Loop-mediated isothermal amplification: Development, validation and application of simple and rapid assays for quantitative detection of species of Arcobacteraceae family- and species-specific *Aliarcobacter faecis* and *Aliarcobacter lanthieri*

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**Keywords**
Arcobacteraceae, loop-mediated isothermal amplification, quantitation, *Aliarcobacter faecis*, *Aliarcobacter lanthieri*.

**Abstract**

Aim: The family Arcobacteraceae formerly genus *Arcobacter* has recently been reclassified into six genera. Among nine species of the genus *Aliarcobacter*, *Aliarcobacter faecis* and *Aliarcobacter lanthieri* have been identified as emerging pathogens potentially cause health risks to humans and animals. This study was designed to develop/optimize, validate and apply Arcobacteraceae family- and two species-specific (*A. faecis* and *A. lanthieri*) loop-mediated isothermal amplification (LAMP) assays to rapidly detect and quantify total number of cells in various environmental niches.

Methods and Results: Three sets of LAMP primers were designed from conserved and variable regions of 16S rRNA (family-specific) and gyrB (species-specific) genes. Optimized Arcobacteraceae family-specific LAMP assay correctly amplified and detected 24 species, whereas species-specific LAMP assays detected *A. faecis* and *A. lanthieri* reference strains as well as 91 pure and mixed culture isolates recovered from aquatic and faecal sources. The specificity of LAMP amplification of *A. faecis* and *A. lanthieri* was further confirmed by restriction fragment length polymorphism analysis. Assay sensitivities were tested using variable DNA concentrations extracted from simulated target species cells in an autoclaved agricultural water sample by achieving a minimum detection limit of 10 cells mL\(^{-1}\) (10 fg). Direct DNA-based quantitative detection, from agricultural surface water, identified *A. faecis* (17%) and *A. lanthieri* (1%) at a low frequency compared to family-level (93%) with the concentration ranging from 2\(\times\)10\(^1\) to 2\(\times\)10\(^5\) cells 100 mL\(^{-1}\).

Conclusions: Overall, these three DNA-based rapid and cost-effective novel LAMP assays are sensitive and can be completed in less than 40 min. They have potential for on-site quantitative detection of species of family Arcobacteraceae, *A. faecis* and *A. lanthieri* in food, environmental and clinical matrices.

Significance and Impact of the Study: The newly developed LAMP assays are specific, sensitive, accurate with higher reproducibility that have potential to
facilitate in a less equipped lab setting and can help in early quantitative detection and rate of prevalence in environmental niches. The assays can be adopted in the diagnostic labs and epidemiological studies.

Introduction

The genus *Arcobacter* was first reported in 1991 (Van-Damme and De Ley 1991). This Gram negative, nonspore forming, motile and spiral-shaped bacteria grows well under microaerophilic condition. However, based on genomic and phenotypic analyses, the family Arcobacteraceae (formerly genus *Arcobacter*), within the class *Campylobacteria*, order *Campylobacteriales*, has recently been reclassified and divided into six genera including *Arcobacter*, *Aliarcobacter*, *Pseudarcobacter*, *Halarcobacter*, *Malacobacter* and *Poseidonibacter* (Pérez-Cataluña et al. 2018; Oren and Garrity 2019; Pérez-Cataluña et al. 2019). Of these, genus *Aliarcobacter* consists of nine species that have been identified as emerging pathogens causing gastroenteritis, bacteraemia, and sepsis in humans, and mastitis, diarrhoea, abortion and reproductive disorders in animals (Ferreira et al. 2016). The epidemiology and pathogenicity of species in family Arcobacteraceae in humans and animals are still not fully understood; therefore, Arcobacteraceae-associated infections are often considered low risk to public health (Besede et al. 2011). This could be due to misidentification with *Campylobacter* which leads to underestimation of the true cases of Arcobacteraceae-associated infection. They are ubiquitously prevalent in aquatic environments such as marine and fresh waters, ground water, wastewater and drinking water reservoirs (Jacob et al. 1993; Moreno et al. 2000; Mori et al. 2000; Mori et al. 2001). Livestock, poultry and wildlife have been considered as main sources of contamination of water (Hausdorf et al. 2013) which indicates that water may play vital role in transmission of the disease to humans and animals (Carter et al. 1987; Noble and Fuhrman 2001; Wu et al. 2011).

Microbial water quality guidelines and standards have been developed for drinking and recreational waters in Canada and other countries where faecal indicator bacteria, including *Escherichia coli* or *Enterococcus* spp., are used as indicators of faecal contamination and prevalence of pathogens in water (Wilkes et al. 2009). However, there is weak to no correlation between indicator and pathogens found (Carter et al. 1987; Noble and Fuhrman 2001; Wu et al. 2011). Collado et al. (2008) reported a correlation between *Arcobacter* spp. and faecal indicator in marine and fresh recreational water. However, the culture-based methods, for such analysis, may require at least 18–96 h to enumerate the number of *E. coli* or enterococci results (Toranzos and McFeters 1997). Moreover the numbers in the water may substantially change in short period of time. Also, the cells may be undetectable and undergo viable but non-culturable state due to injury and cannot be cultured in the laboratory conditions.

