FEMS Microbiology Letters, 366, 2019, fnz042

doi: 10.1093/femsle/fnz042
Advance Access Publication Date: 26 February 2019
Research Letter

RESEARCH LETTER – Pathogens & Pathogenicity

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

HyeSoon Song, YouChan Bae, HyukMan Kwon, YongKuk Kwon and SeongJoon Joh[*,†]

Avian disease division, Animal and Plant Quarantine Agency, HyukSin 8-ro, GimCheon, Republic of Korea

[*]**Corresponding author:** 177, HyukSin 8-ro, GimCheon, Gyeongsangbuk-do, 39660, Republic of Korea. Avian Diseases Division, Animal and Plant Quarantine Agency. Tel: +82 54 912 0815; E-mail: johsj0901@korea.kr

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.

[†]Major: Diagnosis of Avian Diseases

Editor: Wolfgang Kneifel

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; Kolbjørnsen *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,

Received: 14 September 2018; **Accepted:** 24 February 2019

© FEMS 2019. This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com

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 Entero-Common-LAMP assay for simultaneous detection of common enterococcus 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 *Enterococcus* 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. cohnii* (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 (CIAV, 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

Entero-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 *malB* 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 $\times$ 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 Entero-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 $MgCl_2$, 10 $\times$ 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

Table 1. LAMP primers for the detection of *Enterococcus* sp., *E. coli* and *S. aureus*.

Primer set no.	LAMP assay	Target bacterium	Target gene	Primer	Sequence (5′–3′)
1	Entero-Common-LAMP	*Enterococcus* sp.	*rpoB*	F3	GAAGCTGGCGATGAATTATC
				B3	CCTAATGGRTTCAACATGATATC
				FIP(F1c + F2)	TGACGTCCGGCCATTTTATCCCAGGYGTAAAYATGTTAGTTCG
				BIP(B1c + B2)	AATAAAGGGGTTGTTTCCCGTATGGTGTTCCGTCWGGTAAGAA
				LF	CCTTCATGGATTTTACGTTTTTGAACG
				LB	ATGCCGGAAGAAGATATGCC
2	Entero-Specific-LAMP	*Enterococcus faecalis*	Cell surface protein	F3	GAA GGA AAA ACG GTC CAA GA
				B3	TTC CTT TAC CAC TTC TGG TG
				FIP(F1c + F2)	ATT TGT TGT CTG TGT TTT ATC TTC ACA GCG TCA ATT AGC AGA AAC C
				BIP(B1c + B2)	GAG AAG ATG GAG TGG TTT CTT TCC GCT TCA ACA AAT AAA TAG GCT T
				LF	GCG ATC GGT TTT CTA TTT GTT GCA C
				LB	ATT AGC TAG CAA AGA TTC GCA GCA
3		*Enterococcus faecium*	Cell wall protein	F3	ATG TCT AAT TGG CTA CAC AGG
				B3	TTG GAC ATC TGC CTT TGA AT
				FIP(F1c + F2)	CTG TGT CAG ATT CTC ATT GAT TGG CGG TAC GCA AAT GAA ATT ATT T
				BIP(B1c + B2)	ATC ATA CAT TGA CAG ATA AAG AGC TTG CAA TGT CTG TTC TTT TTG TGC
				LF	ATC AAT ATC TGT CTC TCC ACC CCA
				LB	GCG ATT ACA GAT CTT ACC GCT CG
4		*Enterococcus hirae*	*ftsW*	F3	TAG AAA TGA CCG CAG TGT TC
				B3	TAA GAT CAA GCT AGC AAT CGC
				FIP(F1c + F2)	CTC CCG CAA TTT TAG GTT GAA CTT CAT ATA CCA GTA TTG ATC GT
				BIP(B1c + B2)	CTG ATG ATT TTA GCT ATT GCT GGA AAT TAT CAG CCC TTT TTT AAT GGG
				LF	CAA AAC AAG CAC GGC GAT TCC
				LB	GCT ATC TTT TGG GCT GCA GCT AT
5		*Enterococcus gallinarum*	*atpA*	F3	TGA AGG TGA CAA AGT AAA ACG T
				B3	CTT TCA ATC CAG TCT GCA TTG
				FIP(F1c + F2)	CAT CGA TTG GTT GTC CTA ACG GAA AAA TCA TGG AAG TTC CTG TTG
				BIP(B1c + B2)	AGG TCC AAT CGA TAC AGA TAA ATC GAA ACG GAT TTA CGT TGC ATA ACA
				LF	CAC TAC TCG TCC AAT CAA GGC ATC
				LB	GGT AGA AGC AGC TGC ACC T
6		*Enterococcus avium*	*ddl*	F3	TTT GTC AAA CCG GCG AAT A
				B3	AAT GCT TCT TCC TTT ACG ACC
				FIP(F1c + F2)	AGC TCT TGA ATC ATA GCG ATA AGC GGG CTC TAG CGT AGG AAT TTC ACG
				BIP(B1c + B2)	TTA GTT GAG CAA GGC ATC GAT GCT ATG TCG TCC GCA CAT CGT CAT T
				LF	CAA TGC GTT TTG CAG TTC TTC GC
				LB	GTG AGA TTG AAG TTG CGG TTT TAG GC
7		*Enterococcus durans*	Amino acid permease	F3	ATA ACA CCA GAA GCA TCT GAA G
				B3	TTA TGA TGG GTG GCT AGG T
				FIP(F1c + F2)	ACA GTC GTT TAT CTA TTG ATC AAC CAG CAT TGA GAT TCC CTG CAA TTT
				BIP(B1c + B2)	ACA AAA CTC AAG CCC AAA ATA AAT GGC TGG TGA GAT GAA GCA T
				LF	GTG TTC CTA AAA ACA CTG CCT ATC GAT
				LB	TGC TTT CGG TAG GTC CTT TTC TG

