Establishment and validation of a loop-mediated isothermal amplification (LAMP) assay targeting the trtRSBCA locus for rapid detection of *Salmonella* spp. in food

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**A R T I C L E   I N F O**

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**A B S T R A C T**

*Salmonella* infection represents a common foodborne zoonosis of public concern worldwide. Rapid and user-friendly nucleic acid-based detection methods like loop-mediated isothermal amplification (LAMP) can contribute to the improvement of food safety and prevention of foodborne illness. In the present study, a LAMP assay was established and validated as a specific and rapid tool for detecting *Salmonella* spp. in various food matrices. Primers targeted a region within the *trtRSBCA* locus and enabled 100% inclusivity of the 88 tested *Salmonella* strains, while no false positive signal occurred in any of the 92 tested non-*Salmonella* strains. After 20-h enrichment of artificially contaminated minced meat and ready-to-eat salad, initial contamination levels of 1 CFU/25 g were detectable via LAMP. The establishment of a simplified DNA-isolation method, which was adapted for field applications, enabled similar detection probability after 20-h enrichment of artificially contaminated food samples. During validation, 82 variously processed food samples underwent comparative analysis by LAMP and the standard culture method. The LAMP assay was further validated by the investigation of reference material of unknown *Salmonella* contamination status. Results showed that the *trtRSBCA*-based LAMP detection of *Salmonella* spp. is a suitable tool for rapid sample screening within 24 h including first enrichment. Additionally, all *Salmonella* isolates obtained during cultural examination of naturally contaminated samples were correctly identified by LAMP, thus making it a suitable confirmation method for official applications.

**1. Introduction**

Non-typhoidal salmonellosis is still reported as one of the four key global causes of diarrhoeal disease (WHO, 2018). Although diarrhoea usually involves a self-limiting process, it can become life threatening due to dehydration, renal insufficiency and electrolyte imbalances, especially in young children, the elderly or immunosuppressed people (Farthing et al., 2013). Bacteraemia occurs in approximately 5% of the infected persons and can lead to a fatal outcome (Acheson & Hohmann, 2001). Stanaway et al. (2019) showed that especially in developing countries, non-typhoidal *Salmonella* invasive disease is an emerging global health threat, causing as many cases of death and disability as *Salmonella* enterocolitis. In an epidemiological study which considered data from several publications, it was estimated that 86% of the described salmonellosis cases were caused by foodborne infections (Majowicz et al., 2010). The presented facts show that there is a global need for approaches to prevent *Salmonella*–associated foodborne illness by providing rapid, robust, cost-effective and reliable investigation procedures. In the official food control, presence of *Salmonella* spp. in test samples is usually determined by means of the standard culture method DIN EN ISO 6579–1:2017 (Anonymous, 2017a). This examination takes four to five days and therefore is not applicable for fast sample screening. In contrast, nucleic acid amplification techniques like the polymerase chain reaction (PCR) constitute a useful tool to reduce investigation time. However, although PCR usually provides results within 1-2 h after template preparation from enriched food samples, an

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expensive and non-portable thermocycler device is needed to carry out the reaction. In addition, several studies have shown that PCR is susceptible to some inhibitors occasionally occurring in food samples, and thus requires complex and time-consuming template preparation for gaining reliable results (Schrader et al., 2012). At the turn of millennium, Notomi et al. (2000) introduced loop-mediated isothermal amplification (LAMP), a novel method that overcomes the drawbacks of PCR. Since amplification takes place continuously under isothermal conditions, a heating block or water bath is sufficient to carry out the reaction. LAMP is characterised by its high degree of robustness and its efficiency to deal with simplified template preparations from different matrices (Francois et al., 2011). In recent years, a lot of LAMP assays for detecting Salmonella spp. have been developed. Most of them were based on the target gene invA (Yang et al., 2018), whose expression represents a virulence factor necessary for host cell invasion (Galán et al., 1992). It could be shown that this gene is occasionally lacking in some isolates, particularly concerning S. enterica subsp. enterica serotypes Kentucky, Litchfield and Senftenberg (Gnocchio et al., 1997; Turki et al., 2012). In the present study, a LAMP assay targeting a region within the ttrRSBCA locus was established and validated. The ttrRSBCA locus was found to be crucial for anaerobic metabolism and survival of Salmonella (Hensel et al., 1999) and can therefore be expected to be genetically more stable than invA or other potential virulence factor-encoding target genes. The suitability of this region for detecting Salmonella spp. has already been demonstrated in PCR assay development (Malorny et al., 2004). Previous investigations revealed that this part of the Salmonella genome is also eligible for LAMP detection, since it provides optimal properties of inclusivity and exclusivity of Salmonella and non-Salmonella strains, respectively (Kreitlow et al., 2020). The aim of this study was to establish and validate a rapid, specific, sensitive and robust LAMP assay which enables reliable detection of Salmonella spp. in food, and copes with restricted conditions as expected for field operations. To the best of our knowledge, this is the first LAMP study targeting the ttrRSBCA locus for Salmonella detection in food samples.