With the evolving technology, genus and species-specific molecular assays such as conventional and real-time polymerase chain reaction (PCR) have been developed for detection and enumeration of species of Arcobacteraceae family using 16S rRNA, 23S rRNA, housekeeping, virulence and toxin genes (Bastyns et al. 1995; Harmon and Wesley 1996; Houl et al. 2000; Whiteduck-Levellée et al. 2016a, 2016b; Khan et al. 2017; Zambri et al. 2019; Miltenburg et al. 2020). However, these conventional PCR assays are labour and time intensive as well as require post-PCR agarose gel electrophoresis for confirming the specific amplification reactions. On the other hand, real-time PCR assay is comparatively rapid for quantitative analysis of pathogens where target amplicons can be observed with the progression of the assay; however, this technique requires more specialized equipment and skilled labour as well as expensive with the fluorescent probes (Liang et al. 2009).

The loop-mediated isothermal amplification (LAMP) has been developed for on-site specific and rapid detection and identification of target nucleic acid (Notomi et al. 2000; Mori et al. 2001). The main advantage of the LAMP assay is that it only requires a heating block or water bath to carry out amplification reactions. This technique involves two or three sets of different primers that recognize distinct regions in the target sequence while *Bst* DNA polymerase enhances the amplification reaction, at constant temperature, and produces a large amount of high molecular weight DNA in a short period of time (Niesen et al. 2012; Wong et al. 2018). However, this technique has some limitations as compared to PCR such as unsuitable for cloning since the amplified product consists of a large DNA chain that makes it less useful. Moreover, it has low success rate than PCR when apply for multiplex assay due to difficulty in the experimental design and procedures (Dhama et al. 2014; Sahoo et al. 2016).

LAMP assay has been applied as a simple and rapid tool for the detection of various pathogens ranging from bacteria to virus, protozoa and fungi in various food and environmental niches (Liang et al. 2009; Niesen et al. 2012; Wong et al. 2018).
LAMP assays for rapid quantitative detection of Arcobacter spp.

2012; Wong et al. 2018). Similarly, Wang et al. (2014) developed a LAMP assay for detection of A. butzleri, A. cryaerophilus and A. skirrowii and compared them with conventional mono- and multiplex PCR assays (Houf et al. 2000; Douidah et al. 2010). Recently, our lab has developed a conventional multiplex PCR assay for distinctive identification of six species including two (Aliarcobacter faecis and Aliarcobacter lanthieri) new human-associated species of genus Aliarcobacter (formerly genus Arcobacter) (Whiteduck-Leveillee et al. 2015, 2016a; Khan et al. 2017).

Considering the high specificity, sensitivity and rapid application of LAMP assay as a diagnostic tool, this study developed/optimized, validated and applied simple, rapid and sensitive family- and species-specific quantitative LAMP assays for rapid identification of species of Arcobacteraceae family, A. faecis and A. lanthieri in a pure and mixed culture as well as direct DNA-based quantitation of total number of cells from complex matrices such as agricultural water samples.

Materials and methods

Bacterial reference strains and field cultures

For testing the specificity of primers and optimizing three LAMP protocols for amplification reactions, A. butzleri ATCC 49616T, A. faecis LMG 28519T and A. lanthieri LMG 28516T along with six other Aliarcobacter spp., and 18 species from genus Arcobacter, Halarcobacter, Malacobacter, Poseidonibacter and Pseudarcobacter as well as 50 other bacterial reference species and strains were also included as a control in the study (Table 1). For the assessment of specificity of LAMP assays, additionally, A. butzleri (n = 20), A. cryaerophilus (n = 10), A. faecis (n = 10), A. lanthieri (n = 12), A. skirrowii (n = 4) and A. cibarius (n = 5) field strains, previously isolated from various environmental sources in our lab and identified by using multiplex PCR assay, were also included in this study (Khan et al. 2017). All bacterial cultures were grown according to their respective selective growth media and incubation conditions (Zambri et al. 2019). The DNA of each culture was extracted using a boiling method (Khan et al. 2013) by suspending a purified single colony of each reference and field strain as well as putative mixed culture isolates recovered from agricultural water and various faecal sources in 1 × TE (10 mmol L⁻¹ Tris-HCl, 1 mmol L⁻¹ EDTA, pH 8-0) buffer. The mixed culture was obtained by scraping an ASIA media plate with a sterile loop and suspended in 1 × TE buffer followed by a 10-min boiling at 100°C. The tube was centrifuged at 10,000 × g for one minute, and the supernatant containing DNA was transferred to a sterile tube. The DNA was quantified using Qubit® dsDNA HS Assay kit and Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA). The DNA was stored at −20°C for further LAMP assays.

LAMP primer design

Initially, three Arcobacteraceae family-specific primer sets, two outer (F3 and B3), two inner (FIP and BIP) and two Loop (LF1 and LB1) primers, were designed from conserved regions (between V6 and V8) of 16S rRNA gene using Primer Explorer software (Eiken Chemical Co., Tokyo, Japan). Furthermore, two outer, two inner and one forward loop species-specific primer sets for each A. lanthieri and A. faecis, from variable regions of gyrB gene were designed. All primer sets were selected based on alignment of all available 16S rRNA and gyrB gene sequences of species of family Arcobacteraceae using the MegAlign program (DNASTAR Inc., Madison, WI). The primers were synthesized by Eurofins Genomics GmbH (Ebersberg, Germany). The Arcobacteraceae family- and species-specific target genes and primer sequences are listed in Table 2.

