Lactobacillus crispatus colony identification with colorimetric Loop mediated isothermal amplification (LAMP) protocol

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Here we present a protocol and validation for a test that allows the rapid identification of *Lactobacillus crispatus* colonies grown from vaginal samples. In our project we aim to use this method in a citizen science context (see more information on crispatus.org). This method could be adjusted for the identification of other microbes and could also be used in low-resource settings.

Introduction

The Loop-mediated isothermal amplification method (LAMP) is a DNA amplification method with high specificity, amplification efficiency and allows a reaction to take place at a constant temperature. It uses 4 primers and a DNA polymerase with strain displacement activity. The 4 primers recognise 6 different regions on the target DNA allowing for a rapid amplification process (1) To accelerate the reaction one or two additional primers, called Loop Forward (LF) and Loop Backward (LB), are added to the mix. The WarmStart® Colorimetric Master Mix that is used in this protocol, has a low buffer concentration and contains a pH indicator (phenol red) that changes colour from red to yellow due to a drop in pH by the protons that are released during the amplification process. The amplified product can be visually detected by this colour change (2).

A previous study designed unique LAMP primers for *Lactobacillus crispatus* and showed that the LAMP test can detect very diluted *Lactobacillus crispatus* template DNA (3). To prevent lactic acid-producing bacteria from giving a false positive result, we introduced a washing step to reduce the lactic acid associated with the colony by diluting it in water.

Materials

- WarmStart® Colorimetric LAMP 2x Master Mix (DNA&RNA) New England Biolabs
- *L. crispatus* primers (4)**,** prepare a 1 mM working stock
  - F3 ACGAGTTGTGAAGAGGAGTGA
  - B3 TGTTTGGGCCTATTCACTGC
  - FIP CGGTTTGCGGTACGGGTATGTCGTGTGGTAATCACACTGCCA
  - BIP AGGAACTCGGCAAAATGACCCCGGCTAACCAATCTCTTGGCT
  - LB TAACTTCGGAAGAAGGGGTGCT
- MilliQ
-
- Water bath
- Plastic toothpick
- PCR tubes

Procedure

1. Set the temperature of the water bath to 65˚C.

2. Determine how many samples you will be analysing and the number of the PCR tubes you might need, including the positive and negative controls. Use a known *crispatus* containing

material (culture, strain or vaginal sample) as a positive control. Use water as a negative control.

	Volume per reaction
Warmstart	12,5 µl
Primer mix	2,5 µl
MilliQ	10 µl
Total	**25 µl**

Prepare the master mix in PCR tubes as follows:

3. Gently scoop up a single well-isolated colony with the plastic loop or tip and suspend it in a 1,5 ml tube containing 200 micro litres of water. Spin the loop between thumb and index finger to dislodge the bacteria. The water will become turbid.

4. Add a small amount of this bacterial suspension, by dipping a second plastic toothpick in the solution and transfer to the LAMP master mix. Mix the solution with the toothpick. If there are drops at the top of your tube, gently slide them down with your toothpick.

5. Incubate the PCR tubes in the water bath for 30 minutes at 65˚C. If after 30 minutes there are samples that are orange, let them incubate for another 10 minutes.

Result analysis

6. The samples can be assessed by colour. The negative sample should remain red. The starting mix will turn from red to yellow if it is positive for *L. crispatus.* An orange colour is questionable and should be reanalysed.

When this method is used for *Lactobacillus crispatus* isolation, continue by restreaking the remaining colony on a new MRS (de Man, Rogosa and Sharpe (4)) agarose plate and incubate anaerobically for a minimum of 48 hours. The LAMP protocol could be repeated to reaffirm the previous results. Colonies from this secondary plate are then inoculated into MRS liquid medium. After incubating the culture for 24-48 hours (anaerobically) and we prepare a glycerol stock in a cryovial. We used a 60% (v/v) glycerol stock solution by pipetting 1 ml of liquid culture and 500 µl of 60% glycerol in a cryotube vials (the final glycerol concentration is 20%), flash freeze them in liquid nitrogen and store them at -80˚C.

