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A Loop-Mediated Isothermal Amplification-Based Method for Analysing Animal Feed for the Presence of *Salmonella*

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Abstract A loop-mediated isothermal amplification (LAMP)-based method has been developed for the analysis of animal feed for the presence of *Salmonella* spp. The method is compatible with the standard culture-based method ISO 6579:2002. Samples comprising 100 g feed are pre-enriched in 900 ml buffered peptone water, and then 2× 100-μl aliquots are taken, one for secondary enrichment in a LAMP-friendly broth and the other for secondary enrichment according to ISO 6579:2002. A 3-μl aliquot of the LAMP-friendly culture is added directly into a LAMP reaction. LAMP-positive signals are considered presumptive of the presence of *Salmonella* spp.; confirmation is obtained by continuation of ISO 6579:2002. Negative LAMP reactions can be considered as signifying that the sample does not contain viable *Salmonella*. The LAMP assay incorporates an internal amplification control (IAC); the target and IAC signals are distinguished by the difference in annealing temperatures of the amplicons. The method provides simple and reliable screening of feedstuffs for *Salmonella* spp., with uncontaminated samples being identified in 24 h less than using the current ISO standard method alone. The method was used to analyse 79 samples of animal feed and identified 13 *Salmonella*-positive samples.

Keywords Salmonella · LAMP · IAC · Feed · Screening · Confirmation

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Introduction

It has long been recognised that a link exists between contamination of animal feeding stuffs with *Salmonella* and human disease (Jones 2011). Surveys of animal feed have indicated that the presence of this pathogenic zoonotic bacterium is a significant issue for the feed industry (Foley et al. 2008; Papadopoulou et al. 2009; Li et al. 2012). Animal feeds are routinely analysed for the presence of this agent, with the analysis being generally based on the use of conventional culturing of *Salmonella*. However, nucleic acid amplification (NAA)-based methods can offer increased diagnostic capabilities, such as the ability to rapidly identify uncontaminated samples.

An NAA technique which is becoming increasingly reported in the scientific literature is loop-mediated isothermal amplification (LAMP), and several LAMP methods for detection of *Salmonella* have been reported (Kokkinos et al. 2014). Amplification of DNA does not per se provide an indication of bacterial viability, and methods based solely on NAA may not necessarily be completely acceptable to industry (D'Agostino and Rodriguez-Lazaro 2009), which very often prefers to rely on established international standard culture-based methods. Any target signal obtained using NAA is considered to only represent the presumptive presence of the target. There is, nonetheless, an approach which embodies the benefits of identification of viable contaminants by conventional culturing and rapid screening by NAA (D'Agostino and Rodriguez-Lazaro 2009). This entails using the initial stages of a standard culture-based method to enrich a sample and then dividing the enrichment into two subsamples: one of which is taken for analysis by NAA and the other by continuation of the standard culture-based method. NAA is so sensitive and specific that if no target signal is obtained, the target can be considered to be absent. Thus, the analysis of the subsample by NAA screening for target-negative samples and the analysis of the sample using the culture-based method confirm NAA presumptive target-

positive samples. This approach ensures that screening and confirmation are performed on the same sample.

We report a novel rapid method for analysing animal feed for the presence of *Salmonella*, which is based on the screening for *Salmonella*-negative samples using a LAMP assay, with confirmation of presumptively positive signals using a standard culture-based method (International Organisation for Standardization 2002).

Materials and Methods

Salmonella Strains *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 and *S. enterica* subsp. *enterica* serovar Cerro NCTC 5801 were used for the development of the method. For artificial contamination of the test samples with *Salmonella* Typhimurium, one Lenticule® disc (Public Health England) containing a mean of 1–5 colony-forming units (cfu) was used per test sample.

Animal Feed Soya meal was used as the matrix during the development of the method; it was kindly supplied by John Thompson and Sons Ltd., Belfast. One hundred gram soya meal was used as test sample size, and 100-g soya meal samples without artificial contamination were also tested.

Culture-Based Confirmation of Salmonella spp. Detection The method used was based on that described in "ISO 6579:2002 - Microbiology of food and animal feeding stuffs—horizontal method for the detection of *Salmonella* spp." (2002) with the following exceptions:

1. For primary enrichment of the sample, 100 g soya meal was added to 900 ml buffered peptone water (BPW; Oxoid, Basingstoke, UK).
2. Secondary enrichment was performed using only Rappaport-Vassiliadis medium with soya (RVS broth; Oxoid, Basingstoke, UK).
3. Plating out was performed using only xylose lysine desoxycholate agar (XLD; Oxoid, Basingstoke, UK).

Biochemical characterisation of presumptively positive colonies was performed using the API 20E kit (bioMérieux, UK).

LAMP-Friendly Secondary Enrichment One hundred microlitres of the primary enrichment was added into 100 ml of Yersinia-PCR-compatible enrichment (YPCE) broth (Knutsson et al. 2002) and incubated at 37 °C 24 h. Three microlitres was used directly in the LAMP reaction.