Optimization of LAMP assays

Initially, an Arcobacteraceae family-specific LAMP assay was optimized using a real-time fluorometer (Genie® II, Optigene, Horsham, UK) system with a 25 µL reaction mixture consisted of: 15 µL Isothermal Master Mix ISO-001 (Optigene), 2.5 µL 10× primer mix containing 0-2 µmol L⁻¹ F3/B3, 0-8 µmol L⁻¹ FIP/BIP and 0-4 µmol L⁻¹ LF/LB, 2.5 µL nuclease-free water and 5 µL DNA template. The amplification reaction was run at 65°C for 20 min, followed by a melting process initiated and finished at 98 and 80°C with a 0-05 per s ramp rate. Similarly, LAMP assays for A. faecis and A. lanthieri were optimized by setting up a 25 µL reaction containing 15 µL Isothermal Master Mix ISO-001, 5 µL DNA template, 2.5 µL nuclease-free water and primer mix (0-2 µmol L⁻¹ F3/B3, 2-0 µmol L⁻¹ FIP/BIP, 1-0 µmol L⁻¹ LF/LB) with annealing temperatures 67 and 68.5°C respectively for 20 min followed by melting temperature (Table 3).

The specificity of gyrB gene-based LAMP amplification reaction was further confirmed by restriction fragment length polymorphism (RFLP) analysis. The specific restriction sites in the LAMP products were analysed and selected with NEB cutter V2.0 (http://tools.neb.com/NEBcutter2). The assay was carried out by selection of restriction enzyme AluI (New England Biolabs GmbH, Frankfurt, Germany) targeting AGGT restriction site located between F1 and B1 primer annealing positions.
| Sr. # | Species                                    | Source                           | Strain ID   |
|------|--------------------------------------------|----------------------------------|-------------|
| 1    | Aliarcobacter (Arcobacter) lanthieri       | Pig manure                       | LMG 28516   |
| 2    | Aliarcobacter faecis                       | Human waste                      | LMG 28519   |
| 3    | Aliarcobacter butzleri                     | Human diarrheic stool            | ATCC 49616  |
| 4    | Aliarcobacter skirrowii                    | Lamb feces                       | ATCC 51322  |
| 5    | Aliarcobacter cryaerophilus                | Bovine aborted fetus             | LMG 24486   |
| 6    | Aliarcobacter thereuis                     | Organs of aborted porcine        | NCTC 11885  |
| 7    | Aliarcobacter trophiarum                   | Feces of fattening pigs          | LMG 25534   |
| 8    | Aliarcobacter cibarius                     | Broiler carcasses                | LMG 21996   |
| 9    | Aliarcobacter lacus                        | Wastewater treatment plant        | LMG 29062   |
| 10   | Arcobacter nitrofigilis                    | Roots                            | ATCC 33309  |
| 11   | Halarcobacter (Arcobacter) bivalviorum     | Shellfish                        | LMG 26154   |
| 12   | H. ebronensis                              | Estuarine sediment               | LMG 31560   |
| 13   | H. anaerophilus                            | Mussels                          | LMG 27922   |
| 14   | Poseidonibacter (Arcobacter) lekithochrous | Larvae of scallop                | DSM 100870  |
| 15   | Malacobacter (Arcobacter) halophilus       | Hypersaline lagoon               | ATCC BAA-1022|
| 16   | M. marinus                                 | Mix seawater, starfish and seaweed| LMG 25770   |
| 17   | M. molluscum                               | Mussels and oysters              | LMG 25693   |
| 18   | M. mytilii                                 | Mussels                          | LMG 24559   |
| 19   | M. canalis                                 | Wastewater channel               | LMG 29148   |
| 20   | Pseudarcobacter (Arcobacter) defluvii      | Sewage                           | LMG 25694   |
| 21   | P. ellii                                   | Mussels                          | LMG 26115   |
| 22   | P. pacificus                               | Seawater surface                 | LMG 26638   |
| 23   | P. caeni                                   | Wastewater treatment plant        | LMG 29151   |
| 24   | P. suis                                    | Pork meat                        | LMG 26152   |
| 25   | P. aquimarinus                             | Seawater                         | LMG 27923   |
| 26   | P. cloacae                                 | Sewage water                     | LMG 26153   |
| 27   | P. venerupis                               | Shellfish                        | LMG 26156   |
| 28   | Aeromonas allosaccharophila                | Diseased elvers                  | ATCC 51208   |
| 29   | A. bestiarum                               | Infected fish                    | ATCC 51108   |
| 30   | A. caviae                                  | Epizootic of young guinea pigs   | ATCC 15468   |
| 31   | A. hydrophila                              | Ditch water                      | ATCC 13444   |
| 32   | A. jandaei                                 | Human faeces                     | ATCC 49568   |
| 33   | A. media                                   | Marine fish                      | CDC 0435-84 |
| 34   | A. popoffii                                | Drinking water production plant   | ATCC BAA-243 |
| 35   | A. salmonicida                             | Freshwater                       | CDC 0434-84 |
| 36   | A. schubertii                              | Skin                             | ATCC 43700   |
| 37   | A. sobria                                  | Sludge                           | ATCC 35994   |
| 38   | A. trota                                   | Human faeces                     | ATCC 49658   |
| 39   | A. veronii                                 | Red-leg frog                     | ATCC 9071   |
| 40   | A. bv. veronii                             | Amputation Wound                 | ATCC 35625   |
| 41   | Campylobacter jejuni                       | Human faeces                     | ATCC 33291   |
| 42   | C. jejuni                                  | Human faeces                     | ATCC 29428   |
| 43   | C. jejuni                                  | Human faeces                     | ATCC 33291   |
| 44   | C. jejuni                                  | Human faeces                     | ATCC 33292   |
| 45   | C. jejuni subsp. doylei                    | Human faeces                     | ATCC 49349   |
| 46   | C. coli                                    | Swine                            | ATCC 43136   |
| 47   | C. coli                                    | –                                | ATCC 49941   |
| 48   | C. coli                                    | Marmoset faeces                  | ATCC 43478   |
| 49   | C. lari                                    | Human faeces                     | ATCC 43675   |
| 50   | C. helveticus                              | Cat                              | ATCC 51210   |
| 51   | C. fetus subsp. fetus                      | Blood                            | ATCC 15296   |
| 52   | C. hyointestinalis                         | Intestine of swine               | ATCC 35217   |
| 53   | C. lanienae                                | –                                | CCUG 44467   |
| 54   | C. upsaliensis                             | Dog faeces                       | ATCC 43954   |