Table 1. Continued.

Primer set no.	LAMP assay	Target bacterium	Target gene	Primer	Sequence (5′–3′)
8		*Enterococcus cecorum*	*rpoA*	F3	CCT TAA AAT TGT ACG GAT CTG AAG
				B3	TTC GTC AGC TTG AAC GTA A
				FIP(F1c + F2)	GTT TAA GAT TTC AAC ATC AGA ATC GAA ATC GAT ATT ACT GGT CCA GCA
				BIP(B1c + B2)	GAT ATG TAC ATT TGT ACA GTC AGT ACC TGC TTT CAC TTT TAG GCG
				LF	CGA TAA TGT CAC CTG CAG TTA CAA
				LB	GAA GGT GCT ACA TTC CGT GC
9	*Escherichia coli*-LAMP	*Escherichia coli*	*malB*	F3	CAC CTT CAT GGA TAT CGA GAT T
				B3	TGG AGG ATT TAA GCC ATC TC
				FIP(F1c + F2)	CGA GCG TAC AGC TGC AAA ATG ATA TCT TTC GAT ACC ACG ACC T
				BIP(B1c + B2)	CCC TTC TCC CTT TGT AAC AAG ATG ACG CAT AGT CAG CCC AT
				LF	TAA CGA AAG CCT GGG GCG
				LB	CCT GTC ATC GAC AGC AAC ATT CA
10	*Staphylococcus aureus*-LAMP	*Staphylococcus aureus*	*nuc*	F3	CCA ACA GTA TAT AGT GCA ACT TC
				B3	TTG CAT TTT CTA CCA TTT TTT TCG
				FIP(F1c + F2)	AAT GTC ATT GGT TGA CCT TTG TAC AAT TAC ATA AAG AAC CTG CGA C
				BIP(B1c + B2)	GAC TAT TAT TGG TTG ATA CAC CTG ACA CTT GCT TCA GGA CCA TAT T
				LF	AAC CGT ATC ACC ATC AAT CGC
				LB	CAA AGC ATC CTA AAA AAG GTG TAG AGA

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.

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 Enterococcosel 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.

