Loop-mediated isothermal amplification: rapid molecular detection of virulence genes associated with avian pathogenic *Escherichia coli* in poultry

Polona Kogovšek ,[*,1] Jerneja Ambrožič-Avguštin,[†] Alenka Dovč,[‡] Tanja Dreo,[*] Hristo Hristov,[†] Uroš Krapež,[‡] Maja Ravnikar,[*,§] Brigita Slavec,[‡] Marjetka Lotrič,[*] Jana Žel,[*] and Olga Zorman Rojs[‡]

[*]*National Institute of Biology, 1000 Ljubljana, Slovenia;* [†]*Biotechnical Faculty, University of Ljubljana, 1000 Ljubljana, Slovenia;* [‡]*Veterinary Faculty, Institute for Poultry, Birds, Small Mammals and Reptiles, University of Ljubljana, 1000 Ljubljana, Slovenia; and* [§]*Wine Research Centre, University of Nova Gorica, 5000 Nova Gorica, Slovenia*

ABSTRACT Infections with pathogenic *Escherichia coli* can lead to different animal- and human-associated diseases. *E. coli* infections are common in intensive poultry farming, and important economic losses can be expected during infections with avian pathogenic *E. coli* (APEC) strains followed by colibacillosis. Loop-mediated isothermal amplification (LAMP) assays were developed for rapid detection of 3 APEC-associated virulence genes: *sitA*, *traT,* and *ompT*. All 3 LAMP assays are shown to be specific, repeatable, and reproducible. High sensitivities of the assays are shown, where as few as 1,000 bacterial cells/mL can be detected in different matrices. On-site applicability of this LAMP method is demonstrated through testing of different sample types, from animal swabs and tissues, and from environmental samples collected from 6 commercial poultry farms. All 3 virulence genes were detected at high rates (above 85%) in samples from layer and broiler chickens with clinical signs and, interestingly, high prevalence of those genes was detected also in samples collected from clinically healthy broiler flock (above 75%) while lower prevalence was observed in remaining 3 clinically healthy chicken flocks (less than 75%). Importantly, these virulence genes were detected in almost all of the air samples from 11 randomly selected poultry houses, indicating air as an important route of *E. coli* spread. Three LAMP assays that target APEC-associated virulence genes are shown to be sensitive and robust and are therefore applicable for rapid on-site testing of various sample types, from animal swabs to air. This on-site LAMP testing protocol offers rapid diagnostics, with results obtained in <35 min, and it can be applied to other important microorganisms to allow the required prompt measures to be taken.

Key words: APEC-associated virulence genes, isothermal amplification LAMP, on-site detection, colibacillosis

2019 Poultry Science 98:1500–1510
http://dx.doi.org/10.3382/ps/pey516

INTRODUCTION

In poultry, infections with avian pathogenic *Escherichia coli* (**APEC**) can cause colibacillosis, which is an acute and mostly systemic disease that results in significant economic losses. Avian colibacillosis is characterized by multiple organ lesions. Infections of the reproductive tract include salpingitis and peritonitis, which is very common in layer chickens, with effects from the start of egg production (Landman and van Eck, 2015). Omphalitis and yolk-sac infections can occur in ovo during egg formation and incubation or through fecal contamination of eggs. The systemic form of colibacillosis of respiratory origin induces colisepticemia, which is often followed by fibrinopurulent airsacculitis, pericarditis, and perihepatitis (Nolan et al., 2013; Guabiraba and Schouler, 2015).

Although it is believed that colibacillosis is a secondary disease and that APEC strains are opportunists, increasing evidence has shown that some APEC strains are well adapted and can cause severe health problems. APEC strains can carry a diverse set of virulence factors, including adhesins, protectins, iron uptake or transport systems, capsules, elements for evasion of immune responses, toxins, and invasins. Many studies have demonstrated that several combinations of different virulence factors might be characteristic of pathogenic strains, although these virulence factors are rarely all present in the same isolate. However, combinations of certain virulence genes appear to be good predictors of APEC strains (Schouler et al., 2012; Arabi

Received July 12, 2018.
Accepted October 18, 2018.

[1]Corresponding author: polona.kogovsek@nib.si

et al., 2013; Kemmett et al., 2013; Dissanayake et al., 2014).

The golden standard for diagnosis of APEC infections is based on isolation of the pathogen on selective media, with definitive identification of *E. coli* established according to the phenotypic characteristics (Nolan et al. 2013). This whole procedure takes at least 48 h, and the results obtained do not differentiate between virulent and commensal strains of *E. coli*. Under field conditions, presumptive diagnosis of colibacillosis is often made according to clinical signs and pathological lesions only.

To improve and accelerate the detection of *E. coli* strains that harbor virulence genes to allow for prediction or confirmation of colibacillosis, a loop-mediated isothermal amplification (**LAMP**) method was developed and applied here. First, 104 *E. coli* isolates from samples collected on different poultry farms were tested for the presence of 19 virulence genes using PCR. The data produced were evaluated statistically, and 2 genes that showed the highest statistical significance in diseased animals were selected for the design of the LAMP assays. With in silico analysis of the literature, we selected an additional APEC-associated virulence gene that was detected in high percentage in different experimental settings. The performance of the LAMP assays was then evaluated on dilution series of *E. coli* isolates prepared in chicken heart and liver tissues to imitate actual samples that can be tested on site. In addition, the on-site applicability of this method is demonstrated by testing 116 environmental and animal samples from 6 independent flocks with different health status.

MATERIALS AND METHODS

Escherichia coli and Salmonella Isolates

The *E. coli* isolates used for PCR testing and validation of the LAMP assay were obtained from poultry farms in Slovenia. Selection of the genes used in the LAMP assays was based on comparisons of associations between different virulence genes in *E. coli* isolates from different types of samples obtained from commercial poultry flocks over a period of 2 yr (2015 and 2016). Of the total of 104 *E. coli* isolates included, 59 were from different organs obtained during pathological examinations of chickens (Additional File 1). Another 45 isolates were environmental isolates that were obtained from fecal and air samples taken during the monitoring of the poultry farms, with 29 of fecal origin and 16 from air samples. The fecal samples were collected using boot swabs after walking the whole length of the poultry house. The air samples were taken using an air sampler (Coriolis Delta; Bertin Technologies, Montigny-le-Bretonneux, France). The sampler was used to aspirate 1,000 L of air (100 L/min, 10 min), and the airborne particles were transferred into 15 mL phosphate-buffered saline (**PBS**) using a centrifugal vortex of air.

In addition, some *E. coli* strains isolated from other animal species were included: 2 cattle and 4 pig isolates, and 1 isolate each from of a mouflon, an antelope, and a horse.

All of the *E. coli* were isolated directly on chromogenic medium (UriSelect 4; BioRad, France) at 37°C, except for those from the fecal samples, which were first diluted 1:10 in buffered peptone water (Biolife, Italy). After a 24-h incubation, suspect colonies were identified by matrix-assisted laser desorption/ionization, time of flight mass spectrometry (Bruker, Germany). All of the *E. coli* strains were subcultured onto blood agar (Blood agar base No. 2; Thermo Scientific, UK) and stored at –80°C until further testing.

The *Salmonella* strains used for determination of the specificity of LAMP assays were obtained from poultry (broiler and layer chickens, turkeys) and pigs during official Food Business Operator monitoring. The *Salmonella* strains were isolated and the strains confirmed according to standard procedures (ISO 6579:2002/Amd 1:2007, 2007; ISO 6579–1:2017, 2007), using selective/enrichment media. The isolates obtained were serotyped according to the antigenic formulae of the *Salmonella* serovars of the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2008). They were then stored in the internal bacterial culture collection at the Institute of Microbiology and Parasitology, Veterinary Faculty of Ljubljana University (Slovenia). For the purpose of the present study, they were recultivated onto blood agar.