(Continued)
using SeqBuilder Pro (DNASTAR Inc., ver. 15.3, Madison, WI, USA). A 5 μL amplified product was subjected to restriction digestion in 50 μL solution using digestion conditions specified by the manufacturer. The DNA fragments were separated by electrophoresis on 2% agarose gels.

Assessment of assay specificity, sensitivity and quantitation

To assess the specificity of primers, degree of sensitivity of optimized LAMP protocols and quantitation of total number of cells present in a given field sample, a simulation assay was carried out where known number of cells of *A. butzleri*, *A. faecis* and *A. lanthieri* reference strains were spiked in a highly turbid agricultural water to measure the purity of DNA and detection of least number of cells mL⁻¹. For this purpose, initially, the cells of each reference strain were grown in Arcobacter broth media, and incubated at 30°C under microaerophilic (85% N₂, 10% CO₂ and 5% O₂) conditions with continuous shaking at 125 rev min⁻¹ for 17 h to obtain exponential growth phase and 0-02 optical density at 420 nm. The cells were collected and resuspended in 1 mL 1× TE buffer. The cell concentration of each target species was measured on Arcobacter selective isolation agar (ASIA) and incubated under conditions as mentioned above. The known cell concentration (10⁷ cells mL⁻¹) of each target species was spiked in an autoclaved agricultural water sample and 10-fold serially diluted (10⁷–10¹ cells mL⁻¹). Each serially diluted spiked water sample with known cell concentration was filtered through a 0.22 μmol L⁻¹ sterile nitrocellulose filter. The autoclaved agriculture water sample was also filtered and used as a negative control. The spiked filters were further processed for DNA extraction using DNeasy PowerSoil kit (Qiagen; formerly MoBio PowerSoil DNA Isolation Kit) according to the manufacturer’s instructions. The purity and concentration of DNA was measured by Qubit 3.0 fluorometer using Qubit dsDNA HS Assay kit. The extracted DNA was stored at −20°C for further use.

Validation, application and quantitative analysis of optimized LAMP assays

The optimized LAMP assays were further validated by testing 40 mixed cultures of family Arcobacteraceae species recovered from agricultural water and various faecal samples on ASIA media. For further application of these assays, the spiked water DNA of each target species was added with decreasing equivalent number (from 10⁵ to 10¹) of cells mL⁻¹ corresponding to varying concentrations (ranging from 10 pg to 10 fg) of DNA mL⁻¹ to use for quantitation of total number of cells. The specificity

| Sr. # | Species                              | Source                | Strain ID       |
|-------|--------------------------------------|-----------------------|-----------------|
| 55    | *Escherichia coli* O157:H7           | Environmental isolate | –               |
| 56    | *E. coli*                            | Canine                | ATCC 35218T     |
| 57    | *Enterococcus avium*                 | Clinical isolate      | ATCC 49464T     |
| 58    | *E. casseliflavus*                   | –                     | ATCC 700327T    |
| 59    | *E. durans*                          | Human faeces          | ATCC 6056T      |
| 60    | *E. faecalis*                        | Meat                  | ATCC 7080T      |
| 61    | *E. faecium*                         | Human faeces          | ATCC 6569T      |
| 62    | *E. gallinarum*                      | Chicken intestines    | ATCC 49573T     |
| 63    | *E. hirae*                           | –                     | ATCC 8043T      |
| 64    | *E. saccharolyticus*                 | Straw bedding         | ATCC 43076T     |
| 65    | *Pseudomonas shigelloides*           | Environmental isolate | –               |
| 66    | *Salmonella enterica* subsp. arizonae| –                     | ATCC 13314T     |
| 67    | *S. enterica* subsp. diarizonae      | –                     | ATCC 12325T     |
| 68    | *S. enterica* subsp. houtenae        | –                     | ATCC 29932T     |
| 69    | *Helicobacter pylori*                | Human gastric antrum  | NCTC 11637T     |
| 70    | *H. typhlonius*                      | Human caecum          | CCUG 48335T     |
| 71    | *H. gannani*                         | Intestines of mice    | CCUG 43526T     |
| 72    | *H. marmotae*                        | Woodchuck liver       | CCUG 52419T     |
| 73    | *H. cetorum*                         | Baluga whale faeces   | ATCC BAA-429T   |
| 74    | *Klebsiella pneumoniae*              | Human serotype 3      | ATCC 13883T     |
| 75    | *Staphylococcus aureus*              | Clinical isolate      | ATCC 25923T     |
| 76    | *S. epidermidis*                     | Clinical isolate      | ATCC 12228T     |
| 77    | *Streptococcus pyogenes*             | Clinical isolate      | ATCC 19615T     |
Table 2 Arcobacteraceae family- and species-specific oligonucleotide primer sequences designed and used for LAMP assays