RESULTS

Optimal primer sets screening and LAMP assays

Several LAMP primer sets targeting specific genes (including *rpoB*, *ftsW*, *atpA*, *rpoA* and *ddl* of *Enterococcus* sp.; *malB* of *E. coli*; and *nuc* of *S. aureus*) were screened using a DNA amplification kit according to the manufacturer's instructions. After the LAMP amplification under isothermal conditions, a color change from violet to sky blue indicated a positive reaction and the negative reaction remained violet. The sequences of the optimal LAMP primer sets are shown in Table 1.

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

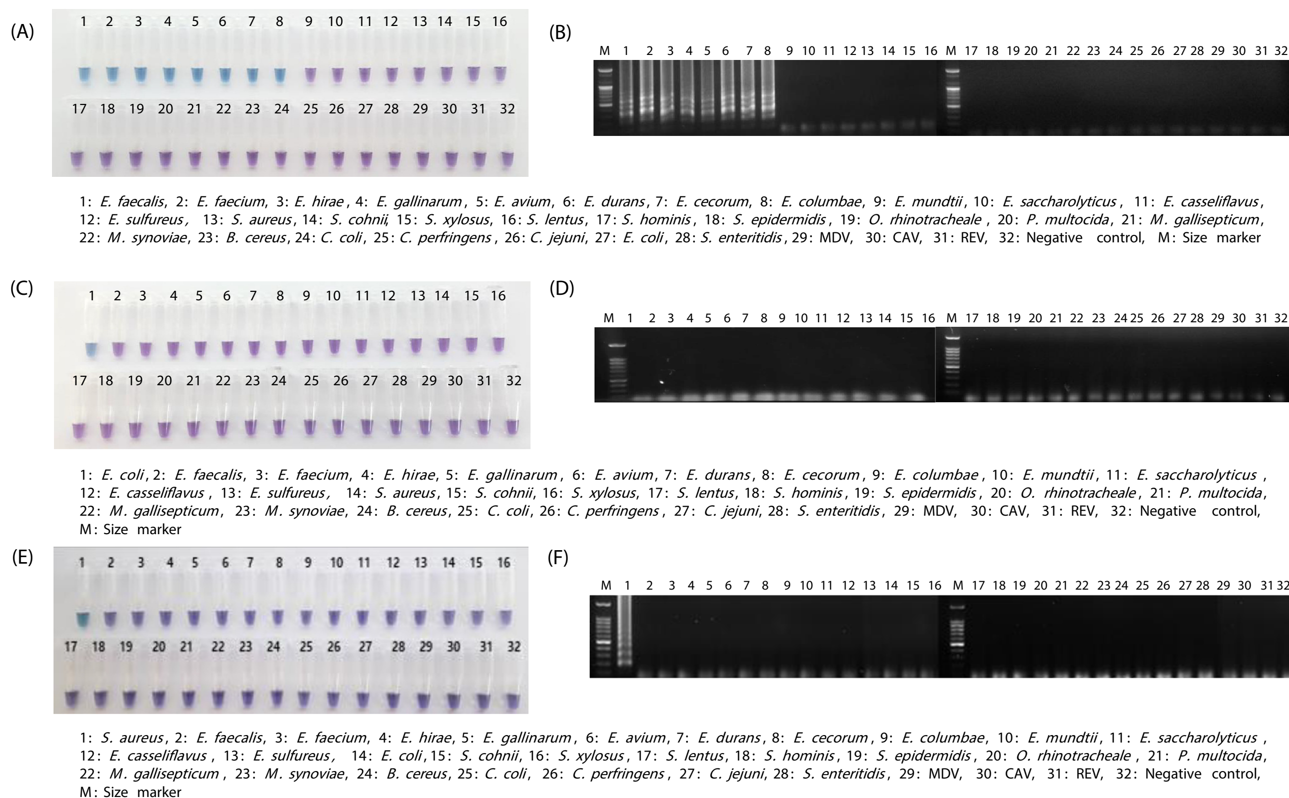

Figure 1. Specificity of the Entero-Common-LAMP, *E. coli*-LAMP and *S. aureus*-LAMP assays: (**A**) Entero-Common-LAMP; (**C**) *E. coli*-LAMP; and (**E**) *S. aureus*-LAMP. A color change from violet to sky blue indicated a LAMP-positive reaction, while the color of a LAMP-negative reaction mixture remained violet. The LAMP products were also resolved by 1.5% agarose gel electrophoresis: (**B**) Entero-Common-LAMP; (**D**) *E. coli*-LAMP; and (**F**) *S. aureus*-LAMP.