The bacterial samples used for PCR and LAMP testing were prepared according to 2 different approaches: (i) pellets from 1-mL overnight broth cultures or bacterial colonies were resuspended in 200 μL sterile water or 500 μL water, respectively, followed by incubation at >95°C for 10 min. The air samples collected in PBS (500 μL) were also incubated at >95°C for 10 min. The supernatants obtained were transferred to fresh tubes. These samples were used to determine the LAMP specificity and reproducibility and for the PCR reactions. (ii) Bacteria were incubated in PEG buffer (60% PEG 200, 20 mM KOH, pH 13.3) for 15 min at room temperature, and then diluted 10-fold in TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA). The PEG-assisted approach is more applicable for on-site testing, and it was therefore introduced into the testing protocol, with these samples used for determination of the sensitivity, selectivity, and repeatability of the LAMP assays.

PCR Analysis

A total of 104 *E. coli* isolates were screened using PCR for the presence of 19 genes that encode virulence factors involved in adhesion (*fimH*, *papC*, *papG*II, *hra*), iron acquisition (*fyuA*, *irp2*, *iroN*, *sitA*, *iucD*, *iutA*), increased serum resistance (*iss*, *traT*), toxin production (*hlyA*, *vat*, *tsh*, *astA)*, outer membrane protein synthesis *(ompT*), production of microcin V (*cvi*), and capsule

Table 1. Oligonucleotide primers used for the PCR amplifications.

Primer name	Primer sequence	Size of PCR product (bp)	Source
AstA-F	5′-TGCCATCAACACAGTATATCC-3′	116	Kemmett et al. (2014)
AstA-R	5′-TCAGGTCGCGAGTGACGGC-3′		
Cvi-F	5′-TGGTAGAATGTGCCAGAGCAAG-3′	1181	Dozois et al. (1992)
Cvi-R	5′-GAGCTGTTTGTAGCGAAGCC-3′		
FimH-1	5′-AACAGCGATGATTTCCAGTTTGTGTG-3′	465	Erjavec et al. (2011)
FimH-2	5′-ATTGCGTACCAGCATTAGCAATGTCC-3′		
FyuA-F	5′-TGATTAACCCCGCGACGGGAA-3′	880	Johnson and Stell (2000)
FyuA-R	5′-CGCAGTAGGCACGATGTTGTA-3′		
hlyA-F	5′-AACAAGGATAAGCACTGTTCTGGCT -3′	1177	Yamamoto et al. (1995)
hlyA-R	5′-ACCATATAAGCGGTCATTCCCGTCA-3′		
Hra-F	5′-TACGGTATTCAGTGGCGGTATC-3′	471	Erjavec et al. (2011)
Hra-R	5′-TCGTCCTTGTAACTCACACTGC-3′		
IroN-F	5′-AAGTCAAAGCAGGGGTTGCCCG-3′	668	Johnson et al. (2000)
IroN-R	5′-GACGCCGACATTAAGACGCAG-3′		
Irp2-F	5′-AAGGATTCGCTGTTACCGGAC-3′	286	Schubert et al. (1998)
Irp2-R	5′-TCGTCGGGCAGCGTTTCTTCT-3′		
Iss-F	5′-ACGATACTCCGTAGCCAGAGAT-3′	793	Erjavec et al. (2011)
Iss-R	5′-ATGAACAGTGCAGATGAGCTCC-3′		
iucD/F	5′-ACAAAAAGTTCTATCGCTTCC-3′	711	Janßen et al. (2001)
iucD R	5′-CCTGATCCAGATGATGCTC-3′		
IutA-F	5′-GGCTGGACATCATGGGAACTGG-3′	302	Johnson et al. (1998)
IutA-R	5′-CGTCGGGAACGGGTAGAATCG-3′		
KpsMTII-F	5′-GCGCATTTGCTGATACTGTTG-3′	270	Johnson and Stell (2000)
KpsMTII-R	5′-CATCCAGACGATAAGCATGAGCA-3′		
OmpT-F	5′-CAGAGTATCTGTCGGTGCCTCA-3′	581	Trkov et al. (2014)
OmpT-R	5′-TACGGTTCCATGTTCCTTCGAC-3′		
papC-F	5′-TGATATCACGCAGTCAGTAGC-3′	501	Franck et al. (1998)
papC-R	5′-CCGGCCATATTCACATAA-3′		
PapGII-F	5′-GGGATGAGCGGGCCTTTGAT-3′	190	Johnson and Brown (1996)
PapGII-R	5′-CGGGCCCCCAAGTAACTCG-3′		
TraT/F	5′-GGTGTGGTGCGATGAGCACAG-3′	290	Johnson and Stell (2000)
TraT-R	5′-CACGGTTCAGCCATCCCTGAG-3′		
Tsh-F/Hbp-F	5′-GGCGGACAATAAAGGACAGG-3′	829	Trkov et al. (2014)
Tsh-R/Hbp-R	5′-GGAGTTATCTGCCTGGATGG-3′		
sitA-F	5′-AGGGGGCACAACTGATTCTCG-3′	608	Rodriguez-Siek et al. (2005)
sitA-R	5′-TACCGGGCCGTTTTCTGTGC-3′		
Vat-F	5′-GAACACAGTTCATCTGATCTCC-3′	419	Parham et al. (2005)
Vat-R	5′-GAATATATCAAATTGGTCCCCC-3′		

F, forward; R, reverse

synthesis (*kpsMT*II) (Table 1). The PCR amplifications were performed in a total volume of 25 μL, which contained 1 μL bacterial sample, 12.5 μL PCR Master mix (Thermo Scientific), and 20 pg of each primer. The reaction conditions were different for the different primer sets, depending on the amplicon length and melting temperatures of the primers (see Table 1).

LAMP Primer Design and Reactions

Sequences for selected APEC-associated virulence genes (*sitA*, *ompT*, *traT*) and an *E. coli* specific gene (*lamB*) were retrieved from NCBI and aligned in the VectorNTI software (InforMax). Regions of homology that were specific for *E. coli* were determined. Two LAMP primer sets were designed to detect the APEC-associated virulence genes and the *E. coli* specific gene using the LAMP Designer software (Premier Biosoft), and were synthesized by Integrated DNA Technologies. The LAMP reactions were performed in a 10-μL reaction volume that contained 4-μL bacterial lysate. The reaction mix contained 2 × Isothermal Master Mix (OptiGene), 0.2 μM F3 and B3 primers, 2 μM FIP and BIP primers, and 1 μM F-loop and B-loop primers. The LAMP reactions were performed in 8-well strips, or 96-well or 384-well plates, in a GenieII (Optigene) or in a Roche LC480 instrument, respectively, at 65°C. For determination of the LAMP product annealing temperature (Tm), the fluorescence was detected (FAM channel for the Roche LC480) during the cooling of the samples from 98 to 80°C (GenieII) or during the heating of the samples from 62 to 98°C (Roche LC480).