2. Material and methods

2.1. DNA isolation from bacterial strains

A total of 180 bacterial strains were included in LAMP assay establishment and validation (Table 1). This selection consisted of reference strains provided by the American Type Culture Collection (ATCC), the Culture Collection University of Gothenburg (CCUG), the German Collection of Microorganisms and Cell Cultures (DSMZ) and the National Collection of Type Cultures (NCTC), as well as field isolates from our in-house culture collection. Bacterial cultures were generally grown under aerobic conditions for 24 h at 37 °C on Columbia agar with sheep blood (COLS) (Oxoid GmbH, Wesel, Germany). Differing from this, Campylobacter spp. and Arcobacter spp./Helicobacter pylori were grown under microaerobic conditions for 24-48 h at 42 °C and 37 °C, respectively, whereas Bacillus spp. were cultured in 10 ml brain heart infusion (BHI) broth (Oxoid GmbH). DNA of each strain was extracted from five to ten colonies from plate cultures or 1 ml enriched BHI broth using the DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer’s instructions. In each eluate, the DNA concentration was measured three times with the spectrophotometer NanoDrop 2000c (Thermo Fisher Scientific GmbH, Dreieich, Germany). Subsequently, DNA concentration was adjusted to 0.1 ng/µl in each template to standardise the test conditions during analytical specificity testing.

2.2. LAMP assay design and optimisation

LAMP primers used in this study were based on segments within the Salmonella genome-specific ttrRSBCA locus and ordered from Eurofins Genomics Germany GmbH (Ebersberg, Germany). Associated sequences

| Table 1 | Strains used for determining analytical specificity of the LAMP assay. |
|---|---|
| Strains | No. of strains | LAMP results |
| SALMONELLA STRAINS | | |
| *Salmonella enterica* subsp. *enterica* | 2 | + |
| Serotype Agona | 2 | + |
| Bovismorbificans | 3 | + |
| Braenderup | 2 | + |
| Brandenburg | 2 | + |
| Chester | 2 | + |
| Derby | 3 | + |
| Dublin | 1 | + |
| Enteritidis (incl. DSM 14221) | 6 | + |
| Essex | 1 | + |
| Hadar | 2 | + |
| Haifa | 1 | + |
| Hato | 1 | + |
| Heidelberg | 2 | + |
| Infantis | 1 | + |
| Kentucky | 2 | + |
| Kisangani | 1 | + |
| Goeln | 2 | + |
| Moulten | 1 | + |
| Muunchen | 2 | + |
| Napoli | 2 | + |
| Newport | 2 | + |
| Oranienburg | 2 | + |
| Paratyphi B | 3 | + |
| Saintpaul | 3 | + |
| Schlesheim | 2 | + |
| Senftenberg (CCUG 37886) | 1 | + |
| Stanley | 2 | + |
| Tennessee (CCUG 12658) | 1 | + |
| Typhimurium (incl. DSM 19587) | 9 | + |
| Virchow | 2 | + |
| *Salmonella enterica* subsp. *enterica* (not serotyped) | 3 | + |
| *Salmonella enterica* subsp. *salamae* | 4 | + |
| *Salmonella enterica* subsp. *arizonae* | 3 | + |
| *Salmonella enterica* subsp. *diarizonae* | 3 | + |
| *Salmonella enterica* subsp. *houtenae* | 3 | + |
| *Salmonella enterica* subsp. *indica* | 3 | + |
| *Salmonella bongori* | 3 | + |
| Non-Salmonella strains | | |
| Aeromonas hydrophila (DSM 30187) | 1 | – |
| Arcobacter butzleri (DSM 87397) | 1 | – |
| Arcobacter cryaerophilus (DSM 7289) | 1 | – |
| Arcobacter skrornini (DSM 7302) | 1 | – |
| Bacillus cereus (incl. ATCC 11778) | 6 | – |
| Bacillus licheniformis | 1 | – |
| Bacillus mycoides (CCUG 26678) | 1 | – |
| Bacillus subtilis (incl. DSM 347) | 2 | – |
| Bacillus thuringiensis (incl. CCUG 74297) | 3 | – |
| Bacteroides fragilis (CCUG 4856) | 1 | – |
| Brochothrix thermosphacta (CCUG 35132) | 1 | – |
| Campylobacter coli | 12 | – |
| Campylobacter fetus subsp. fetus (CCUG 50940) | 1 | – |
| Campylobacter helveticus (CCUG 34042, CCUG 34092) | 2 | – |
| Campylobacter jejuni (incl. NCTC 12662) | 10 | – |
| Campylobacter lari (CCUG 29405, CCUG 29406) | 2 | – |
| Campylobacter upsaliensis (CCUG 74242) | 1 | – |
| Clostridium braakii | 1 | – |
| Clostridium freundii | 2 | – |
| Clostridium koseri (DSM 4595) | 1 | – |
| Clostridium youngae | 1 | – |
| Escherichia coli (DSM 1103, DSM 22665, DSM 22311, DSM 22316) | 4 | – |
| Enterobacter cloacae (NCTC 13464) | 1 | – |
| Enterococcus faecalis (DSM 13591, NCTC 8727) | 2 | – |
| Enterococcus faecium (DSM 25389, DSM 25390) | 2 | – |
| Hafnia alvei (DSM 30163) | 1 | – |
| Helicobacter pylori (CCUG 47164) | 1 | – |
| Klebsiella oxytoca | 1 | – |
| Klebsiella pneumoniae (NCTC 13465) | 1 | – |
| Lactobacillus casei (DSM 20011) | 1 | – |
| Lactococcus lactis (DSM 20481) | 1 | – |
| Listeria grayi (DSM 20596) | 1 | – |

