory samples with Murray–Washington

115   classification degrees of IV (10–25 epithelial cells and >25 leukocytes per field using a low

116   magnification lens (x100)), V (≤10 epithelial cells and >25 leukocytes) or VI (≤10 epithelial cells and

6

117   ≤10 leukocytes) were processed for culture. Specimens not fulfilling these criteria were not

118   considered to be representative of distal airways and were not processed for culture.

119   Good-quality respiratory specimens were quantitatively plated on blood and chocolate

120   agar. Isolated bacteria were identified by matrix-assisted laser desorption/ionization time-of-flight

121   mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany). Susceptibility testing

122   was performed according to EUCAST guidelines (www.eucast.org). Potential pathogenic bacteria

123   included *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, Gram-

124   negative bacilli, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Non-potential pathogenic

125   microorganisms   included   Viridans   group   streptococci,   *Neisseria*   spp,   *Candida*   spp,

126   *Corynebacterium* spp, *H. parainfluenzae* and coagulase-negative staphylococci and were not

127   considered as clinically significant. These samples were informed as having normal or mixed flora.

128   ***Extraction of DNA***

129   Two protocols depending on the type of sample were performed, and they are described in

130   Figure 1. Briefly, samples were concentrated by centrifugation and boiled in DireCtQuant 100W

131   buffer (FrontexBioMed, Ltd. / DireCtQuant, Lleida, Spain) to obtain extracted DNA. This extract

132   was later on used for the LAMP test.

133   ***LAMP protocol***

134   Previously described primers (17) were used for *P. aeruginosa*, *A. baumannii*, *K.*

135   *pneumoniae*, *E. coli*, *S. maltophilia*, and *S. aureus*. The LAMP was performed in 25 µL of reaction

136   mixture: 5 µL primers (0.2µM outer, 1.6µM inner and 0.4µM loop primers), 15 µL Isothermal

137   Master Mix (Optigene) and 5.0µL of extraction product. Once the reaction mix is ready, gentle

138   vortex and centrifugation must be performed. The reaction was conducted in a Versant kPCR

139   (Siemens) at 65°C for 40 minutes for BAL samples and in a Lightcycler (Roche) at 65ºC for 30

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140   minutes for EA/BAS. Both protocols can be implemented independently of the thermocycler, but

141   we performed them in two different machines because they were done in different times and

142   changes in laboratory equipment occurred.

Determination of the limits of detection

144   Each microorganism was inoculated into negative BAL samples to a final concentration

145   ranging from 10^7 to 10^2 CFU/mL. For EA/BAS it was not possible to perform the same study for

146   sensitivity due to the consistency of the samples and the physical impossibility of obtaining

147   homogeneous matrix for all the dilutions. Instead, positive samples were used to perform serial

148   10-fold dilutions in saline buffer. Final concentration for each dilution was based on the

149   approximate initial concentration determined by culture.

Statistical analysis

151   Concordance between culture and LAMP results was studied. Major errors were defined as

152   result discrepancies where the microorganism identified by LAMP was completely different from

153   that identified in culture or the detection of a pathogen when the culture had none. Minor errors

154   were defined as concordant results for the major pathogen identification but LAMP identified

155   additional microorganisms.

156   Diagnostic performance was based on sensitivity, specificity, negative- and positive-

157   predictive values, and accuracy defined as described elsewhere (19, 20). Accuracy was calculated

158   as concordant results over total number analyzed. Cohen's kappa coefficient was also calculated.

159   Statistical analyses were performed using Stata version 15 (Texas, USA).

Ethical aspects.

161   The Ethical Committee of the Hospital Clinic of Barcelona, Spain approved the study

162   protocol. The study is registered as HCB/2016/0294.