for *E. faecium* and *E. columbae,* 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 Entero-Common-LAMP primers F3 and B3 were 50 pg/μl for *E. faecalis*, *E. faecium*, *E. durans* and *E. columbae*, 1.25 ng/μl for *E. hirae,* 250 pg/μl for *E. gallinarum,* 10 pg/μl for *E. avium* and 250 pg/μl for *E. cecorum* (Fig. 5A–H). 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 2 pg/μl *E. gallinarum*, *E. durans* and *E. cecorum* DNA, under isothermal conditions, within 30 min, with assessment by the naked eye (Fig. 4). 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* (Fig. 5I–O). The detection limits for *E. coli* and *S. aureus* using LAMP assays were 2 pg/μl and 400 fg/μl DNA, respectively; however, the detection limits of PCR were 50 pg/μl for *E. coli* and 10 pg/μl for *S. aureus* (Fig. 5P and Q).

In conclusion, the sensitivity of the Entero-Common-LAMP and Entero-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; Stalker *et al.* 2010; Kense and Landman 2011; Kolbjørnsen *et al.* 2011; Jung and Rautenschlein 2014). Enterococci, including *E. faecalis*, *E. faecium*, *E. avium*, *E. durans* and *E. gallinarum*, are frequently isolated from the litter, feed, deadshell, 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