(continued on next page)
corresponded to previously described trRSBCA primers (Kreitlow et al., 2020) and are available in the supplementary data.

LAMP reactions were carried out using the real-time fluorometer Genie® II. For optimising reaction conditions, different reaction temperatures and primer concentrations were examined. Two primer mixes each consisting of forward and backward outer primers (F3/B3), forward and backward inner primers (FIP/BIP) as well as forward and backward loop primers (LF/LB) were prepared with different single primer concentrations in accordance with the recommendations of Kreitlow et al. (2017b). Per reaction mixture, concentrations of F3/B3, FIP/BIP and LF/LB were 0.2 μM, 0.8 μM and 0.4 μM, respectively, using the standard primer mix, and 0.2 μM, 2.0 μM and 1 μM, respectively, using the concentrated primer mix. A temperature gradient was set on the instrument so that both primer mixes could be tested at reaction temperatures ranging from 62 to 69 °C (ΔT = 1 °C). Each LAMP reaction mixture with a total volume of 25 μl contained 15 μl GspSSD isothermal mastermix ISO-001 (OptiGene Ltd.), 2.5 μl nuclease-free water (Qiagen GmbH), 2.5 μl primer mix, and 5 μl DNA template of S. enterica subsp. enterica serotype Typhimurium DSM 195887 (0.1 ng/μl). Per run, one reaction mixture with 5 μl nuclease-free water instead of DNA template served as a negative control. After a 40-min amplification period, a melting curve was generated within descending temperatures between 98 and 80 °C using a ramp rate of 0.05 °C/s. The optimal reaction temperature was determined by means of shortest detection times, considering the strength of fluorescence signals and standard deviation of measurements. Regarding DNA-based analytical sensitivity, ten-fold serially diluted DNA templates of S. Typhimurium DSM 19587 with DNA concentrations ranging from 10 fg/μl to 10 ng/μl were tested with both standard and concentrated primer mix at the previously determined optimal reaction temperature. For selecting the most appropriate primer concentrations, detection times and analytical sensitivity achieved by each primer mix were evaluated. Experiments were performed in triplicate. In the final LAMP assay, composition of reaction mixtures, assay duration and settings for creating melting curves were maintained as previously described. All following runs included one reaction with 5 μl DNA template of S. Typhimurium DSM 195887 (0.1 ng/μl) and one reaction with 5 μl nuclease-free water instead of DNA template as positive and negative control, respectively.

### 2.3. Analytical specificity

DNA from a total of 180 bacterial strains was investigated to determine the analytical specificity of the LAMP assay (Table 1). Eighty-eight Salmonella strains used for inclusivity testing were selected to represent the known Salmonella species, subspecies and several epidemiologically relevant serotypes. Exclusivity of the assay was verified by means of 92 non-Salmonella strains which have close genetic relation to Salmonella spp. or the ability to grow in the same environment or under the same conditions as Salmonella spp. For each amplification product, melting temperatures were recorded to substantiate reaction specificity. All test strains underwent confirmation by MALDI-TOF analysis using the microflex LT/SH device (Bruker Daltonik GmbH, Bremen, Germany) according to the manufacturer’s instructions. Before spotting, Listeria spp., Bacillus spp. and Staphylococcus aureus strains were treated with ethanol/formic acid extraction while the direct transfer method was used for other strains (Bastin et al., 2019).

