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Loop-mediated isothermal amplification assays for Enterococcus sp., Escherichia coli and Staphylococcus aureus in chicken

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One sentence summary: We developed Entero-Common-LAMP, seven types of Entero-Specific-LAMP, E. coli-LAMP and S. aureus-LAMP assays for detection of Enterococcus sp., E. coli and S. aureus, respectively.

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ABSTRACT

Bacterial chondronecrosis with osteomyelitis (BCO) is a major cause of lameness in broiler chicken, and results in serious economic losses worldwide. Although the pathogenesis mechanism leading to lameness is not entirely understood, some strains of Enterococcus sp., avian pathogenic Escherichia coli or Staphylococcus aureus have been long recognized as important causative pathogens. To prevent the progression of Enterococcus sp., avian pathogenic E. coli or S. aureus infections, we developed rapid, sensitive and convenient diagnostic assays using loop-mediated isothermal amplification (LAMP). Entero-Common-LAMP assays were developed for simultaneous detection of eight Enterococcus species. To target specific microorganisms, seven Entero-Specific-LAMP assays for E. faecalis, E. faecium, E. hirae, E. gallinarum, E. avium, E. durans and E. cecorum were developed, as well as E. coli-LAMP and S. aureus-LAMP assays. Considering the prevalence and economic impact of Enterococcus sp., E. coli and S. aureus, the 10 different LAMP assays which were developed have considerable potential as routine diagnostic methods in the field or in resource-limited environments.

Keywords: LAMP; Enterococcus sp.; E. coli; S. aureus

INTRODUCTION

Bacterial chondronecrosis with osteomyelitis (BCO) in broiler chicken compromises chicken welfare and causes serious economic losses to the poultry industry worldwide because of reduced chicken productivity and death (Kestin et al. 1992). BCO, including osteomyelitis, femoral head necrosis, long bone necrosis, proximal femoral degeneration, bacterial chondritis with osteomyelitis and bacterial chondronecrosis, is an important cause of lameness in broiler chicken (McNamee and Smyth 2000; Kolbøjensen et al. 2011). BCO was first reported in 1972, and the incidence of lameness with BCO has increased significantly in Australia, USA, Canada and Europe over the past two decades, with recent reports indicating that over 1% of all broilers grown to heavy processing weights may be affected after 5 wk of age (Kestin et al. 1992; Stalker et al. 2010; Wideman 2016). An investigation in Bulgaria revealed the significant scale of the problem, with lameness accounting for 10% of mortality in lame chickens,
and BCO accounting for more than 90% of these cases (Dinev 2009). Although the complex pathogenicity mechanism of BCO is not entirely understood, Enterococcus sp., avian pathogenic Escherichia coli and Staphylococcus aureus are recognized important pathogens associated with BCO (McNamee et al. 1999; Dinev 2009; Jiang et al. 2015; Wideman 2016; Wijesurendra et al. 2017). These bacteria are ubiquitous in poultry environments where the birds are hatched, reared, or processed; they are transmitted to chicks from breeder parents, contaminated eggs, or hatchery sources by opportunistic infection (McNamee and Smyth 2000; Wideman 2016). Further, BCO appears to occur when Enterococcus sp., E. coli or S. aureus infects the broilers via the integument, respiratory system or gastrointestinal tract, circulates in the bloodstream and forms micro-abscesses that cause infarction and local metaphyseal bone necrosis (McNamee et al. 1999; Wideman 2016). The condition of broiler chicken affected by BCO usually progresses fairly rapidly from mild to severe lameness.

Development of a rapid and specific method for the detection of Enterococcus sp., E. coli or S. aureus infection in the field and in resource-limited environments is important for the prevention of the progression of BCO. Loop-mediated isothermal amplification (LAMP) is a highly specific, efficient and rapid method based on 2–3 sets of primers that target a gene under isothermal conditions, with no special equipment for DNA amplification required. After the LAMP reaction, a positive result is detected by assessing increase in sample turbidity (determined using a real-time turbidity meter) caused by the formation of a magnesium pyrophosphate byproduct, or by visual inspection (color change); there is no need for agarose gel electrophoresis (Notomi et al. 2000; Nagamine, Hase and Notomi 2002; Goto et al. 2009).

In the current study, by targeting highly conserved genes of BCO-associated bacteria, we successfully developed an Enteroc-Common-LAMP assay for simultaneous detection of common enterococci genes of eight Enterococcus sp. (E. faecalis, E. faecium, E. hirae, E. gallinarum, E. avium, E. durans, E. cecorum and E. columbae), seven types of Entero-Specific-LAMP assays for specific detection of seven Enterococci sp. (E. faecalis, E. faecium, E. hirae, E. gallinarum, E. avium, E. durans and E. cecorum), as well as E. coli-LAMP and S. aureus-LAMP assays.