| Target bacteria | Target gene | Primers | Sequences (5’-3’) | bp |
|-----------------|-------------|---------|------------------|----|
| Arcobacteraceae spp. | 16S rRNA | Arco-1-F3 | GCT CGT GTC GTG AGA TG | 17 |
|                  |             | Arco-1-B3 | CCG CTT CCA ATG AGT TCA | 18 |
|                  |             | Arco-1-FIP | TCA CCT TCC TCC TAC TTG CGT ACT CGT TAG TTG CTA ACA | 42 |
|                  |             | Arco-1-BIP | AGT TCG GAT TGT AGT CGT CTC ACC GTC ACG TAG CTC ATG | 43 |
|                  |             | Arco-1-LF | CGT TAG AGT TCT CAG CCG AA | 20 |
|                  |             | Arco-1-LB | CAT GAA GTT GGA ATC GCT AGT AAT C | 25 |
|                  |             | Faecis-1-F3 | ACA AAA ATA ACA AGT GAT GT | 23 |
|                  |             | Faecis-1-B3 | GCT GCC ATT AAA GAT TTG TCC | 21 |
|                  |             | Faecis-1-FIP | TCC CAA TTT TCC TTT TGT TTG TCC TGA GAA GTT ATG GCT GTT GT | 47 |
|                  |             | Faecis-1-BIP | GCT CTT ATG TTA GAC CAA TTG TCT GC TTT TTC ATG AGT TGG ATT | 45 |
|                  |             | Faecis-1-LF | TTG AGG TTC AGG AAC TTT AAC AGA A | 25 |
|                  |             | Lanth-1-F3 | ACA CCA AAA AGG AAA ATT AGG TAG | 24 |
|                  |             | Lanth-1-B3 | ACT TTT GCA ACA ATC TGC AAG TT | 20 |
|                  |             | Lanth-1-FIP | CCA TTA TAG CTT TTG CAT GAA CTG GCC TAT TGC TCA AAA ATT AAC TGG T | 49 |
|                  |             | Lanth-1-BIP | GGC AGC AAG AGG AAG AGA AGC GGA AGT GTT CCA ACA CTC AT | 41 |
|                  |             | Lanth-1-LF | GCT AAA AAG AGA GGA AGA TTA ACT AGA A | 28 |

Table 3 Optimized LAMP primer concentrations and protocols for Arcobacteraceae family- and species-specific detection and identification

| Primer conc. (µmol L⁻¹) | Assay conditions | Melt Temp. (°C) |
|-------------------------|------------------|-----------------|
| F3/ B3                  | FIP/ BIP         | LF/ LB          | Annealing | |
| Arcobacteraceae spp.    |                  | 65°C for 20 min | 86          |
| A. faecis               | 0.2              | 0.8             | 0.4        |
| A. lanthieri            | 0.2              | 2.0             | 1.0        |
| A. butzleri             | 0.2              | 2.0             | 1.0        |
| A. faecis               | 0.2              | 2.0             | 1.0        |
| A. lanthieri            | 0.2              | 2.0             | 1.0        |

Optimization and specificity of LAMP assays

In the first phase of the study, the designed family- and species-specific primers and LAMP protocols were optimized by adjusting optimal annealing temperature (ranging from 60 to 70°C) and tested with the reference species including A. butzleri, A. faecis and A. lanthieri. All primers specifically amplified at their specific annealing (65, 67 and 68°C) and melting temperatures (86, 81 and 82°C), respectively (Table 3), by showing typical amplification curves. Furthermore, a total of 17 reference species of five newly classified genera and 61 field isolates were tested for genus-specificity where all DNA typically amplified at 65°C with the melting curve observed at 86°C. On the other hand, A. faecis and A. lanthieri species-specific primers only amplified each target species including reference and field strains by showing amplification of target gene at 67 and 68.5°C and melting curves at 81 and 82°C respectively. All three LAMP assays were further tested for their specificity using Arcobacteraceae closely-related or other human-associated bacterial species. Of the total 27 species of family Arcobacteraceae, all 16 species of genus Arcobacter, Aliarcobacter, Malacobacter and Poseidonibacter showed positive amplification reactions as compared to genus Pseudarcobacter and

and quality of amplified products were confirmed by analysing melting temperatures and compared with melting temperature obtained for A. butzleri, A. faecis and A. lanthieri amplicons. Furthermore, to evaluate and apply the optimized LAMP primers and protocols, 173 agricultural surface water samples were collected from South Nation River, Ontario, Canada. The watershed is mainly dominated by the dairy farming and mix-uses of cash crops (Wilkes et al. 2009). A total of nine sites were selected and surface water samples were collected from May to November in 2017 and 2018. One litre water sample from each site was collected in a sterile polyethylene bottle and delivered to the AAFC-ORDC lab for microbiological analysis. For the detection and quantitation of total number of species of family Arcobacteraceae (representing species of six newly classified genera), A. faecis and A. lanthieri, the water samples were processed by membrane filtration using 0.22 µm filters. The filters were further processed for total genomic DNA extraction using DNeasy PowerSoil kit. The DNA was quantified and tested for detection and quantification of total number of cells 100 mL⁻¹.