163   **Results**

164   ***BAL samples.***

165   The limit of detection of LAMP for the different pathogens tested in BAL was 10^2 CFU/mL

166   for *S. aureus*, *E. coli*, *P. aeruginosa* and *K. pneumoniae*, and 10^4 CFU/mL for *S. maltophilia* and *A.*

167   *baumannii*. No cross-reaction was identified using these primers while testing spiked samples with

168   the other microorganisms included in the study. Regarding the evaluation with clinical samples,

169   the concordance between culture and LAMP results is shown in Table 1. A total of 58 positive BAL

170   samples were tested. All major errors happened due to a low concentration of bacterial in the

171   sample, except for one case in which *A. baumannii* was not detected although with high

172   concentration, and even after repeating the technique. The accuracy of the LAMP assay was of

173   77.6% or 87.9% if the minor errors were considered as a non-true or true result, respectively

174   (Table 2). In addition, calculation of the clinical sensitivity and specificity yielded 100% (95%CI;

175   59% to 100%) specificity, a sensitivity of 86.3% (95%CI; 73.7% to 94.3%), a PPV of 100% and a NPV

176   of 50% (95%CI; 33.5% to 66.6%) when minor errors where treated as a true positive sample. The

177   statistical data considering minor errors as a false positive sample is shown in Table 2.

178   ***BAS/EA samples.***

179   The estimated limit of detection of the LAMP assay to detect pathogens directly from BAS

180   or EA samples was 10^2 CFU/mL for *P. aeruginosa, K. pneumoniae* and *A. baumannii,* 10^3 CFU/mL

181   for *E. coli*, and 10^4 CFU/mL for *S. aureus* and *S. maltophilia*. No cross-reaction was identified using

182   these primers while testing spiked samples with the other microorganisms included in the study.

183   Regarding the evaluation with clinical samples, a total of 83 samples were tested. The accuracy

184   between culture and LAMP results is shown in Table 2 and it was of 95.2% or 72.3%, respectively

185   when the minor errors where considered as true positives or not. In Table 3, the concordance

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186 between the LAMP assay and bacterial culture is shown. All major errors happened due to a low

187 concentration of bacterial in the sample, except for two cases: *S. aureus* with more than 10,000

188 CFU/mL in culture and *A. baumannii* with 300,000 CFU/mL in culture.

189   Regarding possible amplification inhibitors, a mix of lipidic, hematic, viscous and mucous

190 samples were used and inoculated with the different microorganisms. No evidence of interference

191 was observed in any of these prepared samples.

192   Most positive results appeared before 30 minutes, and together with the sample handling

193 time, the results could be obtained within one-hour time.

194   The sensitivity, specificity, negative and positive predictive values of the LAMP assay to

195 detect pathogens directly from BAS/EA samples are shown in Table 2. When minor errors were

196 considered as false positive results, the sensitivity was 68.9% (95%CI; 57.1% to 79.2%), the

197 specificity was 100% (95%CI; 66.4% to 100%), and the PPV and NPV were of 100% and 28.1%,

198 respectively. However, when minor errors were considered as true positive the statistical

199 indicators were: 94.6% (95%CI; 86.7% to 98.5%) sensitivity, 100% (95%CI; 66.4% to 100%)

200 specificity, 100% PPV and 69.2% (95%CI; 46.5% to 85.4%) NPV (Table 2).

201   The time to positivity of the LAMP test was strongly correlated with the number of

202 bacterial CFU/ml in culture in EA/BAS samples (r= - 0.71, P<0.01) but not in BAL samples (Figure 2).

203 These results suggest that, at least in EA/BAS samples, the time to positivity of LAMP could be

204 used as a semi-quantitative measure.

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Discussion

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206   Although new preventive measures have led to a reduction of HAP incidence, it remains

207   associated with important morbi-mortality (21). Therefore, it is necessary to introduce new

208   methods to improve an early diagnosis. Here, we have evaluated the use of LAMP as a rapid

209   diagnostic tool to identify the main pathogens involved in HAP with promising results.