**MATERIALS AND METHODS**

**Bacterial and viral strains**

Enterococcus strains E. faecalis (ATCC 29 212), E. faecium (ATCC 19 434), E. hirae (ATCC 8043), E. gallinarum (ATCC 49 573), E. avium (ATCC 14 025), E. durans (ATCC 19 432), E. cecorum (ATCC 43 198), E. columbae (ATCC 51 263), E. mundtii (ATCC 43 186), E. saccharolyticus (ATCC 43 076), E. casseliflavus (ATCC 25 788) and E. sulfureus (ATCC 49 903), Escherichia coli (ATCC 25 922), Staphylococcus strains S. aureus (ATCC 25 923), S. cholni (ATCC 35 662), S. xylosus (ATCC 29 971), S. lentus (ATCC 49 574), S. hominis (field isolate) and S. epidermidis (field isolate), Ornithobacterium rhinotracheale (field isolate), Pasteurella multocida (field isolate), Mycoplasma gallisepticum (ATCC 19 610), Mycoplasma synoviae (ATCC 25 204), Bacillus cereus (ATCC 14 579), Campylobacter coli (ATCC 33 559), Clostridium perfringens (ATCC 13 124), Campylobacter jejuni (ATCC 33 560), Salmonella enteritidis (ATCC 31 194), chicken infectious anemia virus (GIAV, field isolate), reticuloendotheliosis virus (REV, field isolate) and Marek’s disease virus (MDV, ATCC VR-624) were from the American Type Culture Collection, and were used as reference strains in the current study.

**Isolation of DNA**

Total bacterial and viral DNA was extracted from the microorganisms using QIAamp DNA mini kit (Qiagen, Germany) and QIAamp DNeasy Blood and Tissue kit (Qiagen), according to the manufacturer’s instructions.

**Design of universal and species-specific LAMP primers**

Enteroc-Common-LAMP primers for simultaneous detection of E. faecalis, E. faecium, E. hirae, E. gallinarum, E. avium, E. durans, E. cecorum and E. columbae were designed based on the published rpoB gene sequences of the Enterococcus sp. Additional LAMP primers, specific to E. faecalis, E. faecium, E. hirae, E. gallinarum, E. avium, E. cecorum and E. durans, were designed to target specific variable regions by using Primer Explorer V4 software (Eiken Chemical Co. Ltd., Japan). The mrlB and nuc genes were selected as target genes for the detection of E. coli and S. aureus. These LAMP primers included the following: a forward outer primer F3; a reverse outer primer B3; a forward inner primer FIP (harboring the F2 region sequence at its 3’-end and the F1c region sequence at its 5’-end); a reverse inner primer BIP (harboring the B2 region sequence at its 3’-end and the B1c region sequence at its 5’-end); a forward loop primer LF; and a reverse loop primer LB. These primers recognized eight conserved regions within their target genes (Table 1).

**LAMP assays**

The LAMP reactions were performed in 25 μl reaction volumes using a Mmiso DNA amplification kit (Mmonitor, South Korea), in accordance with the manufacturer’s instructions. The reaction mixtures contained 1 μl of bacterial genomic DNA, 2 × reaction buffer, 8 U of Bst DNA polymerase, 2.5 pmol of the outer primers (F3 and B3), 20 pmol of the forward and reverse inner primers (FIP and BIP), and 10 pmol of the loop primers (LF and LB). The LAMP assays were performed under isothermal conditions at 63 °C for 30 min, followed by heating to 80 °C for 5 min in a heating block, to terminate the reaction.

**The specificity and detection limits of LAMP**

The specificities of the optimized Enteroc-Common-LAMP, seven types of Entero-Specific-LAMP assays (i.e. E. faecalis-LAMP, E. faecium-LAMP, E. hirae-LAMP, E. gallinarum-LAMP, E. avium-LAMP, E. durans-LAMP and E. cecorum-LAMP), E. coli-LAMP and S. aureus-LAMP assays were tested using all bacteria and viruses. Each LAMP reaction was performed using 25 ng of genomic DNA, at 63 °C for 30 min, and terminated by heating to 80 °C for 5 min in a heating block. The assay detection limits were determined by testing 5-fold serial dilutions of bacterial DNA of E. faecalis, E. faecium, E. hirae, E. gallinarum, E. avium, E. durans, E. cecorum, E. columbae, E. coli and S. aureus.