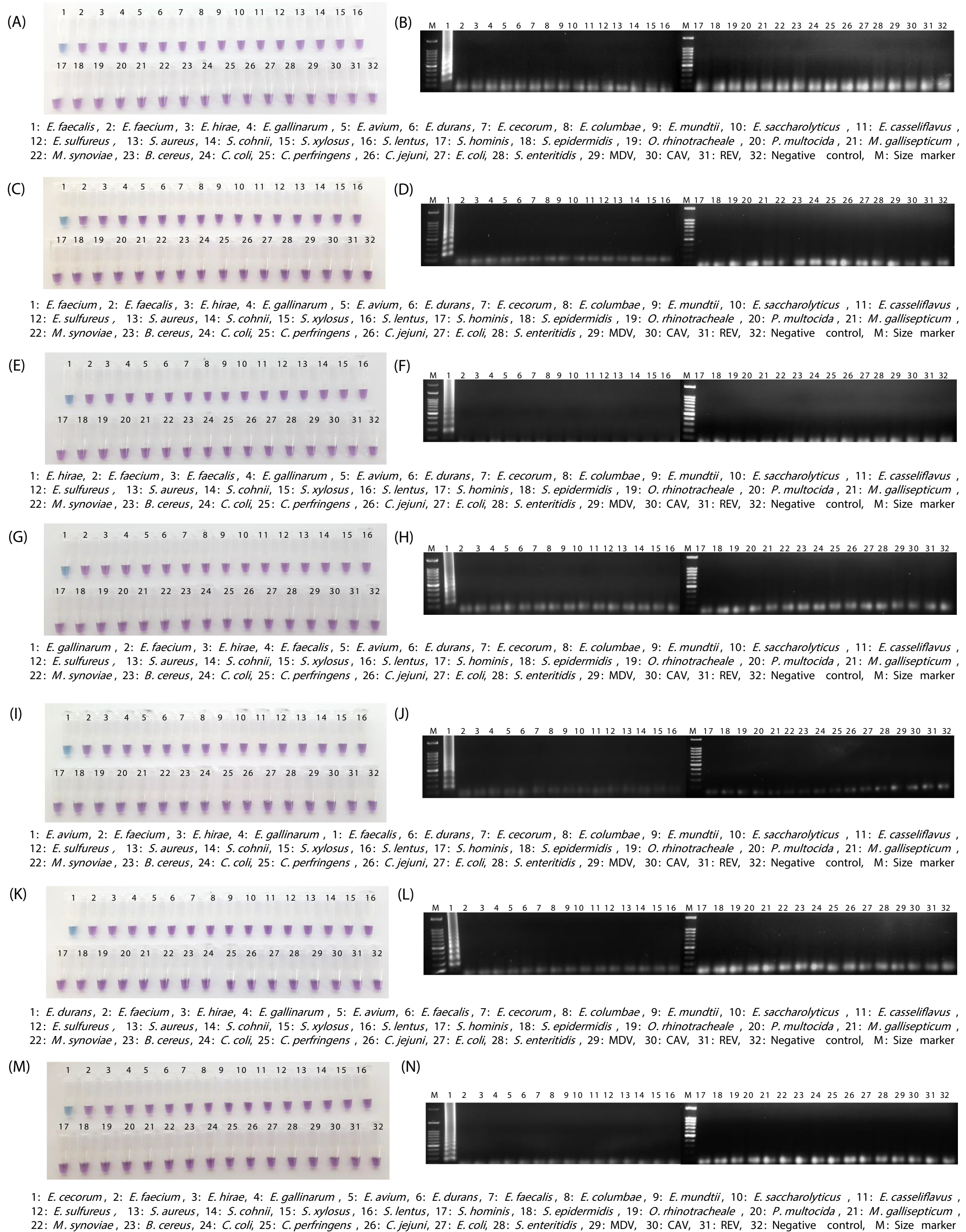

Figure 2. Specificity of seven types of the Entero-Specific-LAMP assays: **(A)** *E. faecalis*-LAMP; **(C)** *E. faecium*-LAMP; **(E)** *E. hirae*-LAMP; **(G)** *E. gallinarum*-LAMP; **(I)** *E. avium*-LAMP; **(K)** *E. durans*-LAMP; and **(M)** *E. cecorum*-LAMP. In these assays, a color change from 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.