Purification of Salmonella Genomic DNA Nucleic acid was purified from 100 ml culture using a commercial DNA extraction kit (Qiagen, Manchester, UK). Three microlitres was used as a template in the LAMP assay. The concentration and purity of the DNA were determined using a NanoDrop instrument (Thermo Scientific, UK). The number of genome copies in the extract was determined by dividing the amount of DNA present by the weight of one genome copy.

LAMP Assay The LAMP assay was adapted from that of Hara-Kudo et al. (2005) which targets a region of the *invA* gene (accession number embl|U43237). The reaction contained 1× *Tin* isothermal master mix, 1 μM each of primers *inv*ASalm FIP and *inv*ASalm BIP, 500 nM each of primers *inv*ASalmF-loop and *inv*ASalmB-loop, 200 nM each of primers *inv*ASalmF3 and *inv*ASalmB3 and 1 μl of internal amplification control (IAC) in a 22 μl volume; 3 μl of the template was added. The thermal parameters of the reaction were 95 °C for 2 min (cell lysis), followed by 65 °C for 50 min (nucleic acid amplification). Amplicon annealing profiling was performed by heating to 98 °C and then cooling to 80 °C at a rate of 0.05 °C s^{-1}. The samples were analysed using a Genie II instrument (OptiGene Ltd., Horsham, UK).

IAC Design The IAC was designed to be amplified by the same primer set used for target amplification, by incorporating the target sequences into the IAC sequence. To ensure that the annealing temperature of the IAC amplicon was higher than the target amplicon, the sequence between the FIP and BIP primer regions was modified from the original *ttatcttgattgaagccgatg* to *gcatctggagcgcagccgatgcgcaggcctc* to increase the G:C content of the IAC amplicon to 50 % (target amplicon 46 %), thereby increasing the annealing temperature by 1.6 °C according to the Oligo Calc: Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools/OligoCalc.html).

IAC Construction The IAC was supplied by Eurofins MWG Operon (Ebersberg, Germany). The IAC sequence was cloned into plasmid pEX-A, which was linearised using *ScaI*.

IAC Optimisation and Demonstration The optimal amount of IAC per reaction, i.e. which will consistently produce a signal in the absence of target nucleic acid, was determined according to the procedure of Cook et al. (2012). To demonstrate that the IAC will not produce a signal in the presence of substances in the feed sample which can be inhibitory to the LAMP reaction and is thus able to identify a failed reaction, a flask containing 900 ml BPW plus 100 g soya meal was inoculated with 1 ml of a mid-log phase culture of *Salmonella* Typhimurium. The culture was incubated at 37 °C for 24 h. Three microlitres of the culture was added directly as a template to a LAMP reaction mix, and the reaction performed as described above. To demonstrate that the IAC will produce a signal when the reaction has performed correctly, 100 g soya

meal was added to 900 ml BPW and incubated at 37 °C for 24 h. One hundred microlitres of the primary enrichment was added into 100 ml of YPCE broth and incubated at 37 °C 24 h. Three microlitres was used directly in the LAMP reaction.

Detection Limit of the LAMP Assay Purified *Salmonella* Cerro DNA was diluted to give concentrations of 3.3×10^4, 3.3×10^3, 3.3×10^2 and 3.3×10^1 genome copies per μl. Three microlitres of each dilution was then used as a template in the LAMP assay. Triplicate reactions were performed for each dilution.

Survey of Animal Feeds Seventy-nine samples of animal feed were analysed. The types of feedstuff analysed included rapemeal, maize, corn, soya bean, wheat, sunflower pellets, distiller grains, whole rape, microalgae, palm kernel expeller and other meals for poultry, fish, etc. All the samples were screened using the LAMP-based method with confirmation of positive samples by following ISO 6579:2002.

Results

A Loop-Mediated Amplification-Based Method for Analysing Animal Feed for the Presence of *Salmonella*

One hundred grams of each of the animal feed samples were added to 900 ml BPW. These were incubated at 37 °C for 16–20 h and tested using the LAMP-based method. One hundred microlitres of primary enrichment culture was added to 100 ml of YPCE broth pre-warmed to 37 °C, and simultaneously, 0.1 ml of primary enrichment culture was added to 10 ml Rappaport-Vassiliadis (RV) medium. The LAMP-based method and the standard method were then both followed (see Fig. 1). A positive signal obtained using the LAMP-based method was considered as presumptively positive, and the ISO standard method was continued to be confirmed. A negative target signal and a positive IAC signal obtained using the LAMP-based method were considered to demonstrate that the sample was uncontaminated by *Salmonella* spp. The method could identify *Salmonella*-negative test samples of soya meal in less than 48 h.

Annealing Temperatures of IAC and Target The amplicon annealing temperatures observed after the amplification process were sufficiently different to distinguish between target and IAC signals. Figure 2 shows the signal produced using 10^4 genome copies of *Salmonella* Typhimurium DNA.