**PCR**

To compare the detection limits of the PCR and LAMP assays, the target genes from E. faecalis, E. faecium, E. hirae, E. gallinarum, E. avium, E. durans, E. cecorum, E. columbae, E. coli and S. aureus were PCR-amplified in individual reactions. The reaction volume was 20 μl, and the reactions contained 2.5 mM each dNTP, 1.5 mM MgCl2, 10 × reaction buffer, 1 U of Taq polymerase, 10 pM LAMP F3 and B3 primers, and 1 μl of serial dilutions of template DNA. The thermal cycler (Eppendorf, Germany) was set to the
| Primer set no. | LAMP assay                  | Target bacterium                  | Target gene | Primer | Sequence (5′–3′)                      |
|---------------|----------------------------|----------------------------------|-------------|--------|--------------------------------------|
| 1             | Entero-Common-LAMP          | Enterococcus sp.                 | rpoB        | F3     | GAAGCTGCGCGATGAATTATC                 |
|               |                            |                                  |             | B3     | CCTAAAGGRTTCAACATGATATC               |
|               |                            |                                  |             | FIP(F1c + F2) | TGACGTCGCGCCATTTTATCCCGAGTGTAATAATCGTGG |
|               |                            |                                  |             | BIP(B1c + B2) | AAAAAAGGGTTTCTCCGTTATGTGTTCCGGTCWGTAAGAA |
|               |                            |                                  |             | LF     | GTTCACTATTGAAATTGTTTTGTAAGG          |
|               |                            |                                  |             | LB     | ATGCGCGAGAAGATGATACCG                |
| 2             | Entero-Specific-LAMP        | Enterococcus faecalis            | Cell surface protein | F3     | GAA GGA AAA ACG GTC CAA GA         |
|               |                            |                                  |             | B3     | TCC CTT TAC CAC TTT GC              |
|               |                            |                                  |             | FIP(F1c + F2) | ATTTGTTGCTGTTTATCCGACAATCGTAATTAGAAGAGC |
|               |                            |                                  |             | BIP(B1c + B2) | GAG AAG ATG GAG TGG TTT CCT GCT TCA ACA AAT TAG GCT T |
|               |                            |                                  |             | LF     | CGG ATG TGT TTT CTA TTT GCA C     |
|               |                            |                                  |             | LB     | ATT AGC TAG CAA AGA TTT GCC         |
| 3             | Entero-Common-LAMP          | Enterococcus faecium             | Cell wall protein | F3     | ATG CTT ATT TGG CTA CAC AGG         |
|               |                            |                                  |             | B3     | TTT GAG ATC TGC CTT TGA AT         |
| 4             | Entero-Common-LAMP          | Enterococcus hirae               | ftsW        | F3     | TGG CTA CAG ATT CTC ATT GAT TGG GCG TAC GCA AAT GAA ATT ATT T |
|               |                            |                                  |             | B3     | CTA ATC TCC ACC ACC GCA            |
| 5             | Entero-Common-LAMP          | Enterococcus gallinarum          | atpA        | F3     | TGA AGG TGA CAA AGT AAA AGG A      |
|               |                            |                                  |             | B3     | TTT CTA ATC CAG TCT GCA TTTG      |
| 6             | Entero-Common-LAMP          | Enterococcus avium               | ddl         | F3     | CAT GGA TTT GGT GTC CTA AGC GAA     |
|               |                            |                                  |             | B3     | AAA TCA TGG TGA TTT GCA AAA TCA    |
| 7             | Entero-Common-LAMP          | Enterococcus durans              | Amino acid permease | F3     | ATA ACA CGA GAA CTA TCT GAA         |
|               |                            |                                  |             | B3     | TTT GGA TGG GAG TTT GCT TGG AGG AAG |

The table lists the LAMP primers for the detection of Enterococcus sp., E. coli and S. aureus. Each row corresponds to a specific primer set, indicating the target bacterium, target gene, primer set, and the primer sequences. The sequences include forward (F3) and reverse (B3) primers, as well as the forward inner (FIP) and reverse inner (BIP) primer pairs, with specific sequences tailored to detect individual bacterial strains or genes.
### RESULTS

#### Detection of *Enterococcus* sp., *E. coli* and *S. aureus* in field samples

The Entero-Common-LAMP, seven types of Entero-Specific-LAMP, *E. coli*-LAMP and *S. aureus*-LAMP assays were used to analyze *E. faecalis*, *E. faecium*, *E. hirae*, *E. gallinarum*, *E. avium*, *E. cecorum*, *E. coli* and *S. aureus*, which were isolated from the livers, femurs and joints of broiler chickens with lameness using Enterococcus agar, MacConkey agar and Mannitol salt agar. The isolated *Enterococcus* sp., *E. coli* and *S. aureus* strains were cultivated on the tryptic soy agar. Genomic DNA was extracted and used in the nine types of LAMP assays to compare the results of 16S rRNA sequencing, PCR and LAMP assays.

#### Specificity of LAMP assays

The specificity of optimal LAMP primer sets was examined using 25 ng of genomic DNA extracted from 28 different bacteria representing various genera and species, and three viruses. As shown in Fig. 1A, it was only in tubes containing eight strains of *Enterococcus* genomic DNA and specific primers that the reaction mixture color changed from violet to sky blue, while the mixtures in other tubes remained violet. Likewise, the LAMP primers specific to *E. coli*, *S. aureus*, *E. faecalis*, *E. faecium*, *E. hirae*, *E. gallinarum*, *E. avium*, *E. durans* and *E. cecorum* yielded amplification products only in those reaction tubes that contained the specific target genomic DNA (Fig. 1C and E; Fig. 2A, C, E, G, I, K and M). The positive reactions were also confirmed by the presence of ladder-like DNA bands on 1.5% TAE agarose gels (Fig. 1D and F; Fig. 2B, D, F, H, J, L and N).