LAMP Assay Selection and Optimization

The performances of individual LAMP assays were initially tested on a small set of samples (selected samples that tested positive or negative with PCR; Additional File 1). Different primer concentrations and combinations (e.g., excluding loop primers), amplification temperatures (65°C, 67°C), and reaction and DNA volumes were tested to determine the optimal reaction conditions. From all of the assays developed and tested (for the individual targets), the assays that showed specific amplification, gave the shortest time to positivity

Table 2. LAMP primer sequences selected for detection of the *sitA*, *traT, ompT,* and *lamB* genes.

Primer	Sequence (5′-3′)
sitA-F3	CAGAGCATTATCTGGCGAC
sitA-B3	CCATAACCAAGCCTGGTG
sitA-FIP	AATTGTCTCTTCGGGTGTGACGG GTTAGGTTTGCCCTCATAG
sitA-BIP	TTCTGGAACCCCATTGAGATGCT GATTCTCGCCAATGGTATG
sitA-LoopF	WGTAGGGATCACCGAAGGR
sitA-LoopB	GCGTTGGAACCACAATTCC
traT-F3	GATGGTGGAAGATGTGAACTAT
traT-B3	CTTCGAGAACAGGCTTCG
traT-FIP	GGCAACATTGTCCGTTGTCACG ATCACGGATGTRCAGATT
traT-BIP	CTGCGTCAGGGCACATCAGGTCT GGTATTTATGCTGGTT
traT-LoopF	CGTTGCCTTAGTACGCTCT
traT-LoopB	TTCAGACCAGTACTGAAACAGG
ompT-F3	GATAGTCTGGTTGATTCAGGAG
ompT-B3	GGGTTTATCTGCCTGAAGAAA
ompT-FIP	GGAGGCAATATGGTGGACAGG TTACTCTCATCAGTCCAGGTT
ompT-BIP	ACGACCAGCTAATGTTGTCCAC GGCATTTAACTGGGATCTGT
ompT-LoopF	TGGCTGGATACGAGCAATC
ompT-LoopB	CTGAGGCACCGACAGATAC
lamB-F3	TACTGACTCGATGACCTCG
lamB-B3	ATGATTGGCGTCCACTTG
lamB-FIP	CATGGAGATCGCACCGTGGGCC TACAATATCAAYAACAACG
lamB-BIP	GGCGACAACTGGGACATGAT GTTGTCGTTATCCCAGTTGA
lamB-LoopF	CGAGGATACGCAGCATGT
lamB-LoopB	TACGTGGGTATGTACCAGGATA

(Tpos), and showed no background fluorescence were selected for further testing (Table 2).

Assessment of the LAMP Assays

The analytical specificities of the 3 APEC-associated virulence gene-specific LAMP assays were analyzed by testing 49 *E. coli* isolates that originated from different animal and environment samples (Additional File 3). The specificities of the *E. coli* specific LAMP-lamB assays were evaluated by testing 28 *E. coli* isolates. The specificities of all of the LAMP assays were evaluated by testing 10 isolates of *Salmonella enterica* subsp. *enterica*. The analytical sensitivities of the APEC-associated virulence gene-specific LAMP assays were evaluated by testing dilution series of *E. coli* lysed in PEG buffer. Furthermore, the sensitivities of these assays were evaluated by testing *E. coli* dilution series spiked into 1 g chicken liver and heart tissue, separately, with 3 mL PEG buffer added. Each sample was analyzed in triplicate. The selectivities of the LAMP assays were evaluated by testing samples of various chicken tissues and environmental samples (e.g., feces, air, feed) prepared in PEG buffer. Two to 3 replicates of each sample were tested for determination of specificities and selectivities.

The repeatabilities of the assays were evaluated by analyzing 3 replicates of individual samples with various titers of bacterial DNA prepared by dilution in different matrices (3 altogether for sensitivity evaluation). For testing the reproducibilities, analyses were performed on up to 17 different days with new/freshly prepared reaction mix, by 2 different operators, and on 2 different devices (GenieII, Roche LC480), where one or more parameters were changed per repetition (e.g., operator, device, day).

On-site Analysis

The applicability and performance of the LAMP method were tested on 6 unrelated poultry farms, as 2 commercial chicken laying farms, 2 broiler farms, and 2 healthy chicken flocks of experimental animals kept for diagnostic purposes. In both of the chicken flocks for the commercial layers, higher mortality and drop in egg production occurred at the age of 45 (flock D) and 49 (flock C) wk. Three dead birds were collected from each of these flocks. The necropsy analyses indicated fibrinous oophoritis, peritonitis, aerosacculitis, and pneumonia. *E. coli* was identified by conventional bacteriological examination in the heart, ovary, liver, and lungs. Cloacal and tracheal swabs and different organ tissues (Additional File 5) with pathological lesions of colibacillosis were taken for testing with the LAMP assays. In both these flocks, concomitant infections with *Mycoplasma synoviae* were confirmed, whereas the chickens from flock C were also positive for *Ornithobacterium rhinotracheale*. In the broiler flock B lameness, depression, respiratory disorders, and increased mortality occurred at the age of 7 d. Tracheal and cloacal swabs were taken from 5 sick broilers. The necropsy analyses showed severe inflammation of air sacs, fibrinous peritonitis, and pericarditis. *E. coli* isolates were identified in lungs, livers, heart, and tibiotarsal swabs by conventional bacteriology. No disease symptoms were observed on a broiler flock G, used as control group. Samples from flocks of clinically healthy experimental chickens (flocks A and E) were included in the study as control groups as well, which were kept for diagnostics proposes at the Veterinary Faculty, University of Ljubljana (Slovenia, approved by the Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection; N° U33401–18/2014/4). Organs and swabs (Additional File 5) of the 2 chickens from flock A euthanized according to animal welfare norms were collected, and 2 additional cloacal and tracheal swabs were taken from 2 live animals. Cloacal swabs were taken from 7 animals from flock E. At least 1 environmental sample (air, feed, feces) was taken from all of the farms. All of these samples were prepared in PEG buffer, with approximately 1 g material (feces, feed) or 1 mL liquid (air samples) incubated with 3 mL PEG at room temperature for 15 min. Swabs were incubated in 1 mL PEG. All of these samples were 10-fold diluted and tested directly in the LAMP assays as at least 2 replicates. An additional 11 air samples were included in the study.

The air was sampled in the field for 11 flocks (5 flocks of meat-type turkeys; 2 flocks of broiler breeders; 4 flocks of broilers) of different ages (1 to 10 wk) for 8 different locations in Slovenia (Additional File 5). These air samples were collected in PBS and were boiled and centrifuged before being directly tested in the LAMP assays as 2 replicates. Samples were deemed positive when for at least 2 replicates a time to positivity (Tpos) and the target gene-specific melting temperature (Tm) were obtained in the LAMP reactions.

Statistical Analysis

Comparisons of associations between different virulence genes in the *E. coli* isolates from different types of samples were performed using chi-square exact tests. The various classes of samples were compared using Bonferroni's tests for column proportions. Statistical significance was set at $P < 0.05$.

RESULTS

Detection of Virulence-associated Genes and Statistical Analysis

Altogether, 104 *E. coli* isolates were tested for the presence of the 19 selected virulence-associated genes using PCR. Almost all of these samples were positive for the *fimH* gene regardless of type and source (Additional File 1). The 3 genes *sitA*, *traT,* and *ompT* were present at high frequencies in the *E. coli* isolates from the organ samples. Interestingly, only a few samples were positive for the *hlyA*, *papC,* and *papG*II genes. Statistical analyses were carried out for easier interpretation of the data and selection of the genes with the highest occurrences (Table 3). The presence of the *ompT*, *iss*, *traT*, *iroN,* and *cvi* genes in the *E. coli* isolates obtained from the organs of the dead animals was determined as statistically significant (Bonferroni's tests).