### 2.4. Bacterial cell-based detection limit

DNA was extracted from Salmonella cell suspensions of different concentrations to determine the cell-based detection limit of the LAMP assay. Therefore, S. Typhimurium DSM 19587 was cultured in 10 ml buffered peptone water (Oxoid GmbH) for 20 h at 37 °C under aerobic conditions. Subsequently, a ten-fold serial dilution of the overnight culture was prepared in accordance with DIN EN ISO 6887–1:2017–07 (Anonymous, 2017b). Buffered peptone water was used as dilution medium and kept at 4 °C to inhibit intermediate cell proliferation. Viable cell counts were obtained by means of the plating method on tryptone soya agar (TSA) (Oxoid GmbH) (Arunrat et al., 2018). Plates that showed between 10 and 300 CFU were used for colony counting, whereby the initial cell concentration was calculated as the weighted mean from two successive dilutions. From each cell suspension, DNA was isolated in triplicate using a slightly modified boiling extraction method as described by De Medici et al. (2003). Briefly, 1 ml of each dilution was transferred to a 1.5-ml reaction tube and centrifuged for 5 min at 10,000 g. After supernatants had been carefully decanted, cell pellets were washed in 300 μl nuclease-free water. Following a further 5-min centrifugation step at 10,000 g, supernatants were removed, and cell pellets were resuspended in 200 μl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) (Alfa Aesar, Thermo Fisher Scientific GmbH, Schwerte, Germany). Cell suspensions underwent boiling for 15 min at 100 °C. After thermic cell disruption, reaction tubes were immediately cooled on ice and then centrifuged for 5 min at 14,000 g and 4 °C. Supernatants were transferred to new 1.5-ml reaction tubes and directly used as templates for LAMP.

### 2.5. Real-time PCR assay

A real-time PCR assay was used as comparative method to LAMP in the examination of artificially contaminated food samples. Reaction conditions as well as trRSBCA locus-targeting PCR primers and probe (Eurofins Genomics GmbH) were derived from an official method published in the Food, Feed and Consumer Goods Code of the Federal Republic of Germany (Anonymous, 2007). The composition of reaction mixtures was modified for the purposes of this study as described in the following: each reaction mixture with a total volume of 25 μl contained 12.5 μl FastStart Essential DNA Probes Master (Roche Diagnostics GmbH, Mannheim, Germany), 7.875 μl nuclease-free water, 2.5 μl DNA template, 1 μl of each oligonucleotide primer (10 μM) and 0.125 μl Salmonella-specific target probe (25 μM). Consequently, oligonucleotide primer and probe concentrations in a reaction mixture were 0.4 μM and 0.125 μM, respectively. The device LightCycler®96 (Roche Diagnostics GmbH) was used to carry out the reactions. Initial denaturation of DNA took place at 95 °C for 10 min and was followed by 45 cycles including denaturation at 95 °C for 15 s and annealing/synthesis at 60 °C for 60 s.

### 2.6. Artificial contamination of food samples

Minced beef and ready-to-eat (RTE) salad containing different types of lettuce as well as radicchio and carrots were purchased from a local supermarket and tested for the absence of Salmonella spp. in accordance
with the standard culture method DIN EN ISO 6579–1:2017 (Anonymous, 2017a). For this purpose, 25-g samples of each matrix were homogenised with 225 ml buffered peptone water by stomacher (Seward Ltd., Worthing, West Sussex, United Kingdom) with subsequent enrichment for 20 h at 37 °C under aerobic conditions. Selective enrichment was done in Mueller-Kauffmann tetrazionate novobiocin (MKTTN) broth (Oxoid GmbH) and Rappaport-Vassiliadis soya peptone (RVS) broth (Oxoid GmbH). Brilliance Salmonella agar (Oxoid GmbH) was used as second selective plate culture medium. Samples were confirmed to be Salmonella-free when no typical colonies appeared on selective agar within 24 h aerobic incubation at 37 °C.