#### Comparison detection limits of LAMP and PCR assays

To compare the detection limits of the 10 types of LAMP assays and that of conventional PCR, 5-fold serially diluted genomic DNA samples from *E. faecalis*, *E. faecium*, *E. hirae*, *E. gallinarum*, *E. avium*, *E. durans*, *E. cecorum*, *E. columbae*, *E. coli* and *S. aureus* were used. The evaluation of the reaction detection limits was performed using primers LAMP F3 and B3 by PCR (Table 1). As shown in Fig. 3, the detection limits for the Entero-Common-LAMP assay were 2 pg/μl for *E. faecalis* and *E. durans*, 10 pg/μl following PCR conditions: 94°C for 5 min; 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s; with a final elongation at 72°C for 7 min. For the Entero-common-PCR with *E. gallinarum* and *E. avium*-specific-PCR of *E. avium*, the reaction conditions were as follows: an initial denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min and polymerization at 72°C for 1 min; and an extension at 72°C for 7 min. The PCR products were analyzed under UV light after 1.5% agarose gel electrophoresis.

### Table 1. Continued.

| Primer set no. | Primer | Target bacterium | Target gene | Sequence (5’→3’) |
|---------------|--------|-----------------|-------------|-----------------|
| 8             | F3     | *Enterococcus* cecorum | rpoA       | CCT AAT ATG CAC GAT TTC CAA A |
|               | B3     |                 | malB       | TTC GTC AGC TTG AAC GTA A |
|               | LF     |                 | nuc        | FIP (F1c + F2) GTT TAA GAT TTC AAC ATC AGA ATC GAA ATC GAT ATT ACT GGT CCA GCA |
|               | LB     |                 |            | BIP (B1c + B2) GAT ATG TAC ATT TGT ACA GTC AGT ACC TGC TTT CAC TTT TAG GCG |
| 9             | F3     | *Escherichia* coli | malB       | CAC CTT CAT GGA TAT CGA GAT T |
|               | B3     |                 |            | TGG AGG ATT TAA GCC ATC TC |
|               | LF     |                 |            | FIP (F1c + F2) CGA GCG TAC AGC TGC AAA ATG ATA TCT TTC GAT ACC ACG ACC T |
|               | LB     |                 |            | BIP (B1c + B2) CCC TTC TCC CTT TGT AAC AAG ATG ACG CAT AGT CAG CCC AT |
| 10            | F3     | *Staphylococcus* aureus | nuc        | CAA TAA TGT CAC CTG CAG TTA CAA |
|               | B3     |                 |            | CTA GTC ATC AGC AAC AAG ATC |
|               | LF     |                 |            | FIP (F1c + F2) AAT GTC ATT GGT TGA CCT TTG TAC AAT TAC ATA AAG AAC CTG CGA C |
|               | LB     |                 |            | BIP (B1c + B2) CCC TTC TCC CTT TGT AAC AAG ATG ACG CAT AGT CAG CCC AT |
for E. faecalis and E. coli, 400 fg/μl for E. hirae, 40 pg/μl for E. gallinarum, 4 pg/μl for E. avium and 50 pg/μl for E. cecorum. Therein, the success of the LAMP reaction was detected with the naked eye and by agarose gel electrophoresis. On the other hand, the detection limits of PCR using the Enterococcus species primers and B. cereus were 50 pg/μl for E. faecalis, 4 pg/μl for E. hirae, 4 pg/μl for E. gallinarum, 4 pg/μl for E. avium, 2 pg/μl for E. cecorum and 6.25 ng/μl for E. coli (Fig. 5A-D). Seven types of Enterococcus-specific-LAMP assays detected the target genes from 400 fg/μl for E. faecalis, E. hirae, 10 pg/μl for E. avium, 16 pg/μl for E. cecorum and 2 pg/μl for S. aureus, respectively (Table 2). The detection limits of PCR for the Enterococcus-specific-LAMP primers were 2 pg/μl and 400 fg/μl DNA, respectively; however, the detection limits of LAMP were 50 pg/μl for E. coli and 10 pg/μl for S. aureus (Fig. 5B and Q). In conclusion, the sensitivity of the Enterococcus-specific-LAMP and Enterococcus-specific-LAMP assays (for E. faecalis, E. faecium, E. hirae, E. gallinarum, E. avium, E. durans and E. cecorum), and E. coli-LAMP and S. aureus-LAMP assays, was higher than that of conventional PCR for detecting pathogens associated with the lameness in broiler chicken.

Detection of pathogen-related lameness in the clinical samples

In total, 140 bacterial strains isolated during 2016 and 2017 from the livers, femurs and joints of broiler chickens with lameness from the Animal and Plant Quarantine Agency in Korea were analyzed by LAMP assays, conventional PCR and 16S rRNA sequencing. The nine types of LAMP assays and PCR were congruent (100%). The agreement between the LAMP assays and 16S rRNA sequencing was 92.6%, 83.9%, 95.2%, 0%, 100% and 95% for the detection of E. faecalis, E. faecium, E. hirae, E. gallinarum, E. avium and the avian pathogenic E. coli, respectively (Table 2).