Results

Optimization and specificity of LAMP assays

In the first phase of the study, the designed family- and species-specific primers and LAMP protocols were optimized by adjusting optimal annealing temperature (ranging from 60 to 70°C) and tested with the reference species including A. butzleri, A. faecis and A. lanthieri. All primers specifically amplified at their specific annealing (65, 67 and 68°C) and melting temperatures (86, 81 and 82°C), respectively (Table 3), by showing typical amplification curves. Furthermore, a total of 17 reference species of five newly classified genera and 61 field isolates were tested for genus-specificity where all DNA typically amplified at 65°C with the melting curve observed at 86°C. On the other hand, A. faecis and A. lanthieri species-specific primers only amplified each target species including reference and field strains by showing amplification of target gene at 67 and 68.5°C and melting curves at 81 and 82°C respectively. All three LAMP assays were further tested for their specificity using Arcobacteraceae closely-related or other human-associated bacterial species. Of the total 27 species of family Arcobacteraceae, all 16 species of genus Arcobacter, Aliarcobacter, Malacobacter and Poseidonibacter showed positive amplification reactions as compared to genus Pseudarcobacter and
Halarcobacter where among 11 species, all except H. ebroensis, P. caeni and P. pacificus showed positive amplification reaction. Overall, the result indicates that the designed primers and optimized LAMP protocol are highly (89%) specific to all target genera and species of family Arcobacteraceae. In addition, all control DNA templates showed negative amplification reaction to A. faecis and A. lanthieri species-specific LAMP assays.

Species-specific LAMP assays were further confirmed using RFLP analysis of the LAMP amplified products of A. faecis and A. lanthieri. After using the restriction enzyme Alul, a single restriction site for A. faecis LAMP amplified product yielded two (104 and 111 bp) fragments as compared to A. lanthieri where three (42, 95 and 108 bp) fragments were obtained (Fig. S1a,b).

Validation and development of standard curves for quantitative analysis

The optimized LAMP assays were further validated by testing putative cultures of family Arcobacteraceae recovered from surface water (n = 9) and various faecal (n = 31) samples. All 40 culture isolates were amplified, yielded typical melting curves and identified to family-level. Whereas, A. faecis was detected in all water and 28 faecal samples compared to A. lanthieri where six water and 13 faecal samples were positively amplified with A. lanthieri primers. The results also showed that both target species were simultaneously detected in six water and 10 faecal samples indicating the common prevalence of these species in environmental niches. After the optimization of three LAMP assays, family- and A. faecis and A. lanthieri species-specific standard curves for quantitation of total number of cells were developed using the spiked DNA with the known cell concentration equivalent (ranging from $10^2$ to $10^5$ cell mL$^{-1}$) corresponding to the DNA concentrations (ranging from 10 pg to 10 fg mL$^{-1}$). Based on the fluorescent signals, typical amplification curves at specific melting temperatures were obtained while negative controls did not show a cross-amplification reaction to the specific LAMP primers and protocols. The least quantification detection limit 10 cells mL$^{-1}$ was achieved (Fig. 1a–c).

LAMP assay application and direct DNA-based quantitation of total cells in environmental samples

The optimized newly developed LAMP family- and species-specific assays were further applied for detection and quantification of Arcobacteraceae family-level as well as A. faecis and A. lanthieri species in 173 agricultural water samples. Of the total 173 samples, 161 (93%) samples showed typical amplification and melting curves comparable to the family Arcobacteraceae standard where the total number ranged from 57 to $2.5 \times 10^5$ cells 100 mL$^{-1}$. The results show that species of family Arcobacteraceae are commonly prevalent in the agricultural water. On the other hand, when DNA was tested for speciation, of the total 161 family-level positive water samples, 27 (17%) and two (1%) showed typical amplification for A. faecis and A. lanthieri, respectively, and melting curves similar to the standards while one sample was positively amplified for both target species. The total number of cells of each target species was ranged from 21 to $3.7 \times 10^4$ cells 100 mL$^{-1}$. The results showed that the newly developed assays are rapid, robust and simple for detection and quantitative analysis of species of Arcobacteraceae family, A. faecis and A. lanthieri in various environmental niches.