2.6.1. Simplified DNA extraction

DNA extraction was adapted to restricted laboratory conditions as expected for field applications of the LAMP assay. Templates were prepared from artificially contaminated minced beef and RTE salad without washing and centrifugation steps. Preparation and enrichment of the samples were performed once as described in Section 2.6. One millilitre enrichment liquid was taken from every sample and mixed with 53 μl TE buffer (200 mM Tris, 20 mM EDTA, pH 8) (Alfa Aesar, Thermo Fisher Scientific GmbH) so that the final concentration of EDTA was 1 mM and 10 mM, respectively. The aliquot with the standard culture method DIN EN ISO 6579–1:2017 (Anonymous, 2017a). For this purpose, 25-g samples of each matrix were homogenised with 225 ml buffered peptone water by stomacher (Seward Ltd., Worthing, West Sussex, United Kingdom) with subsequent enrichment for 20 h at 37 °C under aerobic conditions. Selective enrichment was done in Mueller-Kauffmann tetrazionate novobiocin (MKTTN) broth (Oxoid GmbH) and Rappaport-Vassiliadis soya peptone (RVS) broth (Oxoid GmbH). Brilliance Salmonella agar (Oxoid GmbH) was used as second selective plate culture medium. Samples were confirmed to be Salmonella-free when no typical colonies appeared on selective agar within 24 h aerobic incubation at 37 °C. 

S. Typhimurium DSM 19587 and S. Enteritidis DSM 14221 were used for artificial contamination of food samples. Ten-fold serial dilutions of 20-h cultures were prepared, and bacterial cell counts were determined as described in Section 2.4. Thereafter, the dilution series underwent cold storage for 24 h at 4 °C to stress bacterial cells (Kramer et al., 2011). Twenty-five-grammes of each food matrix were inoculated with 0–1, 1–10 and 10–100 CFU of each bacterial strain using 1-ml aliquots from appropriate dilutions. Two 25-g portions per matrix remained non-inoculated and served as negative extraction controls. All samples were homogenised with 225 ml buffered peptone water for 2.5 min at 230 rpm by stomacher (Seward Ltd.), followed by aerobic incubation for 20 h at 37 °C. After incubation, DNA was extracted from 1 ml first enrichment liquid as described in Section 2.4. All templates were analysed with LAMP and PCR, whereby results of both methods were compared. Artificial inoculation of food samples was repeated three times.

2.7. Examination of naturally contaminated food samples

A total of 82 food samples were purchased from different local supermarkets and directly tested for the presence of Salmonella by LAMP and the standard culture method in accordance with DIN EN ISO 6579–1:2017 (Anonymous, 2017a). In total, 21 chicken carcasses, 28 minced meat samples including pork, beef, lamb, turkey and chicken, and 33 RTE salads containing different types of lettuce as well as carrots, white cabbage, green cabbage, spinach, beetroot, tomatoes, sweetcorn, peas, peanuts, almonds and pumpkin seeds were investigated. Preparation of whole chicken carcasses followed the description of Simmons et al. (2003) and underwent slight modifications for the purposes of this study. Briefly, chicken carcasses were placed in sterile polyethylene bags and rinsed with 450 ml buffered peptone water by vigorous manual shaking for 1 min. Minced meat and RTE salad samples were prepared for cultural examination as described in Section 2.6. After aerobic enrichment for 20 h at 37 °C, DNA was isolated from 1 ml first enrichment liquid of each sample following the conventional and simplified DNA extraction method as described in Sections 2.4. and 2.6.1. All obtained templates were analysed with LAMP, while further cultural examination was conducted as outlined in Section 2.6. Suspect colonies on Xylose Lysine Deoxycholate (XLD) agar and Brilliance Salmonella agar were subcultured on COLS agar for 24 h at 37 °C under aerobic conditions. After ruling out agglutination with 0.85% NaCl-solution, antiserum Anti-Salmonella I (A – E + Vi) and II (F – 67) (sifin diagnostics GmbH, Berlin, Germany) were used to confirm the isolates. Bacterial cultures showing agglutination were verified by MALDI-TOF analysis using the direct transfer method (Section 2.3.). Additionally, DNA was extracted from the agglutinating colonies by the conventional boiling method as described in Section 2.4. and subjected to LAMP analysis.

2.8. Examination of reference material

Encoded reference material (three minced meat samples) of unknown Salmonella spp. contamination status was purchased from the German Reference Office for Proficiency Testing and Reference Materials (DRRR) (Kempten, Germany). All samples were prepared and investigated by LAMP and the standard culture method as outlined in Section 2.7. Results were evaluated by the DRRR.

2.9. Data processing

Analysis of raw data was performed using the software Genie Explorer (OptiGene Ltd.). Calculations and graphics were generated by Microsoft Office Excel 2016 (Microsoft Corporation, Redmond, WA, USA).