DISCUSSION

Lameness with BCO is an important skeletal disease of broiler chicken (Nairn and Watson 1972). Some strains of Enterococcus sp., avian pathogenic E. coli and S. aureus are recognized as important BCO pathogens in the poultry industry, and are isolated from single or mixed cultures from BCO lesions (Wideman et al. 2012; Braga et al. 2016). In addition, E. cecorum and E. hirae are recovered from the joints and femurs of lame birds (Wood et al. 2002; Staliker et al. 2010; Kense and Landman 2011; Kolbjørnsen et al. 2011; Jung and Rautenschlein 2014). Entero- cocci, including E. faecalis, E. faecium, E. avium, E. durans and E. gallinarum, are frequently isolated from the litter, feed, dead-shell, or 1-day-old chicks in poultry farms (Cortes et al. 2004; Deeming 2005).

In one study, avian pathogenic E. coli was recovered from over 90% of bacteriologically tested chickens with lameness...
Figure 2. Specificity of seven types of the Entero-Specific-LAMP assays: (A) E. faecalis-LAMP; (C) E. faecium-LAMP; (E) E. hirae-LAMP; (G) E. gallinarum-LAMP; (I) E. avium-LAMP; (K) E. durans-LAMP; and (M) E. cecorum-LAMP. In these assays, a color change from violet to sky blue was observed only in the tubes containing the target genomic DNA. The products of the LAMP assays were resolved by 1.5% agarose gel electrophoresis: (B) E. faecalis-LAMP; (D) E. faecium-LAMP; (F) E. hirae-LAMP; (H) E. gallinarum-LAMP; (J) E. avium-LAMP; (L) E. durans-LAMP; and (N) E. cecorum-LAMP.
Figure 3. Detection limits of the Entero-Common-LAMP assay. (A) Naked-eye visualization of the LAMP products. The color of LAMP-positive reactions turned sky blue, while the color of LAMP-negative reactions remained violet. (B) Agarose gel electrophoresis of LAMP products. Lane M, 100 bp DNA marker. E. faecalis genomic DNA (lanes/tubes): 1, 10 pg/ul; 2, 2 pg/ul; 3, 400 fg/ul; 4, 80 fg/ul; and 5, 16 fg/ul. E. faecium genomic DNA (lanes/tubes): 6, 50 pg/ul; 7, 10 pg/ul; 8, 2 pg/ul; 9, 400 fg/ul; and 10, 80 fg/ul. E. durans genomic DNA (lanes/tubes): 11, 10 pg/ul; 12, 2 pg/ul; 13, 400 fg/ul; 14, 80 fg/ul; and 15, 16 fg/ul. E. hirae genomic DNA (lanes/tubes): 16, 10 pg/ul; 17, 2 pg/ul; 18, 400 fg/ul; 19, 80 fg/ul; and 20, 16 fg/ul. E. columbae genomic DNA (lanes/tubes): 21, 250 pg/ul; 22, 50 pg/ul; 23, 10 pg/ul; 24, 2 pg/ul; and 25, 400 fg/ul. E. avium genomic DNA (lanes/tubes): 26, 500 pg/ul; 27, 100 pg/ul; 28, 20 pg/ul; 29, 4 pg/ul; and 30, 800 fg/ul. E. gallinarum genomic DNA (lanes/tubes): 31, 5 ng/ul; 32, 1 ng/ul; 33, 200 pg/ul; 34, 40 pg/ul; and 35, 8 pg/ul. E. cecorum genomic DNA (lanes/tubes): 36, 1.25 ng/ul; 37, 250 pg/ul; 38, 50 pg/ul; 39, 10 pg/ul; and 40, 2 pg/ul. Lane 41, negative control.

Figure 4. Detection limits of seven types of the Entero-Specific-LAMP, E. coli-LAMP and S. aureus-LAMP assays. Visual inspection of LAMP products for the detection of (A) E. faecalis, (C) E. faecium, (E) E. hirae, (G) E. gallinarum, (I) E. avium, (M) E. cecorum, (O) E. coli and (Q) S. aureus under natural light. Agarose gel electrophoresis of LAMP products from different LAMP assays: (B) E. faecalis-LAMP, (D) E. faecium-LAMP, (F) E. hirae-LAMP, (H) E. gallinarum-LAMP, (J) E. avium-LAMP, (L) E. durans-LAMP, (N) E. cecorum-LAMP, (P) E. coli-LAMP and (R) S. aureus-LAMP. Lane M, 100 bp DNA marker; lanes (tubes): 1, 250 pg/ul; 2, 50 pg/ul; 3, 10 pg/ul; 4, 2 pg/ul; 5, 400 fg/ul; 6, 80 fg/ul; 7, 16 fg/ul; and 8, negative control.