Discussion

Conventional culture-based methods for recovering species of various species of family Arcobacteraceae have limitations including cultivating time (4–6 days), potential for false-positive or negative inference, reduced bacterial viability between collecting and processing samples due to microaerophilic nature, in-lab environment and growth rate (culturable vs. non-culturable) that may impact obtaining results in timely-manner (Roszak and Colwell 1987). Although the nonculturable cells of Gram-negative bacteria have inability to cause infections, they significantly contribute to the antigen and endotoxin level that may activate immunological response. Therefore, it is important to detect the total number of cells (viable and non-viable cells) without cultivating for rapid screening of environmental, food and clinical samples for pathogen detection. Compared to culture-based identification including morphology and physico–chemical properties and antibody-based assay, molecular methods including PCR and real-time PCR are more reliable and rapid especially when species-level identification is required. Although, these molecular tools are useful and adopted by diagnostic and analytical labs, they require more than 2–3 h excluding culturing time and DNA preparation. Therefore, LAMP assay has advantages over PCR methods where the target pathogen can be rapidly and accurately detected as well as specifically quantified by accumulation of $10^9$ copies of the target in less than 60 min. Moreover amplification reaction of a target nucleic acid can be performed under isothermal conditions that does not require thermal cycle system and can be conducted in a water bath or heated block (Notomi et al. 2000).

Considering the significance of LAMP assay, there was a need to develop a more accurate, reliable and rapid
identification system that can detect species of multiple genera that are potentially pathogenic to humans and animals. In recent years, only one LAMP assay has been developed for quantitative detection of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* (Wang et al. 2014). However, this assay was only tested for three species, and with the substantial increase in the number of species over the last few years, none of the other species of family Arcobacteraceae were investigated for the specificity and sensitivity of LAMP primers and protocol.

In this study, we developed species-specific LAMP assays for *A. faecis* and *A. lanthieri* using variable regions of gyrB housekeeping gene, responsible for performing essential bacterial function, whereas conserved

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**Figure 1** (a) Amplification signals of *A. butzleri* reference strain showing number of cells (ranging from $10^4$ to $10^7$ cells mL$^{-1}$) varying DNA concentrations of a spiked culture in an agricultural water; (b) Annealing curve reactions of different cell numbers of *A. butzleri* reference strain showing a specific relative intensity at 86°C against negative control; (c) Standard curve for *A. butzleri* using genus-specific LAMP-based assay where amplification of 16S rRNA gene-based genus-specific LAMP primers targeting *A. butzleri* DNA with an increasing number of cells (ranging from $10^3$ to $10^6$) corresponding to various DNA concentrations (ranging from 10 pg to 10 fg)
regions of gene encoding 16S rRNA was selected for Arcobacteraceae family-specific primer design. These genes have previously been used for detection of species of family Arcobacteraceae in environmental, clinical and food samples (Douidah et al. 2010; Khan et al. 2017). Wang et al. (2014) developed a LAMP assay to detect species of family Arcobacteraceae in poultry using 23S rRNA as a target gene. To the best of our knowledge, this is the first Arcobacteraceae family- and specific-specific LAMP-based study where 16S rRNA and gyrB genes were selected for designing LAMP primers and a maximum number of species have been tested for optimization, validation of protocols and assessment of specificity and sensitivity of LAMP assays. In order to obtain the best amplification and specificity results, the primers including F3 and B3 were designed with <300 bp DNA fragment size. In addition, loop primers were also designed to accelerate amplification and reduce reaction time (Nagamine et al. 2002). Similarly, for specific and sensitive amplification of target gene and species, optimization of LAMP protocol significantly depends on several important factors including primer and DNA template concentration, annealing temperature and type of DNA polymerase used (Notomi et al. 2000). Therefore, these parameters were carefully applied in order to determine an optimal protocol that can be used for obtaining maximum amplification reaction in terms of specificity and intensity for rapidly detecting target DNA. Various concentrations for each primer set (ranged from 0.1 to 2.0 µmol L\(^{-1}\)) were used for assessing the intensity, specificity and reproducibility of the amplification reaction for each targeted species. The obtained amplification for all primer sets used for family- and species-specific was within the range of primer concentrations tested. In the second step, range of annealing temperatures (from 65 to 70°C) and time (20–25 min) were tested separately for each target for determining suitable temperature and time for specificity with high intensity of amplification reaction. Each developed LAMP assay was able to specifically detect each target DNA with a high intensity at different temperature 65°C (family-specific), 67°C (A. faecis) and 68.5°C (A. lanthieri) in less than 40 min including annealing and melting curve steps. The respective results also confirmed that each newly developed and optimized LAMP assay was efficiently producing specific amplification and melting curves for all species, except H. ebronensis, P. caeni and P. pacificus, of six genera of Arcobacteraceae as well as each targeted species. Although the designed primers for species of Arcobacteraceae family showed homogeneity in the nucleotide sequences where the Blast research results showed ≥99% homology to their corresponding species, it is also possible that there is mismatches in the species tested in the study. Therefore, further research is required to identify degree of heterogeneity to strain-level.