3. Results and discussion

3.1. LAMP assay design and optimisation

In this study, a rapid, sensitive, robust and specific LAMP assay was established and validated for detecting Salmonella spp. in various food matrices. For determining the most appropriate reaction conditions using the ttrRSBCA-targeting LAMP primers, assay parameters were optimised regarding reaction temperature and primer concentrations. Both primer mix compositions tested achieved the shortest detection times at reaction temperatures of 64–66 °C (Fig. 1A). Since lowest scattering of measurements and strongest fluorescence signals within these temperatures constantly appeared at 64 °C, the optimal reaction temperature was set at 64 °C for both standard and concentrated primer mix. Examples of the corresponding amplification curves are shown in the supplementary data. Irrespective of the primer mix composition used, the detection limit of the LAMP assay was 5 pg DNA per reaction mixture. During temperature optimisation and DNA-based analytical sensitivity testing, average detection times provided by the concentrated primer mix were shorter by up to 7.8 min (Fig. 1B). Among users of the real-time fluorometer Genie® II, this primer mix composition is applied frequently since it generally provides higher reaction speed (Abdulmawjood et al., 2014; Sange et al., 2019). Thus, the concentrated primer mix was selected for further LAMP applications.

3.2. Analytical specificity

LAMP primers used in the present study targeted a region within the ttrRSBCA locus. All 88 Salmonella strains tested within this study induced a positive fluorescence signal, whereas no DNA amplification appeared in any of the 92 non-Salmonella strains (Table 1). Of 88 recorded detection times, approximatively 99% were between 7.3 and 11.5 min (Fig. 2A). Specifc melting temperatures of 88.0 ± 0.8 °C were observed in all amplification products (Fig. 2B). Thus, analytical specificity was 100% for the strains tested. Previously it could be shown that the ttrRSBCA region is characterised by its high degree of inclusivity within a broad range of Salmonella species, subspecies and serotypes (Kreitlow et al., 2020). Various studies on the biomolecular detection of Salmonella also reported analytical specificity of 100% when the ttrRSBCA locus was used as target region, referring to its high genetic stability (Dmitric et al., 2018; Malorny et al., 2004; Quintela et al., 2019). As with the findings of these studies, the present LAMP results
support the view that the ttrRSBCA locus is a suitable target region for detecting Salmonella spp.

3.3. Bacterial cell-based detection limit

The cell-based detection limit of the LAMP assay was measured by extracting DNA from ten-fold serial dilutions of overnight cultured S. Typhimurium DSM 19587. Detection probability of 100% was achieved when the cell concentration was $9.82 \times 10^3$ CFU/ml. In two out of three cases, DNA from $9.82 \times 10^2$ CFU/ml was amplified. Thus, the detection limit was 25–250 CFU/reaction. Some published LAMP assays for detecting Salmonella spp. achieved higher analytical sensitivity and partially showed detection limits below 10 CFU per reaction (Tang et al., 2012; Yang et al., 2015, 2018). Up to 50 CFU per reaction led to a detection rate of 100% in a ttrRSBCA-based PCR study (Malorny et al., 2004). This finding is contrary to the general assumption of the LAMP method providing more sensitive reactions than PCR (Law et al., 2015), and might be due to the usage of degenerative primers in this study. It was already shown that consideration of single nucleotide polymorphisms in primer design increases analytical specificity but at the same time has a detrimental effect on analytical sensitivity (Forsman et al., 2005; Fichon et al., 2006). Although the ttrRSBCA-LAMP assay will not be able to directly detect Salmonella spp. at low contamination levels in food, adequate enrichment of samples compensates the detection limit of 25–250 CFU/reaction as described in Section 3.4. Several regions targeted by the previously mentioned highly sensitive LAMP assays revealed shortcomings regarding inclusivity of Salmonella spp. (Boyd & Harri, 1999; Kreitlow et al., 2020; Turki et al., 2012). Due to the relevance of salmonellosis to health, it is a major challenge to develop rapid detection methods that at the same time enable reliable Salmonella identification throughout the diversity of this pathogen as was focused on in the present study.