210   We found an overall accuracy between LAMP and culture of 88% for BAL samples and 95%

211   for BAS/EA samples. Almost all syndromic tests available to identify pathogens causing respiratory

212   tract infections include virus and atypical bacteria (22), but not many include the major bacterial

213   pathogens causing HAP. Furthermore, rapid identification of the pathogen causing VAP is crucial to

214   improve the patient outcome. In this sense, a multiplex PCR-based syndromic panel including 16

215   bacterial and one fungal target as well as 22 antimicrobial resistance markers has been evaluated

216   (23, 24, 25), showing a sensitivity from 60 % to 90% and a turnaround time of circa 4 hours. In our

217   case, as LAMP is much less affected than PCR by possible inhibitors in the sample (26), in the

218   condition of high bacteria concentration, no automatic nucleic acid extraction is required, and

219   therefore we can reduce turnaround including preparation of the sample and LAMP to around 1

220   hour.

221   Kang *et al*. (17) performed a nationwide study in China in which the qLAMP assay was

222   compared with culture to detect eight respiratory bacterial pathogens from sputum, detecting

223   pathogens in 1047 (69.28%) patients from 1533 qualified patients. They used a kit for the nucleic

224   acid extraction.

225   Cost is also important. We have calculated that the LAMP assay (counting both reagents for

226   nucleic acid extraction and LAMP together with plastic material) to detect the six pathogens

227   causing HAP in this study will cost 12€.

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228   LAMP may be useful then as a complementary tool to culture, allowing rapid identification

229   of the microorganism causing the infection. In addition, a semi-quantitative approach can be used

230   extrapolating the time to result with the CFU/ml in BAS and EA. In BAL the data that we had was

231   not sufficient to get a significant result (data not shown). If the quantification is under 10^2-10^3

232   CFU/ml the LAMP will probably not provide a positive result, but in general culture result will be

233   considered as colonization in these cases. Therefore, every positive LAMP result should be taken

234   into consideration, always taking into account the Gram stain, quality of samples and clinical

235   situation of the patient. Culture should not be avoided in any case and LAMP could be

236   implemented as a complement to accelerate the diagnosis of HAP.

237   Furthermore, it also seems promising for the detection of resistance genes (27, 28).

238   Pathogen identification and potential antibiotic resistance is possible with LAMP, both more

239   rapidly identified (1 hour) than with a time consuming (16 to 24 hours) classical phenotypic

240   method. And, it could be even faster when applied directly to samples (29).

241   LAMP method may be used to detect the most frequent bacteria causing HAP. It is a

242   simple, cheap, sensitive, specific and rapid (circa one hour) assay. Multiplexing targets may

243   facilitate the implementation of this technique in routine laboratories. Each laboratory must adapt

244   the targets according to its epidemiology.

245 **Conflicts of interest**

246 The authors declare no conflicts of interest.

247

248 **Acknowledgments**

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348 **Figure legends.**

349

350 **Figure 1. Sample preparation workflow for bronchoalveolar lavage (BAL) and**

351 **bronchoaspirate/endotracheal aspirate (BAS/EA).**

352

353 **Figure 2. Correlation of time to positivity and bacterial load in culture.** Scatter plot shows time to

354 result (in minutes) against number of CFU/mL (log10). Trend line displays best fit of all data points

355 and the 95% CI for the prediction. Vertical reference lines indicate time points. BAS/EA:

356 bronchoaspirate/endotracheal aspirate.

Tables

Table 1. LAMP results from BAL samples according to the result obtained by culture.

Microorganisms identified by culture	N	Concordance	Minor errors	Major errors	Comments
S. aureus	19	15	2[A,B]	2[C,D]	[A]LAMP: SAUR+SMAL detected [B]LAMP: SAUR+PAER detected (GNB in gram stain) [C,D]Culture: Few CFU of SAUR
P. aeruginosa	11	9	-	2[E,F]	[E]Culture: < 1000 CFU of PAER, LAMP: SAUR detected [F]Culture: <1000 CFU of PAER, LAMP negative
S. maltophilia	6	6	-	-	-
K. pneumoniae	4	2	-	2[G]	[G]Culture: <1000 CFU of KPNE (two cases)
E. coli	3	3	-	-	
A. baumannii	2	1	-	1[H]	[H]Culture: 100,000 CFU of ABAU
Negative	7	6	1[I]	-	[I]LAMP: KPNE detected (GNB in gram stain)
Mixed flora	6	3	3[J,K,L]	-	[J]LAMP:PAER detected [K]LAMP:SAUR detected [L]LAMP:KPNE detected
TOTAL	58	45	6	7	