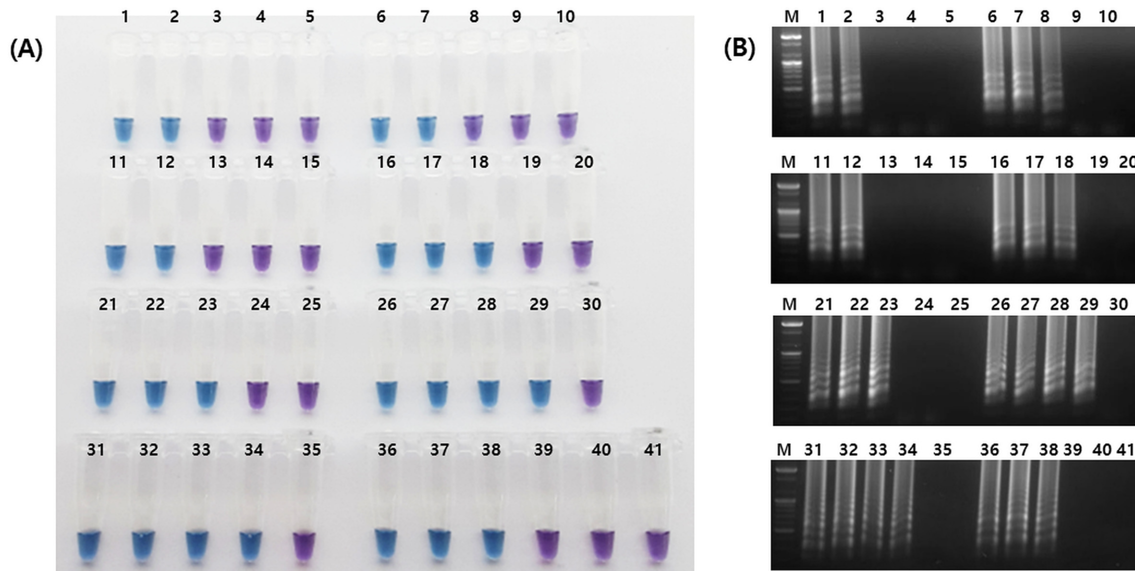

Figure 3. Detection limits of the Entero-Common-LAMP assay. (**A**) Naked-eye visualization of the LAMP products. The color of LAMP-positive reactions turned sky blue, while the color of LAMP-negative reactions remained violet. (**B**) Agarose gel electrophoresis of LAMP products. Lane M, 100 bp DNA 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.

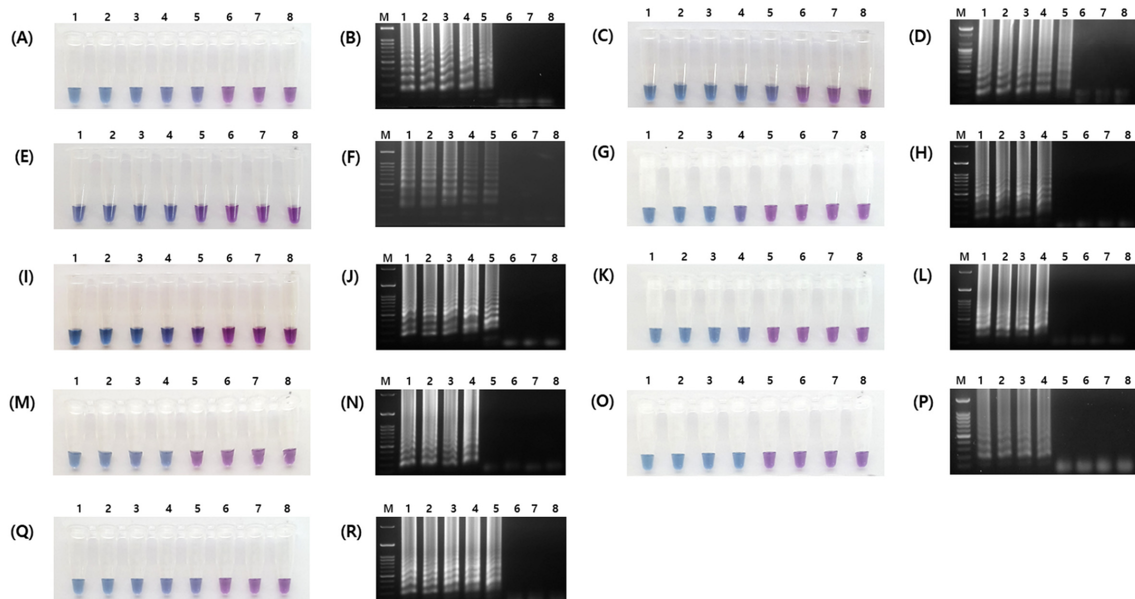

Figure 4. Detection limits of seven types of the Entero-Specific-LAMP, *E. coli*-LAMP and *S. aureus*-LAMP assays. Visual inspection of LAMP products for the detection of (**A**) *E. faecalis*, (**C**) *E. faecium*, (**E**) *E. hirae*, (**G**) *E. gallinarum*, (**I**) *E. avium*, (**K**) *E. durans*, (**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.*