The occurrence of virulence genes associated with APEC was also investigated in silico in the literature, considering different geographical locations, study designs, detection and analysis approaches. Several studies have shown that the *sitA*, *ompT,* and *traT* genes prevailed in the environments tested (Additional File 2).

Based on the statistical approaches and in silico analysis of published data, a set of 3 APEC-associated virulence genes was selected to develop the LAMP method: *sitA*, *ompT,* and *traT*.

Primer Design and Evaluation of the LAMP Assays

Two sets of primers were designed for the *sitA*, *ompT,* and *traT* genes, where regions of sequence specific for *E. coli* were identified. Two additional sets of primers were designed for detection of all *E. coli* (general assays) that targeted the *lamB* gene. Tpos, specificity, sensitivity, and generation of artifacts of each LAMP assay were evaluated through the analysis of a set of samples with different *E. coli* DNA concentrations, along with negative samples. The optimal sets of primers were selected for further tests (Table 2).

LAMP Assay Specificity

The analytical specificities of the LAMP assays were initially evaluated using in silico analysis, which showed high predicted specificities for *E. coli*.

The specificities of all of the LAMP assays were evaluated through testing 59 isolates: 31 *E. coli* isolates from poultry farms (LAMP-lamB evaluated on only 10 isolates), 13 *E. coli* isolates from other hosts, 5 isolates from dishwashers, and 10 *Salmonella* isolates (Additional File 3). The samples were tested in parallel with PCR, and the data were compared. In 57 out of these 59 samples, the presence of the selected APEC virulence genes was confirmed by both methods, with >96.6% diagnostic sensitivity of the APEC-associated virulence gene-specific LAMP assays (Table 4, Additional File 3). The differences between the data acquired by each of these methods can be attributed to the higher sensitivity of the LAMP method over the PCR method. The high Tpos in these samples showed that there were fewer copies of the selected gene in the samples. For *Salmonella typhimurium* (sample code, 145/15) the *traT* gene was detected with PCR, but not with the LAMP assay. Indeed, the primers designed for this PCR can amplify the *S. typhimurium* isolates (according to the BLAST searches), while the LAMP primers were specifically designed to exclude *Salmonella* isolates. The *E. coli* specific LAMP assays showed high specificity for *E. coli* isolates, and did not react with the *Salmonella* isolates.

All of the positive results from all 3 of the LAMP assays were obtained in <25 min of amplification, with a mean Tpos of 5.5. ± 3.6, 3.8 ± 0.8, 3.6 ± 1.1, and 5.0 ± 1.3 min for the *sitA*, *traT*, *ompT,* and *lamB* LAMP assays, respectively. The short time needed to obtain the results of these reactions suggests the possibility of their use for rapid diagnostics.

The anneal temperature (Tm) analysis of the LAMP products showed that all of the signals were specific. The mean Tm for the specific amplicons was 86.9 ± 0.3, 88.6 ± 0.2, 83.1 ± 0.2, and 88.2 ± 0.2 for the *sitA*, *traT*, *ompT,* and *lamB* LAMP assays, respectively.

Analytical Sensitivity of the APEC-associated Virulence Gene-specific LAMP Assays

The sensitivities of the on-site APEC-associated virulence gene-specific LAMP assay protocols were determined by testing an *E. coli* (isolate code, 7007–54 C/O) dilution series prepared in PEG buffer without and with

Table 3. Occurrence of the virulence-associated genes in the *E. coli* isolates from the difference sources.

Gene	Positive/total *E. coli* isolates according to source (%)				
	All	Organs	Faecal	Air	*P*-value
*kpsMT*II	17/104 (16.4)	10/59 (16.9)	3/29 (10.3)	4/16 (25.0)	0.450
fimH	101/104 (97.1)	57/59 (96.6)	29/29 (100)	15/16 (93.8)	0.620
papC	4/101 (4.0)	3/56 (5.4)	1/29 (3.5)	0/16 (0.0)	1.000
*papG*II	3/101 (3.0)	3/56 (5.4)	0/29 (0.0)	0/16 (0.0)	0.560
hra	18/101 (17.8)	9/56 (16.1)	6/29 (20.7)	3/16 (18.8)	0.960
ompT	**76/104 (73.1)**	**49[a]/59 (83.1)**	**19[a,b]/29 (65.5)**	**8[b]/16 (50.0)**	**0.018**
iss	60/104 (57.7)	40[a]/59 (67.8)	15[a,b]/29 (51.7)	5[b]/16 (31.3)	0.020
traT	**82/104 (78.8)**	**52[a]/59 (88.1)**	**20[a,b]/29 (69.0)**	**10[b]/16 (62.5)**	**0.020**
fyuA	34/104 (32.7)	24/59 (40.7)	5/29 (17.2)	5/16 (31.3)	0.079
irp2	33/104 (31.7)	22/59 (37.3)	5/29 (17.2)	6/16 (37.5)	0.133
iroN	67/103 (65.1)	44[a]/58 (75.9)	17[a,b]/29 (58.6)	6[b]/16 (37.5)	0.011
sitA[c]	**80/101 (79.2)**	**48/56 (85.7)**	**20/29 (69.0)**	**12/16 (75.0)**	**0.089**
iucD	50/101 (49.5)	30/56 (53.6)	13/29 (44.8)	7/16 (43.8)	0.694
iutA	54/104 (51.9)	34/59 (57.6)	13/29 (44.8)	7/16 (43.8)	0.490
hlyA	0/101 (0.0)	0/56 (0.0)	0/29 (0.0)	0/16 (0.0)	n.d.
vat	14/104 (13.5)	11/59 (18.6)	2/29 (6.9)	1/16 (6.3)	0.109
tsh	20/101 (19.8)	15/56 (26.8)	4/29 (13.8)	1/16 (6.3)	0.120
astA	19/101 (18.8)	9/56 (16.1)	5/29 (17.2)	5/16 (31.3)	0.398
cvi	32/101(31.7)	22[a]/56 (39.3)	9[a,b]/29 (31.0)	1[b]/16 (6.3)	0.012

P-values calculated with Bonferroni's tests.
Genes selected for development of LAMP assays shown in bold.
[a,b]Different subsets of *E. coli* sources where the column proportions differed significantly from each other ($P < 0.05$; Bonferroni's tests for column proportions).
[c]Gene was selected on a basis of literature search.
n.d., not determined.

Table 4. Correlations between the PCR assays and the APEC virulence gene-specific APEC LAMP assays for the 59 isolates included.

PCR positivity	LAMP Positivity	Isolates with assay correlation (n)		
		sitA	*traT*	*ompT*
Yes	Yes	33	40	32
No	Yes	2	0	1
Yes	No	0	1	0
No	No	24	18	26

the addition of chicken organ tissue (liver, heart). Testing of this *E. coli* dilution series showed that all 3 of the *sitA*, *traT,* and *ompT* LAMP assays detected as low as 10^3 cells/mL with at least 2 replicates identified as positive (Table 5). The same sensitivities were observed in the samples with the added chicken liver and heart tissues.

The linearity of the Tpos and number of cells/mL show the semiquantitative nature of these LAMP assays, especially in the range of 10^6 to 10^4 cell/mL, with the exception of the *traT* LAMP assay, which has a linear range from 10^6 to 10^2 cell/mL, regardless of the sample matrix (Additional File 4).