One of the major objectives of this study was to validate the specificity of the optimized LAMP assays by testing other reference and field strains. Therefore, Arcobacteraceae family- and species-specific LAMP assays were applied to a total of 27 species representing six genera along with 61 field isolates including A. butzleri, A. faecis, A. lanthieri, A. cryaerophilus and A. skirrowii as well as 50 other bacterial reference strains. Each LAMP assay specifically amplified target species and strains and did not show cross-amplification with other tested control species and strains. The specificity of the assays was further evaluated for the detection to family- and species-level in a mixed putatively positive cultures recovered from selective growth media. The LAMP assays correctly detected all putative mixed cultures to family-level whereas 48 and 90% cultures were identified as A. faecis and A. lanthieri respectively. Interestingly, of these, 85% cultures showed positive amplification reaction for both target species which showed incongruence with our previous conventional mPCR study results where only 20% cultures were positive for multiple species (Khan et al. 2017). The results of this study indicate that the newly developed LAMP assays are more specific and sensitive to detect multiple species in a mixed culture especially in those cases where one of the target species is present at a low concentration.

After successful optimization and validation of these novel LAMP assays, the major aspect of this study was to assess the degree of sensitivity of these assays for quantification of total number of cells in a complex environmental sample. Initially, we developed a standard curve from a spiked culture of target species in an autoclaved agricultural water where a minimum detection limit of 10 cells mL\(^{-1}\) was achieved. Our limit of detection is similar to the previous study where Notomi et al. (2000) described that LAMP assay has an ability to detect less than 10 copies of target sequence. The standard curve was further applied to the field samples for evaluating the sensitivity of the assays where a minimum of ≤10 cells mL\(^{-1}\) for Arcobacteraceae family and target species in agricultural water samples was observed compared to a minimum 200 CFU and 2 CFU per reaction in chicken meat and the DNA isolated from cultured bacteria respectively (Wang et al. 2014). This difference could be due to the DNA purity and inhibitors that may interfere and reduce the amplification sensitivity of the assay; therefore, various efforts have been made to obtain highly purified nucleic acid from various environmental and clinical niches (Moreira 1998; Bergallo et al. 2006). The total number of cells based on quantitative detection to genu-
level were ranged from 57 to $2.5 \times 10^5$ cells 100 mL$^{-1}$ and to species-level for *A. faecis* (ranged from 21 to $3.7 \times 10^4$ cells 100 mL$^{-1}$) and *A. lanthieri* (ranged from 45 to $3.2 \times 10^5$ cells 100 mL$^{-1}$), respectively. Although the standard curves were developed from the DNA concentrations (10 pg to 10 fg) against the number of cells ($10^5$–10$^5$ CFU mL$^{-1}$), obtained from the culture-based data, with 10 cells mL$^{-1}$ as a least detection limit, the amplification reaction of total DNA was observed below 10 cells mL$^{-1}$ which indicates the sensitivity and specificity of the assays and purity of the total DNA that could quantify total number of cells lower than the detection limit in field samples. The infectious dose of genus and species of *Arcobacter* has not been determined yet; however, $10^3$ cells mL$^{-1}$ is considered as an infectious dose when ingested at this concentration (USEPA 2012). Hence, the sensitivity-level of detection of these assays has shown that the cell concentration is within the range of infectious dose that can potentially cause health risks. In this context, further research efforts are underway to use these newly developed assays in combination with propidium monoazide (PMA) or ethidium monoazide (EMA) to estimate the number of live/dead (viable and non-viable) cells and compare with culture-based method for accurate enumeration of cells presents in environmental or clinical samples. Recent study has developed PMA-based qPCR assay for the detection of viable cells of Arcobacteraceae spp. in shellfish (Salas-Massó et al. 2019). However, the major disadvantages such as toxic effect and penetration of these dyes into live or dead cells can sometime under- or overestimate cells (Nocker et al. 2006; Taylor et al. 2014). Therefore, it requires optimization of dye concentration and exposure time without impacting on the specificity and sensitivity of these newly developed LAMP assays.

In conclusion, the results of three simple and rapid newly developed LAMP assays showed that assays are more specific, sensitive, accurate and have higher reproducibility than previously developed family- and species-specific molecular assays. Moreover, the study results provide sufficient data that would support in adopting these novel LAMP assays that have potential to facilitate on-site or in a less equipped lab setting for rapid and direct DNA-based quantitative screening of environmental and clinical samples for *A. faecis* and *A. lanthieri*. In compared to our previous real-time PCR assays (Miltenburg et al. 2020), the major advantages of these LAMP assays are that they do not require post-PCR analysis and can be completed in less than 40 min with a minimum to no risk of cross-reaction with other species of closely related genera. These assays can also contribute to improve our ability and understanding on the early quantitative detection and identification, rate of prevalence and ecology of these emerging pathogens in environmental niches as well as can be applied in the diagnostic labs and epidemiological research studies.

**Acknowledgements**

The research funding for this study was provided by Agriculture and Agri-Food Canada under Microbial Biosoystenmatics and Bioinformatics and Canada-European Union Harmonization projects #: J-001609 and J-000157. We thank field crew Emilia Craiovan and co-op students including Mary Gwen Miltenberg, Mark Libby and Linda Liu for their sampling and laboratory assistance.

**Conflict of Interest**

The authors have declared no conflict of interest.

**Authors’ contribution**

IK and AA conceived research project, performed sequence analyses and designed experiments. IK, AA and AB designed LAMP primers and protocols, conducted experiments, analysed data and drafted manuscript. MM, MP, DL, GW and ET coordinated in identifying sampling sites and sample collection procedure as well as contributed in data interpretation and editing of manuscript. All authors reviewed, edited and approved the final manuscript.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** RFLP analysis of LAMP species-specific amplified products using *Alu*I restriction enzyme.