(Dinev 2009), and was the most frequently isolated bacterium from chickens with BCO (Wijesurendra et al. 2017). S. aureus is the major pathogen responsible for bone and joint infections (McCullagh et al. 1998), and is also isolated from the litter, feeders, drinkers and the air in poultry houses (Thompson, Gibbs and Patterson 1980; Sauter et al. 1981; McNamee et al. 1999). Enterococcus sp., avian pathogenic E. coli and S. aureus are also responsible for significant financial losses worldwide. Therefore, they should be detected precisely and as early as possible to eradicate them and prevent their transmission. To achieve this, a simple and rapid diagnostic method for the detection of Enterococcus sp., E.
Figure 5. Electrophoretic analysis of PCR products to compare the detection limits of conventional PCR and LAMP assays. PCR was performed to detect (A) E. faecalis, (B) E. faecium, (C) E. hirae, (D) E. gallinarum, (E) E. avium, (F) E. durans, (G) E. cecorum and (H) E. columbae using universal primers F3 and B3 for Enterococcus species. Gels in (I–Q) show resolved PCR products of specific target genes from (I) E. faecalis, (J) E. faecium, (K) E. hirae, (L) E. gallinarum, (M) E. avium, (N) E. durans, (O) E. cecorum, (P) E. coli and (Q) S. aureus. Lanes: M, 100 bp DNA marker; 1, 6.25 ng/ul; 2, 1.25 ng/ul; 3, 250 pg/ul; 4, 50 pg/ul; 5, 10 pg/ul; 6, 2 pg/ul; 7, 400 fg/ul; 9, 31.3 ng/ul; 10, 6.25 ng/ul; 11, 1.25 ng/ul; 12, 250 pg/ul; 13, 50 pg/ul; 14, 10 pg/ul; 15, 2 pg/ul; 8 and 16, negative control.

Table 2. Outcomes of nine types of LAMP assays of clinical samples, compared with the diagnostic PCR and 16S rRNA sequencing assays

| Species                  | LAMP P/T | N/T | F/T | Sensitivity | PCR P/T | N/T | F/T | Sensitivity | 16S rRNA P/T | N/T | F/T | Sensitivity |
|--------------------------|----------|-----|-----|-------------|---------|-----|-----|-------------|--------------|-----|-----|-------------|
| Enterococcus sp. common  | 87/140   | 53/140 | 0/140 | 100%        | 87/140  | 53/140 | 0/140 | 100%        | NA           | NA  | NA | NA          |
| E. faecalis              | 27/140   | 113/140 | 0/140 | 100%        | 27/140  | 113/140 | 0/140 | 100%        | 25/140       | 113/142 | 2/140 | 92.6%       |
| E. faecium               | 27/140   | 113/140 | 0/140 | 100%        | 27/140  | 113/140 | 0/140 | 100%        | 26/140       | 109/140 | 5/140 | 83.9%       |
| E. hirae                 | 20/140   | 120/140 | 0/140 | 100%        | 20/140  | 120/140 | 0/140 | 100%        | 20/140       | 119/140 | 1/140 | 95.2%       |
| E. gallinarum            | 9/140    | 131/140 | 0/140 | 100%        | 9/140   | 131/140 | 0/140 | 100%        | 0/140        | 131/140 | 9/140 | 0%          |
| E. avium                 | 1/140    | 139/140 | 0/140 | 100%        | 1/140   | 139/140 | 0/140 | 100%        | 1/140        | 139/140 | 0/140 | 100%        |
| E. cecorum               | 3/140    | 137/140 | 0/140 | 100%        | 3/140   | 137/140 | 0/140 | 100%        | NA           | NA  | NA | NA          |
| E. coli                  | 38/140   | 102/140 | 0/140 | 100%        | 38/140  | 102/140 | 0/140 | 100%        | 38/140       | 100/140 | 2/140 | 95%         |
| S. aureus                | 10/140   | 130/140 | 0/140 | 100%        | 10/140  | 130/140 | 0/140 | 100%        | NA           | NA  | NA | NA          |

*P, number of true positives; T, number of total samples; N, number of true negatives; F, number of false positives and false negatives; NA, not applicable.

coli and S. aureus in broiler chicken with lameness is necessary. At present, MALDI-TOF, VITEK and 16S rRNA sequencing analyses following bacterial isolation are routinely used for the identification of Enterococcus sp., E. coli and S. aureus. Although bacterial identification after isolation is the most reliable method, it is time-consuming and labor-intensive. PCR-based methods are well optimized with respect to the sensitivity, specificity and repeatability of the amplification of a target gene, and detect pathogens more quickly than bacterial culture. However, these methods require special equipment, such as thermal cyclers.
and skilled labor, and PCR amplicons must be analyzed by electrophoresis (Peters et al. 2004). By contrast, LAMP is a simple, rapid, efficient and cost-effective method, which uses a water bath or block heater to amplify the target DNA under isothermal conditions. The success of the LAMP amplification reaction may be assessed by the naked eye, either as a turbidity change (white precipitate formation), or through a color change (from violet to sky blue), without the need for electrophoretic analysis.