Repeatability and Reproducibility

The repeatabilities of the APEC-associated virulence gene-specific LAMP assays were determined through the analysis of 3 replicates of individual samples with various bacterial DNA concentrations. In the samples with the higher bacterial DNA concentrations ($>10^3$ cells/mL), the assay repeatability was 100%, which means that all of the replicates gave positive results (Table 5). At lower bacterial DNA concentrations, the detection of the individual target genes using the LAMP assays varied, giving positive and negative results. This can be attributed to stochastic effects in the target copy distribution in the replicates. The data were 100% reproducible when tested with the different devices, and by the different operators, on the different days, and with the different reaction mixes (data not shown).

Sensitivity and Selectivity of the on-site LAMP Testing Approach Evaluated on Actual Samples

A total of 116 samples from a total of 6 flocks that showed different health status regarding *E. coli* infections were tested for the presence of all 3 of these APEC-associated virulence genes and *E. coli* specific genes (Additional File 5, Table 6). As expected, no APEC-associated virulence genes nor *E. coli* specific genes were detected in samples collected from the internal organs of the experimental animals (muscle, liver, spleen, kidney, lungs, heart). Conversely, all of the LAMP assays gave positive reactions with cloaca and (some) trachea swabs from the experimental animals, as well as for both of the air samples collected from the experimental animal farms (flock A).

All of the tissue samples collected from the dead commercial layer chickens with pathological lesions of colibacillosis tested positive for all 3 APEC-associated virulence genes. Interestingly, all 3 of these virulence

Table 5. Analytical sensitivities for time to positivity (Tpos) and target gene-specific melting temperature (Tm) of the *sitA*, *traT,* and *ompT* APEC LAMP assays when tested on *E. coli* suspensions as dilution series (in PEG buffer; n = 3).

LAMP	Dilution	No tissue		Liver tissue		Heart tissue	
gene assay	(cells/mL)	Tpos (min)	Tm (°C)	Tpos (min)	Tm (°C)	Tpos (min)	Tm (°C)
sitA	10^6	4.8 ± 0.3	85.9 ± 0.0	4.3 ± 0.4	85.5 ± 0.6	4.2 ± 0.5	86.0 ± 0.2
	10^5	7.0 ± 0.4	86.7 ± 0.1	5.5 ± 0.7	85.8 ± 0.1	6.0 ± 0.2	85.8 ± 0.1
	10^4	9.0 ± 0.4	86.9 ± 0.0	7.2 ± 0.2	85.8 ± 0.1	6.7 ± 0.9	85.8 ± 0.0
	10^3	10.2 ± 1.6	87.0 ± 0.1	11.0 ± 3.3	85.8 ± 0.1	13.9 ± 1.4	85.9 ± 0.2
	10^2	neg	/	neg	/	21.2[b]	85.8
	10^1	neg	/	neg	/	neg	/
traT	10^6	5.3 ± 0.2	87.5 ± 0.0	3.8 ± 0.6	87.7 ± 0.2	4.8 ± 0.1	87.6 ± 0.2
	10^5	6.6 ± 0.9	88.4 ± 0.1	6.5 ± 0.2	87.5 ± 0.1	5.9 ± 0.6	87.5 ± 0.1
	10^4	9.4 ± 0.4[a]	88.2 ± 0.2	7.6 ± 1.4	87.3 ± 0.1	7.9 ± 0.4	87.4 ± 0.0
	10^3	13.3 ± 3.1	88.7 ± 0.0	11.2 ± 2.9	87.3 ± 0.1	12.4 ± 3.0[a]	87.7 ± 0.1
	10^2	neg	/	14.4[b]	87.7	neg	/
	10^1	neg	/	neg	/	neg	/
ompT	10^6	4.6 ± 0.3	82.1 ± 0.0	4.5 ± 0.1	82.2 ± 0.2	4.3 ± 0.1	82.1 ± 0.1
	10^5	5.8 ± 0.1	82.9 ± 0.0	5.6 ± 0.3	82.0 ± 0.1	5.0 ± 0.4	82.2 ± 0.0
	10^4	8.0 ± 1.6	82.9 ± 0.1	8.4 ± 1.0	81.8 ± 0.0	8.2 ± 1.7	81.9 ± 0.1
	10^3	8.9 ± 1.0[a]	83.6 ± 0.1	11.6 ± 2.7	81.5 ± 0.3	15.6 ± 2.5	81.8 ± 0.0
	10^2	neg	/	17.5 ± 2.6[a]	82.3 ± 0.1	16.0[b]	82.5
	10^1	neg	/	neg	/	9.2[b]	82.4

Data are means ±standard deviation.
[a]Two of 3 replicates positive.
[b]One of 3 replicates positive.
neg, no amplification detected;/, no Tm available.

Table 6. Overview of the time to positivity (Tpos) and target gene-specific melting temperature (Tm) of the *lamB*, *sitA*, *traT,* and *ompT* APEC LAMP assays of the actual samples (see also Additional File 5).

		lamB		*sitA*		*traT*		*ompT*	
LAMP assay	Statistic	Tpos (min)	Tm (°C)	Tpos (min)	Tm (°C)	Tpos (min)	Tm (°C)	Tpos (min)	Tm (°C)
Flocks C and D Commercial layers—colibacillosis	Mean	6.5	86.5	5.2	85.2	7.6	87.1	5.2	81.9
	SD	1.9	0.2	1.7	0.3	5.5	0.2	1.5	0.2
	Minimum	3.6	86.1	2.8	84.7	2.2	86.8	2.5	81.4
	Maximum	13.5	87.1	10.1	85.9	29.0	87.7	11.1	82.5
	Positivity (%)	**100**		**100**		**100**		**100**	
Flock B Broilers—affected	Mean	11.2	86.8	9.2	85.5	9.9	87.2	9.6	82.0
	SD	6.3	0.1	4.5	0.1	6.2	0.2	5.0	0.2
	Minimum	5.0	86.5	5.0	85.3	5.0	86.9	5.0	81.8
	Maximum	22.4	87.0	17.9	85.7	25.9	87.6	19.5	82.3
	Positivity (%)	**100**		**100**		**85**		**92**	
Flock F Broilers	Mean	10.2	86.1	9.0	84.8	9.4	86.7	9.6	81.5
	SD	2.7	0.2	3.9	0.2	4.4	0.2	3.1	0.3
	Minimum	7.3	85.9	6.7	84.6	6.6	86.4	6.7	81.3
	Maximum	12.6	86.3	14.8	84.9	14.4	86.8	12.9	81.8
	Positivity (%)	**75**		**100**		**75**		**75**	
Flock A Control flock—clinically healthy	Mean	11.0	86.8	11.5	85.6	11.4	87.3	12.2	82.1
	SD	5.3	0.2	7.2	0.2	5.6	0.2	5.7	0.2
	Minimum	6.3	86.6	5.9	85.3	6.2	87.0	6.7	81.7
	Maximum	19.5	87.1	30.8	86.2	22.3	87.6	21.6	82.3
	Positivity (%)	**42**		**50**		**38**		**42**	
Flock E Control flock—clinically healthy	Mean	11.7	86.4	13.3	85.1	16.6	87.2	11.4	81.9
	SD	2.5	0.1	2.5	0.1	4.3	0.2	2.4	0.1
	Minimum	8.9	86.1	9.8	85.0	11.7	86.9	9.0	81.7
	Maximum	14.9	86.5	15.3	85.3	21.9	87.4	15.1	82.0
	Positivity (%)	**88**		**50**		**75**		**63**	
Flock C and D Commercial layers—clinically healthy	Mean	14.0	86.3	13.6	84.9	13.8	86.7	14.3	81.7
	SD	6.4	0.4	3.7	0.5	6.8	0.4	5.1	0.5
	Minimum	8.4	85.7	8.1	84.2	5.0	86.2	7.9	81.1
	Maximum	34.2	87.1	21.1	85.7	30.2	87.3	24.7	82.5
	Positivity (%)	**57**		**57**		**64**		**39**	