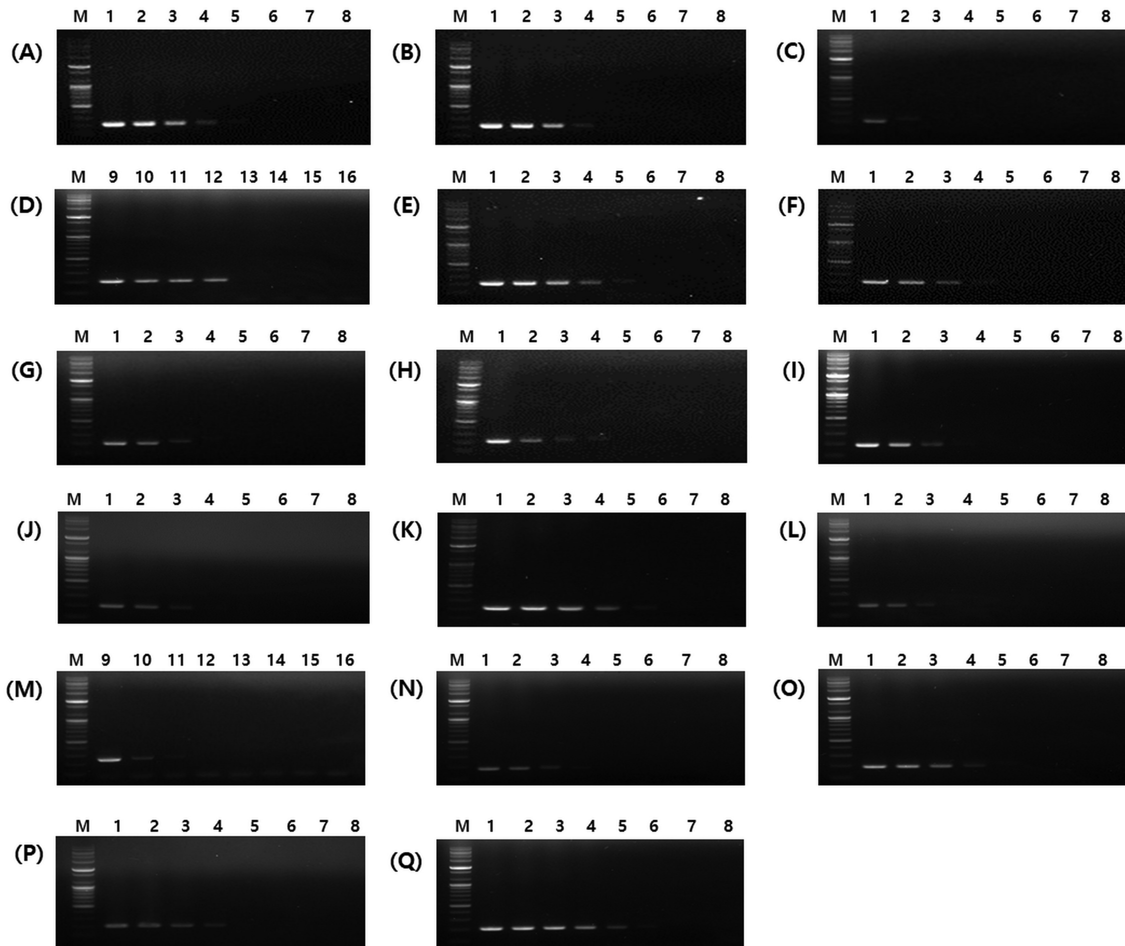

Figure 5. Electrophoretic analysis of PCR products to compare the detection limits of conventional PCR and LAMP assays. PCR was performed to detect (A) *E. faecalis*, (B) *E. faecium*, (C) *E. hirae*, (D) *E. gallinarum*, (E) *E. avium*, (F) *E. durans*, (G) *E. cecorum* and (H) *E. columbae* using universal 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[a]

	LAMP				PCR				16S *rRNA* sequencing			
Species	P/T	N/T	F/T	Sensitivity	P/T	N/T	F/T	Sensitivity	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

[a]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 *ddl* 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.

ACKNOWLEDGMENTS

This work was supported by a research grant (B-1543084–2016-17–04) from the Animal and Plant Quarantine Agency (QIA), Ministry of Agriculture, Food and Rural Affairs, the Republic of Korea.

Conflicts of interest. None declared.

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 hatchling quality of ducklings, chicks and poults. *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 naphthol 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.

Martzy 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.

McCullagh JJ, McNamee PT, 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.

McNamee PT, McCullagh JJ, Rodgers JD *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 ampli-fication 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, Chamings 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.