N: number of samples tested by culture and LAMP; SAUR: *S. aureus*; SMAL: *S. maltophilia;* PAER: *P. aeruginosa*; KPNE: *K pneumoniae*; ECOL: *E. coli;* ABAU*: A. baumannii;* GNB: Gram-negative bacilli*.*

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362 **Table 2. Statistics of the use of LAMP to detect pathogens directly from BAL and BAS/EA**

363 **samples.**

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	Minor error as wrong		Minor error as right	
BAL	**Value (%)**	**95%CI**	**Value (%)**	**95%CI**
Sensitivity	76.5	62.5-87.2	86.3	73.7-94.3
Specificity	85.7	42.1-99.6	100	59-100
PPV	97.5	86.3-99.6	100	
NPV	33.3	21.9-47.2	50	33.5-66.6
Accuracy	77.6	64.7-87.5	87.9	76.7-95
Kappa coefficient	43.3	17.9-68.8	70.9	51.5-90.2
BAS/EA				
Sensitivity	68.9	57.1-79.2	94.6	86.7-98.5
Specificity	100	66.3-100	100	66.4-100
PPV	100		100	
NPV	28.1	21.8-35.5	69.2	46.5-85.4
Accuracy	72.3	61.4-81.6	95.2	88.1-98.7
Kappa coefficient	46.3	29.2-63.5	89.7	80-99.5

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366 BAL: bronchoalveolar lavage; BAS: bronchoaspirate; EA: endotraqueal aspirate; PPV: positive

367 predictive value; NPV: negative predictive value; CI: confidence interval.

368   **Table 3. LAMP results from BAS/EA samples according to the result obtained by culture.**

Microorganisms identified by culture	N	Concordance	Minor errors	Major errors	Comments
K. pneumoniae	13	10	3[A,B]	-	[A]LAMP: KPNE and ECOL detected (two cases) [B]LAMP: KPNE and PAER detected
S. aureus	10	7	2[C,D]	1[E]	[C]LAMP: SAUR and KPNE detected [D]LAMP: SAUR and ECOL detected [E]Culture: <1000 CFU of SAUR
P. aeruginosa	11	9	2[F]	-	[F]LAMP: PAER and ECOL detected (two cases)
E. coli	8	3	5[G,H,I,J]	-	[G]LAMP: ECOL and PAER detected [H]LAMP: ECOL, PAER, SAUR and SMAL detected [I]LAMP: ECOL and KPNE detected [J]LAMP: ECOL, PAER and SMAL detected (two cases)
S. maltophilia	2	2	-	-	-
Polymicrobial	10	3	4	3	See table in supplementary material.
Other	10	7	3[K,L,M]	-	[K]LAMP: ECOL and KPNE detected [L]LAMP: PAER detected [M]LAMP: PAER, SAUR and SMAL detected
Mixed flora	10	10	-	-	-
Negative	9	9	-	-	-
TOTAL	83	60	19	4	

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370   N: number of samples tested by culture and LAMP; KPNE: *K pneumoniae*; ECOL: *E. coli*; PAER: *P.*

371   *aeruginosa*; SAUR: *S. aureus*; SMAL: *S. maltophilia.*

vortex
5 sec

100 µl EA/BAS
+ 900 µl saline solution

600 g
5 min

EA = Endotracheal aspirate
BAS = Bronchoaspirate
BAL = Bronchoalveolar lavage

600 g
5 min

take
supernatant

15,000 g
5 min

Add 100 µL
lysis buffer

Heat 99°C
10 min

900 µl BAL

discharge
supernatant

15,000 g
5 min

**Take 25 µl of supernatant
to perfom LAMP**

EA/ BAS, r=-0.71, P<0.01

Time to Result (minutes)

Number of CFU/mL