SD, standard deviation.

genes were also detected in swabs of clinically healthy animals from these 2 flocks and in 2 control flocks of healthy animals, although lower percentages of positive samples were obtained (Table 6, 39 to 75%). The relative levels of the virulence genes appeared to show estimates that were lower than in the samples obtained from the dead animals (Tpos >13 min for all 3 virulence genes); however, additional studies need to be carried out to confirm this observation. In some of the samples where virulence genes were detected, the *E. coli* specific LAMP assays gave negative results, which implies that there were low levels of the bacteria. Higher incidence of virulence genes was also detected for the broiler flocks (flocks B and G), where in almost all of the samples all 3 of the virulence genes were detected with the LAMP assays. The incidence of the virulence genes was 75 to 100%. All of the additional 11 air samples collected from 11 flocks also tested positive for all of the 3 virulence genes (Additional File 5).

For all of these genes tested, Tpos was <35 min (Additional File 5), which is a sufficient time frame for on-site detection of the APEC-associated virulence genes in actual samples.

The selectivities of the LAMP assays were determined through testing samples of the various chicken tissues and the environmental samples (e.g., feces, air, feed). No differences in detection of the target gene in terms of Tpos were seen for any of these samples. However, differences in Tm were seen between different samples, which varied by up to 1.7°C between individual measurements for samples prepared with the PEG buffer (s*itA*, *traT* LAMP assays). Furthermore, the mean Tm was up to 3.9°C higher in the air samples (Additional File 5).

DISCUSSION

Under field conditions, the diagnosis of colibacillosis relies in many cases on only the clinical picture and post-mortem lesions seen for diseased or dead birds. However, it is well known that other bacterial and viral pathogens can cause similar clinical signs and pathological lesions, especially when they occur in an acute septicemia setting (Nolan et al., 2013). To limit and prevent further spread of the disease, rapid and accurate diagnosis is crucial. Conventional detection and identification approaches do not offer rapid diagnosis, and faster molecular-biology-based methods also require in-laboratory conditions. Although some qPCR cyclers are portable, the DNA extraction process that is needed prior to the qPCR amplification is the limiting step for on-site application. Also, the LAMP method is not sensitive to inhibitory effects from different sample matrices, and so it can be used for direct testing of samples without the need for their purification (Kogovšek et al., 2017; Stratakos et al., 2017) and can be applied on site for fast diagnosis (Tang et al., 2012).

It has been shown that different virulence factors can be used as detection markers for LAMP testing of *E. coli* pathogenic strains, such as Shiga toxin *stx* genes (Stratakos et al., 2017); the intimin *eae* gene (Xue-han et al., 2013); and the O-antigen specific genes *rfbE*, *wzx,* and *wzy* (Wang et al., 2009, 2012), or *invA* gene for detection of *Salmonella* isolates (Youn et al., 2017). Virulence factors connected with APEC strains have been identified, although none of these have been exclusively linked to colibacillosis, as they have been shown to be present also in commensal strains (Dziva and Stevens, 2008). Therefore, presence of several virulence genes needs to be followed to monitor for potential APEC strains. The data presented here on the occurrences of 19 virulence-associated genes in *E. coli* isolates obtained from Slovenian poultry and their environment, as well as the in silico investigations (Additional File 2), indicate that *sitA*, *traT,* and *ompT* can be associated with APEC. The *sitA* gene encodes a periplasmic-binding protein of the SitABCD transport system, which is involved in iron and manganese transport and which provides increased resistance to oxidative stress (Dziva and Stevens, 2008; Kemmett et al., 2013). The *ompT* gene encodes an outer membrane protein and it has been shown that inactivation of this gene decreases the cell adhesion, invasion, colonization, and proliferation potential of APEC strains (Hejair et al., 2017). The *traT* gene is involved in serum resistance mechanisms, and as for the 2 other selected genes, it has an important role in APEC virulence (Nolan et al., 2013; Maciel et al., 2017).

Evaluation of the performance of the individual APEC-associated virulence gene LAMP assays showed high specificity, reproducibility, and repeatability. All 3 of these LAMP assays also showed high sensitivity, with the detection of as few as 10^3 cells/mL, regardless of the sample type. LAMP assays were reported to be more sensitive than classical PCR assays (Ji et al., 2010) and we observed similar effect (Additional File 3). Actual samples were imitated here by addition of liver and heart tissue to the buffer, and these data from all 3 of the LAMP assays show that none of the potential inhibitory factors in these samples affected the LAMP amplification and its sensitivity. Furthermore, actual samples from poultry farms were tested directly with the LAMP assays, and in all types of the samples tested, APEC-associated virulence genes were detected. The applicability of this LAMP method to direct detection of *E. coli* has been reported previously for beef and bovine feces samples (Stratakos et al., 2017), minced meat (Ravan et al., 2016), milk (Yang et al., 2014), lettuce (Xue-han et al., 2013), stool samples (Song et al., 2005; Teh et al., 2014), and others. In the present study, we have shown that this LAMP method is also applicable for direct testing of various environmental samples (e.g., feed, feces, air sampled in PBS buffer) as well as of tissues of the internal organs of chickens. In the latter case, we were able to detect APEC-associated virulence genes in all samples of dead

animals (100% prevalence) showing that LAMP assays can be applied as fast on-site confirmation tool. Similarly, *Salmonella* spp. was detected with the LAMP method in duck liver and spleen homogenates (Tang et al., 2012) and in rinse water of chicken carcasses (Youn et al., 2017).

Although there were no effects of the different tissues and buffers on these amplifications, significant effects on Tm were detected between the samples prepared by boiling of bacterial suspensions and air samples collected in PBS, and those prepared with PEG buffer and diluted in TE. Overall, for the air samples collected in PBS and prepared by boiling, the Tm was higher than for the samples of boiled pure bacterial suspensions. The effects of PBS alone to Tm were evaluated by spiking DNA into PBS, with increased Tm seen (data not shown). On the other hand, the air samples collected in PBS and prepared with the PEG buffer gave lower mean Tm values than those prepared by boiling only. These data demonstrate effects of these different buffers and matrixes on the Tm in all of these LAMP assays tested, as has already been indicated before (Kogovšek et al., 2017). The effects of the buffer components on Tm should be evaluated for each sample preparation protocol and should be monitored using spiking of positive controls.

These data indicate high prevalence of bacteria harboring virulence genes in the affected commercial layers from 2 independent farms (100%) and in an affected broiler flock (85 to 100%). High prevalence of bacteria harboring virulence genes was also detected in healthy broiler flock. On the other side, lower levels of virulence genes (38 to 75%) were determined in clinically healthy animals from the commercial layer chickens from the same farm as the affected layer chickens and in clinically healthy control flocks A and E. The results also show correlation between average Tpos (minutes) and percentage of positive samples in individual flock, indicating higher amount of bacterial DNA in samples from flocks with higher prevalence of the virulence genes and vice versa. A time-spanning on-site experimental study would need to be carried out to further explore the connection between the prevalence of the virulence genes, Tpos, health status of animals, and probability for development of the disease in individual flock.