3.4. Salmonella detection in artificially contaminated food samples

After 20-h enrichment of artificially contaminated food samples, the detection rate of Salmonella strains by LAMP was 100% in both matrices at initial contamination levels of 1–10 CFU/25 g and 10–100 CFU/25 g. When samples were inoculated with 0–1 CFU/25 g, the detection rate was 83.3%, which was concordant with the cell count-based probability for picking one cell out of the corresponding dilution stage. No amplification signal was induced by samples that were not artificially contaminated before incubation. When simplified DNA extraction was applied, all contamination levels remained detectable with a detection rate of 100% by LAMP after 20-h enrichment in all three repetitions. LAMP results were consistently equivalent to those achieved by PCR. Testing of the non-purified DNA templates with LAMP resulted in slightly prolonged detection times and flattened slopes of fluorescence curves with decreased end-point fluorescence (Fig. 3). Similar variation of performance was observable in PCR (data not shown). However, qualitative detection of Salmonella spp. was possible in all inoculated samples. Thus, the ttrRSBCA-based LAMP assay reliably identifies Salmonella spp. even from minimally processed DNA templates. Some LAMP assays revealing higher analytical sensitivity than the ttrRSBCA-LAMP assay coped with shorter sample incubation than 20 h used in the present study and suggested by DIN EN ISO 6579–1:2017 (Li et al., 2016; Zhang et al., 2012). Nonetheless, enrichment was always necessary for Salmonella detection in food matrices. On the one hand, incubation times enabling one-day results are advantageous for effective sample screening, but on the other hand it is questionable whether shortened enrichment, especially between 6 and 12 h, is widely implementable in routine laboratory practice.

![Fig. 1. Detection times achieved by standard and concentrated primer mix depending on different reaction temperatures (A), and primer mix compositions (B); ■ = Standard primer mix; ▴ = Concentrated primer mix.](image1.png)

![Fig. 2. Distribution of detection times (A), and melting temperatures (B) achieved by the ttrRSBCA-LAMP assay during inclusivity testing of 88 Salmonella strains.](image2.png)
3.5. Salmonella detection in naturally contaminated food samples

After establishment, the *ttrRSBCA*-LAMP assay underwent validation by investigating 82 commercially purchased food samples and encoded reference material. Of the 82 food samples, 77 samples were consistently tested negative by LAMP and the standard culture method. Five samples exhibited positive findings as shown in Table 2. In the cultural examination, retail sample No. 2 only yielded one *Salmonella* colony on selective agar plates in total. Presumably, cell concentration was below the detection limit of the LAMP assay after first enrichment since the isolate could be correctly identified via LAMP (Table 3). This finding is contrary to the previously determined detection limit of 1 CFU/25 g after 20-h incubation. The frozen condition of the retail sample in question could explain the deviating result. The research of Dominguez and Schaffner (2009) revealed that *Salmonella* spp. can survive frozen storage but suffer structural damage, which leads to decreased colony counts on selective agar. Moreover, it was shown that frozen storage and the initial presence of low numbers of injured cells result in extended lag phases (Mackey & Derrick, 1982, 1984). Provided that the frozen sample exhibited contamination with injured cells and/or low cell numbers, *Salmonella* replication during 20-h incubation could have been insufficient for detection via LAMP. In the present study, cells used for artificial inoculation only underwent cold stress at 4 °C for imitating consumer practice for storage conditions. This possibly led to unexpected results during assay validation because of overestimated detection probability. Furthermore, modified atmosphere treatment of the investigated non-frozen products must be considered. It could be shown that gases used for modified atmosphere packaging (MAP) appear as bacterial cell stressors, leading to metabolic disorders, reduced cell counts in various food matrices, extended lag phase of growth and a decrease in growth rate during the logarithmic phase (Dasˇ et al., 2006; Djordjević et al., 2018; Farber, 1991). These effects could explain failed detection after simplified DNA extraction from retail sample No. 1. So far, no studies exist that investigated the influence of relevant bacterial cell stressors on detection probability during LAMP investigation of contaminated products. One false positive finding was retrieved for retail sample No. 4 using both the conventional and field DNA extraction method. Detection of DNA from non-culturable cells constitutes a general weak point in nucleic acid amplification-based methods and possibly led to the deviating result (Wolffs et al., 2005). Consequently, in official food control, such positive test results usually need to be confirmed by cultural examination. LAMP investigation of selective enrichment cultures in MKTTN and RVS broth could counteract this inadequacy at the cost of prolonged investigation time. Further research is needed to point out the extent to which associated assay accuracy could be increased. All

![Amplification curves obtained after conventional boiling extraction (A), and simplified boiling extraction (B) of DNA from differently inoculated minced meat samples; NEC = negative extraction control.](image)

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**Table 2**

Results obtained by LAMP and the standard culture method investigating retail and reference samples after first enrichment.