In the current study, we developed different types of LAMP assays to detect Enterococcus sp., E. coli and S. aureus. We designed 10 sets of primers (six primers each) targeting the rpoB gene from eight common Enterococcus species, seven Enterococcus-specific genes (i.e. a cell surface protein gene of E. faecalis, a cell wall protein gene of E. faecium, the ftsW gene of E. hirae, the atpA gene of E. gallinarum, the ddi gene of E. avium, an amino acid permease gene of E. durans and the rpoA of E. cecorum), the malB gene of E. coli and the nuc gene of S. aureus.

We then tested the reaction detection limits and specificity in LAMP reactions performed at 63°C for 30 min. The detection limit of the Entero-Common-LAMP assay was between 50 pg/μl and 400 fg/μl, whereas the detection limit of the conventional PCR using the Entero-Common-LAMP primers F3 and B3 was between 1.25 ng/μl and 10 pg/μl. This demonstrated that the Entero-Common-LAMP assay was 5–10 times more sensitive than the Entero-common-PCR.

Specifically, in the case of E. hirae, the detection limits of Entero-Common-LAMP assay and Entero-common-PCR were 400 fg/μl and 1.25 ng/μl, respectively, which indicated that LAMP was 3125 times more sensitive than the PCR reaction. Further, seven types of Entero-Specific-LAMP assays detected the target genes from 400 fg/μl E. faecalis, E. faecium, E. hirae and E. avium DNA, and from 2 pg/μl E. gallinarum, E. durans and E. cecorum DNA. By contrast, the detection limits of PCR with the Entero-Specific-LAMP primers F3 and B3 were 250 pg/μl for E. faecalis, E. faecium, E. gallinarum and E. durans, 50 pg/μl for E. hirae and E. cecorum, and 6.25 ng/μl for E. avium. The sensitivity of the Entero-Specific-LAMP assays was therefore 25–625 times higher than that of the Entero-specific-PCR reactions. Above all, the sensitivity of the LAMP assay for the detection of E. avium was 15 625 times higher than that of the PCR reaction.

The detection limits of E. coli-LAMP and S. aureus-LAMP were 2 pg/μl and 400 fg/μl, respectively; however, the detection limits of the PCR reactions were 50 pg/μl for E. coli and 10 pg/μl for S. aureus.

Furthermore, the sensitivities of the E. faecalis-LAMP and S. aureus-LAMP assays were superior to those reported previously (Lim, Teh and Thong 2013; Zhao et al. 2013; Wang et al. 2015; Martzy et al. 2017). Collectively, these observations indicated that the sensitivity of the 10 LAMP assays was much higher than that of conventional PCR and of previously devised LAMP assays.

The specificity tests for the Entero-Common-LAMP, the seven types of Entero-Specific-LAMP and the E. coli-LAMP and S. aureus-LAMP assays revealed that the target genes were successfully detected without cross-reactivity with other avian bacterial and viral pathogens.

The practical application of the LAMP assays was evaluated using 140 samples, and the outcomes were compared with those of PCR and 16S rRNA sequencing. The seven LAMP assays and PCR reactions accurately identified all samples of different Enterococcus isolates (including E. faecalis, E. faecium, E. hirae, E. avium and E. cecorum) at the genus and species level.

Further, the LAMP and PCR assays were 100% congruent for both S. aureus and E. coli detection. By contrast, the results of 16S rRNA sequencing indicated 92.6%, 83.9%, 95.2% and 100% agreement in the identification of E. faecalis, E. faecium, E. hirae and E. avium, respectively. Strikingly, E. gallinarum was not identified by using 16S rRNA sequencing, while the identification rate using E. gallinarum-LAMP and E. gallinarum-PCR was 100%. This indicated that 16S rRNA sequencing was less efficient in identifying the Enterococcus species than LAMP assays and conventional PCR. Finally, the VITEK 2 system was used for the detection of three E. cecorum and 10 S. aureus strains, and the congruence of the LAMP assays and VITEK 2 was 100% for both bacteria (data not shown). The results of the nine types of LAMP assays were also confirmed by sequencing. This indicated that the LAMP assays yielded accurate results within 30 min compared with those generated by 16S rRNA sequencing (18–24 h), VITEK 2 (18–24 h) and conventional PCR (3–4 h).

We presented the first-ever Entero-Common-LAMP assay and seven types of Entero-Specific-LAMP assays using new target genes. Additionally, we developed E. coli-LAMP and S. aureus-LAMP assays for detection of E. coli and S. aureus, respectively. The high specificity and amplification ability of the 10 types of LAMP assays allowed an easy and rapid visualization of the amplification success without the need for gel electrophoresis.

In conclusion, the established LAMP detection methods have the potential to become a very useful tool for the prevention of disease transmission or outbreaks in the field or in resource-limited environments.

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REFERENCES

Braga JF, Silva CC, Teixeira MP et al. Vertebral osteomyelitis associated with single and mixed bacterial infection in broilers. Avian Pathol 2016;45:640–8.