Colibacillosis outbreaks are believed to be linked to several independent factors, including environmental conditions and infections with other pathogens (Guabiraba and Schouler, 2015). Indeed, infections with other respiratory pathogens were seen here in the commercial layer chickens. Furthermore, fecal contamination in air and dust has been reported to be an important route for *E. coli* spread through a flock (Guabiraba and Schouler, 2015). This was also confirmed in the present study, as high prevalence of the selected APEC-associated virulence genes was defined for air and fecal samples from the commercial layer flocks. Additionally, in almost all of the air samples taken from different farms (14 samples altogether), APEC-associated virulence genes were detected in different flocks, from layers to broilers and turkeys, and of different ages, regardless of the health status of the flocks.

The LAMP assays developed here are shown to be robust and to allow rapid on-site detection of virulence genes associated with APEC. The performance characteristics of the LAMP method itself also imply its use for various applications in the detection of other important poultry pathogenic microorganisms (Stępień-Pyśniak et al., 2018) in different matrices.

SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online.

ACKNOWLEDGMENTS

The whole study, collection of the samples, analysis and data interpretation, and preparation of the manuscript was supported financially by the Slovene Ministry of Agriculture, Forestry and Food, Slovene Research Agency (Project N° P4-0165, V4-1401) and the FP7-KBBE-2013-7-613908-Decathlon project. Poultry companies and poultry veterinarians who were involved are gratefully acknowledged for their contributions to this study. The authors would like to thank Christopher Berrie (freelance scientific editor, Cepagatti, Italy) for language revision.

REFERENCES

Arabi, S., M. Jafarpour, M. Mirinargesi, S. B. Asl, R. Naghshbandi, and M. Shabanpour. 2013. Molecular characterization of avian pathogenic *Escherichia coli* in broilers bred in Northern Iran. Glob. Vet. 10:382–386.

Dissanayake, D. R. A., S. Octavia, and R. Lan. 2014. Population structure and virulence content of avian pathogenic *Escherichia coli* isolated from outbreaks in Sri Lanka. Vet. Microbiol. 168:403–412.

Dozois, C. M., J. M. Fairbrother, J. Harel, and M. Bosse. 1992. pap- and pil-related DNA sequences and other virulence determinants associated with *Escherichia coli* isolated from septicemic chickens and Turkeys. Infect. Immun. 60:2648–2656.

Dziva, F., and M. P. Stevens. 2008. Colibacillosis in poultry: unravelling the molecular basis of virulence of avian pathogenic *Escherichia coli* in their natural hosts. Avian Pathol. 37:355–366.

Franck, S. M., B. T. Bosworth, and H. W. Moon. 1998. Multiplex PCR for enterotoxigenic, attaching and effacing, and shiga toxin-producing *Escherichia coli* strains from calves. J. Clin. Microbiol. 36:1795–1797.

Grimont, P., and F.-X. Weill. 2008. Antigenic formulae of the Salmonella serovars. WHO Collab. Cent. Ref. Res. Salmonella:1–167 http://nih.dmsc.moph.go.th/aboutus/media/antigenic%20formula%20of%20Salmonella.pdf.

Guabiraba, R., and C. Schouler. 2015. Avian colibacillosis: still many black holes. FEMS Microbiol Lett. 362:fnv118.

Hejair, H. M. A., J. Ma, Y. Zhu, M. Sun, W. Dong, Y. Zhang, Z. Pan, W. Zhang, and H. Yao. 2017. Role of outer membrane protein T in pathogenicity of avian pathogenic *Escherichia coli*. Res. Vet. Sci. 115:109–116 http://dx.doi.org/10.1016/j.rvsc.2017.01.026.

ISO 6579-1:2017. 2007. Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of Salmonella - Part 1: Detection of Salmonella spp. Int.