| Sample No. | Sample matrix | Standard culture method (DIN EN ISO 6579–1:2017) | LAMP Method A Detection time (min:s) | LAMP Method B Detection time (min:s) |
|------------|---------------|-----------------------------------------------|-------------------------------------|-------------------------------------|
| Retail samples | | | | |
| 1 | RTE salad (rocket) | + | 19:54 | - |
| 2 | Chicken carcass (frozen) | + | - | - |
| 3 | Chicken carcass (fresh) | + | 13:05 (38:45) | |
| 4 | Chicken carcass (fresh) | - | 25:02 (34:25) | |
| 5 | Chicken carcass (fresh) | + | 10:31 (34:07) | |
| Reference samples | | | | |
| 1 | Minced meat (frozen) | - | - | - |
| 2 | Minced meat (frozen) | + | 04:51 (10:05) | |
| 3 | Minced meat (frozen) | + | 04:56 (10:44) | |

Method A = conventional boiling extraction of DNA; Method B = simplified boiling extraction of DNA.

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**Table 3**

Confirmation of suspect colonies via antisera, MALDI-TOF and LAMP.

| Sample No. | Sample matrix | Antisera | MALDI-TOF | LAMP |
|------------|---------------|----------|-----------|------|
| Retail samples | | | | |
| 1 | RTE salad (rocket) | + | + | + |
| 2 | Chicken carcass (frozen) | + | + | + |
| 3 | Chicken carcass (fresh) | + | + | + |
| 4 | Chicken carcass (fresh) | / | / | / |
| 5 | Chicken carcass (fresh) | + | + | + |
| Ring test samples | | | | |
| 1 | Minced meat | / | / | / |
| 2 | Minced meat | + | + | + |
| 3 | Minced meat | + | + | + |

/ = no suspect colonies available.
isolates identified as *Salmonella* spp. by the standard culture method were also correctly determined via LAMP. In consideration of 100% inclusivity and exclusivity (see Section 3.2.), these findings qualify the assay for being implemented as an official method for confirmation of suspect colonies obtained during cultural examination (Table 3).

The significance of assay validation was limited by the low availability of native *Salmonella*-contaminated food samples. Thus, performance indicators such as those recommended by AOAC International could not be evaluated statistically (Feldsine et al., 2002). Effective monitoring strategies led to a decreasing trend of human salmonellosis cases (EFSA & ECDC, 2019). In the German retail sector, *Salmonella* prevalences of 0.3–3.59%, 5.57%, 7.63% and 1.22% were calculated among routine samples of minced meat, broiler meat, chicken meat and salad, respectively (Hartung et al., 2019). These data underline the low prevalence of *Salmonella*-positive food samples as reflected during assay validation in the present study. The issue of inappropriate balance between positive and negative samples also affects validation of other performance indicators such as those recommended by AOAC International (Anonymous, 2007). The DRRR confirmed that all reference samples were &-positive food samples as reflected during assay validation because the German version EN ISO 6579-1:2017 (ECDC, 2019). In the German retail sector, 3.59%, 5.57%, 7.63% and 1.22% were calculated for the presence of *Salmonella* results than the standard culture method. Nonetheless, most reliable Salmonella detection was enabled when the conventional boiling method was used for DNA extraction.

### 4. Conclusion

In the present study, a specific and robust LAMP assay targeting the ttrRSBCA locus was established, enabling rapid food sample screening for the presence of *Salmonella* spp. within 24 h. Coping with simplified DNA extraction, it can be applied under restricted laboratory conditions without the requirement of cost-intensive equipment. In addition, the assay turned out to be suitable for official confirmation of isolates obtained during cultural examination of food samples. For improving detection probability and reliability within the context of possible official applications, DNA extraction after selective enrichment in MKTTN and RVS broth should be considered. Deeper evaluation of the LAMP assay by investigating further naturally contaminated food samples will help to confirm diagnostic confidence regarding rapid detection within 24 h. In prospective LAMP assay development, investigation of detection probability in food matrices should consider differently challenged bacterial backgrounds to reveal realistic limitations of assay performance.

**CRediT authorship contribution statement**

**Antonia Kreitlow:** Investigation, Validation, Formal analysis, Methodology, Visualization, Data curation, Writing – original draft.

**André Becker:** Visualization, Methodology, Writing – review & editing.

**Ulrich Schotte:** Methodology, Resources, Writing – review & editing.

**Burkhard Malorny:** Methodology, Resources, Writing – review & editing.

**Madeleine Plotz:** Project administration, Writing – review & editing.

**Amir Abdulmawjood:** Conceptualization, Methodology, Supervision, Funding acquisition, Writing – review & editing.

**Declaration of competing interest**

The authors have no competing interest to declare.

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2021.107973.

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