Cortes CR, Téllez Isaias G, Lopez Cuello C et al. Bacterial isolation rate from fertile eggs, hatching eggs, and neonatal broilers with yolk sac infection. Rev Latinoam Microbiol 2004;46:12–6.

Deeming DC. Yolk sac, body dimensions and hatching quality of ducklings, chicks and pouls. Br Poult Sci 2005;46:560–4.

Dinev I. Clinical and morphological investigations on the prevalence of lameness associated with femoral head necrosis in broilers. Br Poult Sci 2009;50:284–90.

Goto M, Honda E, Ogura A et al. Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy napthol blue. Biotechniques 2009;46:167–72.

Jiang T, Mandal RK, Wideman RF, Jr et al. Molecular survey of bacterial communities associated with bacterial chondronecrosis with osteomyelitis (BCO) in broilers. PLoS One 2015;10:e0124403.

Jung A, Rautenschlein S. Comprehensive report of an Enterococcus cecorum infection in a broiler flock in Northern Germany. BMC Vet Res 2014;10:311.

Kense MJ, Landman WJ. Enterococcus cecorum infections in broiler breeders and their offspring: molecular epidemiology. Avian Pathol 2011;40:603–12.
Kestin SC, Knowles TG, Tinch AE et al. Prevalence of leg weakness in broiler chickens and its relationship with genotype. Vet Rec 1992;131:190–4.

Kolbjørnsen Ø, David B, Gilhuus M. Bacterial osteomyelitis in a 3-week-old broiler chicken associated with Enterococcus hirae. Vet Pathol 2011;48:1134–7.

Lim KT, Teh CS, Thong KL. Loop-mediated isothermal amplification assay for the rapid detection of Staphylococcus aureus. Biomed Res Int 2013;2013:895816.

Martzzy R, Kolm C, Brunner K et al. A loop-mediated isothermal amplification (LAMP) assay for the rapid detection of Enterococcus spp. in water. Water Res 2017;122:62–9.

McNamee PT, McCullagh JJ, Smyth JA et al. The use of pulsed field gel electrophoresis to investigate the epidemiology of Staphylococcus aureus infection in commercial broiler flocks. Vet Microbiol 1998;63:275–81.

McCullagh JJ, McNamee PT, Smyth JA et al. Development of an experimental model of bacterial chondronecrosis with osteomyelitis in broilers following exposure to Staphylococcus aureus by aerosol, and inoculation with chicken anaemia and infectious bursal disease viruses. Avian Pathol 1999;28:26–35.

McNamee PT, Smyth JA. Bacterial chondronecrosis with osteomyelitis (‘femoral head necrosis’) of broiler chickens: a review. Avian Pathol 2000;29:253–70.

Nagamine K, Hase T, Notomi T. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol Cell Probes 2002;16:223–9.

Nairn ME, Watson AR. Leg weakness of poultry: a clinical and pathological characterisation. Aust Vet J 1972;48:645–56.

Notomi T, Okayama H, Masubuchi H et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 2000;28:e63.

Peters RP, van Agtmael MA, Danner SA et al. New developments in the diagnosis of bloodstream infections. Lancet Infect Dis 2004;4:751–60.

Sauter EA, Petersen CF, Steele EE et al. The airborne microflora of poultry houses. Poult Sci 1981;60:569–74.

Stalker MJ, Brash ML, Weisz A et al. Arthritis and osteomyelitis associated with Enterococcus cecorum infection in broiler and broiler breeder chickens in Ontario, Canada. J Vet Diagn Invest 2010;22:643–5.

Thompson JK, Gibbs PA, Patterson JT. Staphylococcus aureus in commercial laying flocks: incidence and characteristics of strains isolated from chicks, pullets and hens in an integrated commercial enterprise. Br Poult Sci 1980;21:315–30.

Wang XR, Wu LF, Wang Y et al. Rapid detection of Staphylococcus aureus by loop-mediated isothermal amplification. Appl Biochem Biotechnol 2015;175:882–91.

Wideman RF, Jr, Hamal KR, Stark JM et al. A wire-flooring model for inducing lameness in broilers: evaluation of probiotics as a prophylactic treatment. Poult Sci 2012;91:870–83.

Wideman RF, Jr. Bacterial chondronecrosis with osteomyelitis and lameness in broilers: a review. Poult Sci 2016;95:325–44.

Wijesurendra DS, Chaming AN, Bushell RN et al. Pathological and microbiological investigations into cases of bacterial chondronecrosis and osteomyelitis in broiler poultry. Avian Pathol 2017;46:683–94.

Wood AM, MacKenzie G, McGiliveray NC et al. Isolation of Enterococcus cecorum from bone lesions in broiler chickens. Vet Rec 2002;150:27.

Zhao X, Li Y, Park M, Wang J et al. Loop-mediated isothermal amplification assay targeting the femA gene for rapid detection of Staphylococcus aureus from clinical and food samples. J Microbiol Biotechnol 2013;23:246–50.