Verification of colorimetric LAMP-test results

We grew two previously isolated and sequenced *L. crispatus* strains RL09 and RL10 (5) on tryptic soy broth (TSB) agar. We also streaked vaginal swabs with different Nugent scores on Tryptic Soy Broth agarose plates supplemented with 10% horse serum, 1g/L tween 80 and 10g/L glucose and pH set to 5 with 10% acetic acid. The inoculated agarose plates were incubated anaerobically at 37˚C for 48-72 hours. We selected the colonies based on their morphology and confirmed identy with the LAMP test. Colony PCR with specific *L. crispatus* primers was used to validate the LAMP test results. The primers used in this assay were derived from Kusters et al. (6) and produced an amplicon of 145 bp.

Forward: AACTAACAGATTTACTTCGGTAATGA
Reverse: AGCTGATCATGCGATCTGC

The identity of the positive and negative colonies were further confirmed by MALDI-TOF biotyping at the Streeklaboratorium Amsterdam.

We tested LAMP on other *Lactobacillus* species such as *L. plantarum, L. delbrueckii* subsp. *bulgaricus* and *L. gasseri* cultures as a control. These species all tested negative as well as on colony PCR. Isolates that were positive on the LAMP test were also positive on the agarose gel electrophoresis of the PCR product (Fig.1).

A

B

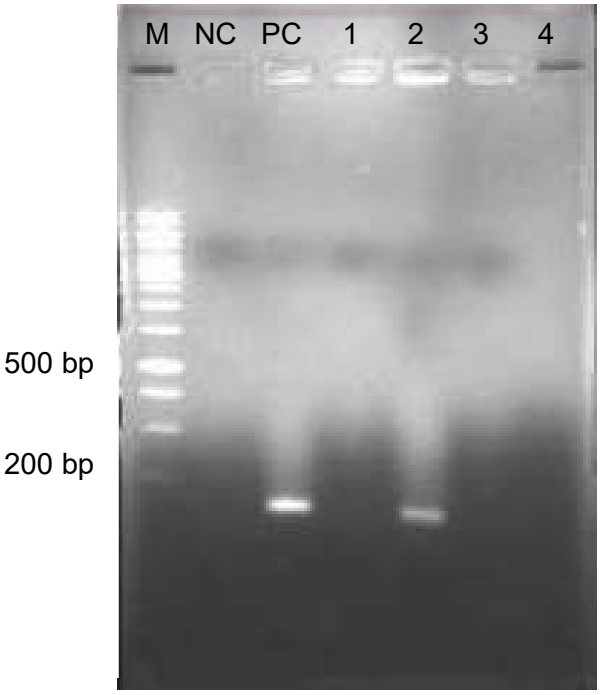

Figure 1. Panel A Visualisation of LAMP test and validation with colony PCR on agarose gel NC, Negative controle; PC, positive control (*L. crispatus* RL10 genomic DNA); 1: *L. plantarum; 2*: *L. crispatus; 3, L. delbrueckii* subsp. *bulgaricus* and 4, *L. gasseri*. as a positive control; .
Panel B Colony pcr products on on agarose gel electrophoresis to validate the LAMP results with various colonies from a vaginal swab. #2 tested positive with the LAMP test as well as with the *crispatus*-specific colony pcr. The bands match the expected 145 amplicon.

Comments

There are different ways a false positive or negative can be obtained. The assay is sensitive to acidic solutions or templates due to the pH indicator in the mix. It is important to eliminate all traces of acidity prior to the assay. Lactic acid producing organisms should first be washed in water to dilute the lactic acid associated with the colony as described in step 3. For a reliable test, make sure to use a non-absorbent material for the inoculation of the master mix. A wooden toothpick would absorb the water content, acid included, and turn the LAMP master mix yellow before incubating your samples, resulting in a false positive result. The samples should not be incubated for longer than 45 minutes. A longer incubation period may lead to false positive results. Consider the size of the colony and the amount of water to wash it in. For smaller colonies the amount of water can be lowered. We have noticed that the use of isolated DNA in Tris-HCl buffer can turn a positive result orange instead of yellow. This may be due to the buffer's neutralising effect.

Citizen science / low resource adjustments

A few replacements that could be introduced to adjust this protocol to low-resource setting:

- Instead of a water bath kitchen equipment could be used to create the constant temperature such as a 'sous-vide.'
- Vittorio Saggiomo has also introduced recycled Nespresso cups filled with phase-change material (T-cup) and a 3d printed tube holder. The phase change material is melted in boiling water, and will stay at the desired temperature for hours. (2)

References

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