Organ. Stand. https://www.iso.org/standard/56712.html (accessed 05/11/2018).
ISO 6579:2002/Amd 1:2007. 2007. Annex D: Detection of Salmonella spp. in animal faeces and in environmental samples from the primary production stage. Int. Organ. Stand. https://www.iso.org/standard/42109.html (accessed 05/11/2018).
Janßen, T., C. Schwarz, P. Preikschat, M. Voss, H.-C. Philipp, and L. H. Wieler. 2001. Virulence-associated genes in avian pathogenic (APEC) isolated from internal organs of poultry having died from colibacillosis. Int. J. Med. Microbiol. 291:371–378 http://www.sciencedirect.com/science/article/pii/S1438422104700466.
Ji, J., Q. M. Xie, C. Y. Chen, S. W. Bai, L. S. Zou, K. J. Zuo, Y. C. Cao, C. Y. Xue, J. Y. Ma, and Y. Z. Bi. 2010. Molecular detection of Muscovy duck parvovirus by loop-mediated isothermal amplification assay. Poult. Sci. 89:477–483 http://dx.doi.org/10.3382/ps.2009-00527.
Johnson, J. R., and J. J. Brown. 1996. A novel multiply primed polymerase chain reaction assay for identification of variant papG genes encoding the Gal(alpha 1-4)Gal-binding PapG adhesins of *Escherichia coli*. J. Infect. Dis. 173:920–926 http://www.ncbi.nlm.nih.gov/pubmed/8603972.
Johnson, J. R., J. J. Brown, U. B. Carlino, and T. A. Russo. 1998. Colonization with and acquisition of uropathogenic *Escherichia coli* as revealed by polymerase chain reaction-based detection. J. Infect. Dis. 177:1120–1124.
Johnson, J. R., T. A. Russo, P. I. Tarr, U. Carlino, S. S. Bilge, J. C. Vary, and A. L. Stell. 2000. Molecular epidemiological and phylogenetic associations of two novel putative virulence genes, iha and iroN (E. coli), among *Escherichia coli* isolates from patients with urosepsis. Infect. Immun. 68:3040–3047.
Johnson, J. R., and A. L. Stell. 2000. Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J. Infect. Dis. 181:261–272 https://academic.oup.com/jid/article-lookup/doi/10.1086/315217.
Kemmett, K., T. Humphrey, S. Rushton, A. Close, P. Wigley, and N. J. Williams. 2013. A longitudinal study simultaneously exploring the carriage of APEC virulence associated genes and the molecular epidemiology of faecal and systemic E. coli in commercial broiler chickens. PLoS One 8:e67749 http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3692481/.
Kemmett, K., N. J. Williams, G. Chaloner, S. Humphrey, P. Wigley, and T. Humphrey. 2014. The contribution of systemic *Escherichia coli* infection to the early mortalities of commercial broiler chickens. Avian Pathol. 43:37–42. http://www.ncbi.nlm.nih.gov/pubmed/24328462.
Kogovšek, P., N. Mehle, A. Pugelj, T. Jakomin, H.-J. Schroers, M. Ravnikar, and M. Dermastia. 2017. Rapid loop-mediated isothermal amplification assays for grapevine yellows phytoplasmas on crude leaf-vein homogenate has the same performance as qPCR. Eur. J. Plant Pathol. 148:75–84. http://dx.doi.org/10.1007/s10658-016-1070-z.
Landman, W. J. M., and J. H. H. van Eck. 2015. The incidence and economic impact of the *Escherichia coli* peritonitis syndrome in Dutch poultry farming. Avian Pathol. 44:370–378.
Maciel, J. F., L. B. Matter, M. M. Trindade, G. Camillo, M. Lovato, S. de Ávila Botton, and A. Castagna de Vargas. 2017. Virulence factors and antimicrobial susceptibility profile of extraintestinal *Escherichia coli* isolated from an avian colisepticemia outbreak. Microb. Pathog. 103:119–122.
Nolan, L. K., H. J. Barnes, J.-P. Vaillancourt, T. Abdul-Aziz, and C. M. Logue. 2013. Colibacillosis. Pages 751–805 in Diseases of Poultry. D. E. Swayne, ed. Ames, Iowa: Wiley-Blackwell.
Parham, N. J., S. J. Pollard, M. Desvaux, A. Scott-Tucker, C. Liu, A. Fivian, and I. R. Henderson. 2005. Distribution of the serine protease autotransporters of the Enterobacteriaceae among extraintestinal clinical isolates of *Escherichia coli*. J. Clin. Microbiol. 43:4076–4082.
Ravan, H., M. Amandadi, and N. Sanadgol. 2016. A highly specific and sensitive loop-mediated isothermal amplification method for the detection of *Escherichia coli* O157: H7. Microb. Pathog. 91:161–165. http://dx.doi.org/10.1016/j.micpath.2015.12.011.
Rodriguez-Siek, K. E., C. W. Giddings, C. Doetkott, T. J. Johnson, and L. K. Nolan. 2005. Characterizing the APEC pathotype. Vet. Res. 36:241–256.
Schouler, C., B. Schaeffer, A. Brée, A. Mora, G. Dahbi, F. Biet, E. Oswald, J. Mainil, J. Blanco, and M. Moulin-Schouleur. 2012. Diagnostic strategy for identifying avian pathogenic *Escherichia coli* based on four patterns of virulence genes. J. Clin. Microbiol. 50:1673–1678.
Schubert, S., A. Rakin, H. Karch, E. Carniel, and J. Heesemann. 1998. Prevalence of the "high-pathogenicity island" of Yersinia species among *Escherichia coli* strains that are pathogenic to humans. Infect. Immun. 66:480–485.
Song, T., C. Toma, N. Nakasone, and M. Iwanaga. 2005. Sensitive and rapid detection of Shigella and enteroinvasive *Escherichia coli* by a loop-mediated isothermal amplification method. FEMS Microbiol. Lett. 243:259–263.
Erjavec, M. S., A. Palandačić, D. Žgur-Bertok, and J. Ambrožič Avguštin. 2011. Genetic background of uropathogenic *Escherichia coli* isolates from Slovenia in relation to fluoroquinolone and sulfamethoxazole/trimethoprim resistance. Acta Biol. Slov. 54:5–13.
Stępień-Pyśniak, D., U. Kosikowska, T. Hauschild, A. Burzyński, J. Wilczyński, A. Kolińska, A. Nowaczek, and A. Marek. 2018. A loop-mediated isothermal amplification procedure targeting the sodA gene for rapid and specific identification of *Gallibacterium anatis*. Poult. Sci. 97:1141–1147 http://dx.doi.org/10.3382/ps/pex420.
Stratakos, A. C., M. Linton, S. Millington, and I. R. Grant. 2017. A loop-mediated isothermal amplification method for rapid direct detection and differentiation of nonpathogenic and verocytotoxigenic *Escherichia coli* in beef and bovine faeces. J. Appl. Microbiol. 122:817–828.
Tang, T., A. Cheng, M. Wang, X. Li, Q. He, R. Jia, D. Zhu, and X. Chen. 2012. Development and clinical verification of a loop-mediated isothermal amplification method for detection of Salmonella species in suspect infected ducks. Poult. Sci. 91:979–986. http://dx.doi.org/10.3382/ps.2011-01992.
Teh, C. S. J., K. H. Chua, Y. A. L. Lim, S. C. Lee, and K. L. Thong. 2014. Loop-mediated isothermal amplification assay for detection of generic and verocytotoxin-producing *Escherichia coli* among indigenous individuals in Malaysia. The Scientific World Journal. Article ID 457839, 6 pages, 2014. https://doi.org/10.1155/2014/457839.
Trkov, M., T. Rupel, D. Žgur-Bertok, S. Trontelj, G. Avguštin, and J. Ambrožič Avguštin. 2014. Molecular characterization of *Escherichia coli* strains isolated from different food sources. Food Technol. Biotechnol. 52:1–6.
Wang, F., L. Jiang, Q. Yang, W. Prinyawiwatkul, and B. Ge. 2012. Rapid and specific detection of *Escherichia coli* serogroups O26, O45, O103, O111, O121, O145, and O157 in ground beef, beef trim, and produce by loop-mediated isothermal amplification. Appl. Environ. Microbiol. 78:2727–2736.
Wang, D. G., F. Liu, G. C. Huo, D. X. Ren, and Y. G. Li. 2009. Development and evaluation of a loop-mediated isothermal amplification method for detecting *Escherichia coli* O157 in raw milk. J. Rapid Methods Autom. Microbiol. 17:55–66.
Xue-han, Z., Y. Qing, L. Ya-dong, L. Bin, I. Renata, and H. Kong-wang. 2013. Development of a LAMP assay for rapid detection of different intimin variants of attaching and effacing microbial pathogens. J. Med. Microbiol. 62:1665–1672.
Yamamoto, S., A. Terai, K. Yuri, H. Kurazono, Y. Takeda, and O. Yoshida. 1995. Detection of urovirulence factors in *Escherichia coli* by multiplex polymerase chain reaction. FEMS Immunol. Med. Microbiol. 12:85–90.
Yang, W., X. Song, J. Wang, Z. Li, M. Ji, and Y. Li. 2014. Detection methods for milk pathogenic bacteria by loop-mediated isothermal amplification. Biosci. Trends 8:316–321.
Youn, S. Y., O. M. Jeong, B. K. Choi, S. C. Jung, and M. S. Kang. 2017. Application of loop-mediated isothermal amplification with propidium monoazide treatment to detect live Salmonella in chicken carcasses. Poult. Sci. 96:458–464. http://dx.doi.org/10.3382/ps/pew341.

APPENDIX

The datasets supporting the conclusions of this article are included within the article and its additional files.

Supplemental file 1: Results of the PCR testing for the presence of 19 virulence associated genes in *E. coli* isolates. 1 stands for positive result and 0 for negative result; nd—not determined.

Supplemental file 2: Overview of studies involving APEC-associated virulence genes occurrence. Percentage of positive *E. coli* isolates isolated from poultry is given, country of the study and reference are added as well. Percentage of positive isolates above 80% are shown in bold.

Supplemental file 3: Results of the LAMP assays *lamB*, *sitA*, *ompT,* and *traT*. The samples were tested in parallel with PCR method and the results are compared.

Supplemental file 4: Diagrams showing the linearity of the LAMP assays. *sitA, traT,* and *ompT* LAMP assays were tested on a dilution series of the bacterial isolate added to PEG buffer only (no tissues) and to PEG buffer with liver and heart tissue. The time of positivity (Tpos, min) shows linear response in relation to the concentration of the bacteria in the sample (log concentration, cells/mL).

Supplemental file 5: Results of testing of real samples collected from different holdings. Six flocks (A, B, C, D, E, F) were extensively sampled (healthy and diseased animals, environmental samples) and tested, while on additional 8 locations only air was sampled and tested (11 air samples altogether). Tpos (min) and Tm (°C) are given for positive samples;/– negative result. Mean, standard deviation, and minimal and maximal values are calculated for each LAMP assay.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

This study included a control group, as samples from a flock of clinically healthy experimental chickens kept for diagnostics purposes at the Veterinary Faculty, University of Ljubljana (approved by the Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection, approval number U33401–18/2014/4).