IAC Optimisation The optimal amount of IAC per reaction was determined at 40 copies, as this quantity (and higher quantities; not shown) yielded amplicon signals at a similar time (Fig. 3a), whereas the signals obtained from 20 copies were obtained at differing times, indicating inconsistent amplification (Fig. 3b).

Demonstration of Correct and Failed Assays Neither target nor IAC signal was obtained when neat primary enrichment

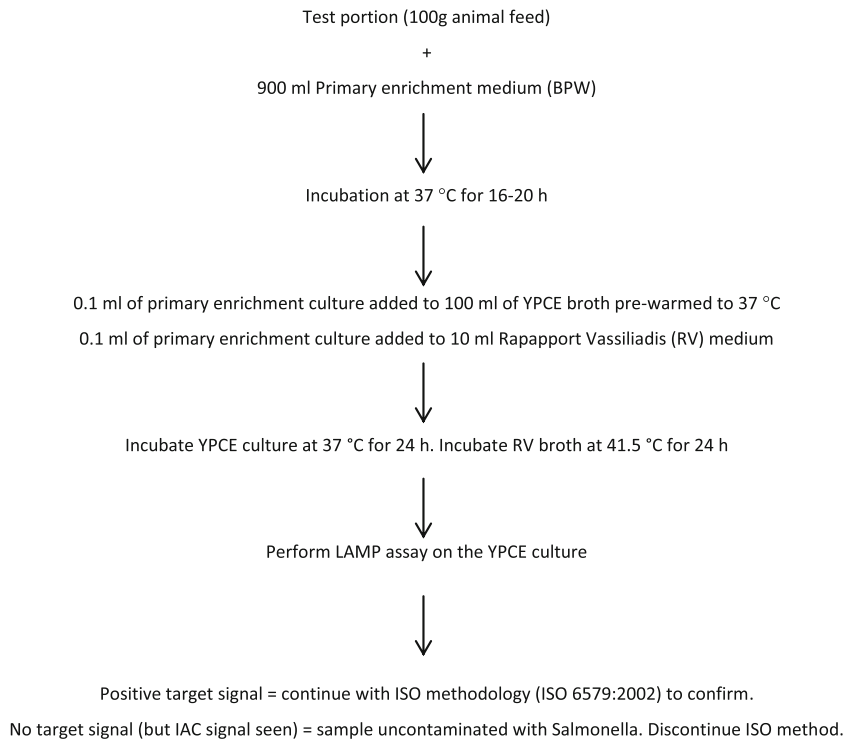

Fig. 1 Flowchart of the LAMP-based/ISO method for the detection of *Salmonella* spp. in animal feed

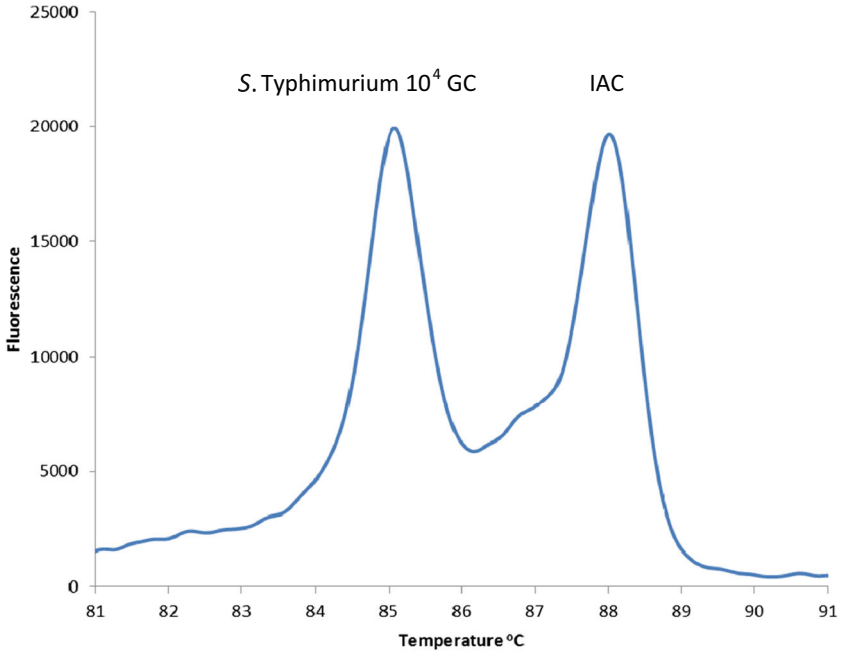

Fig. 2 The 10^4 genome copies of *Salmonella* Typhimurium with 40 copies of IAC

culture was added directly to the LAMP reaction (Fig. 4a, b); this assay had therefore failed.

When the culture was diluted at 1:10, the target signal was restored (Fig. 4c, d).

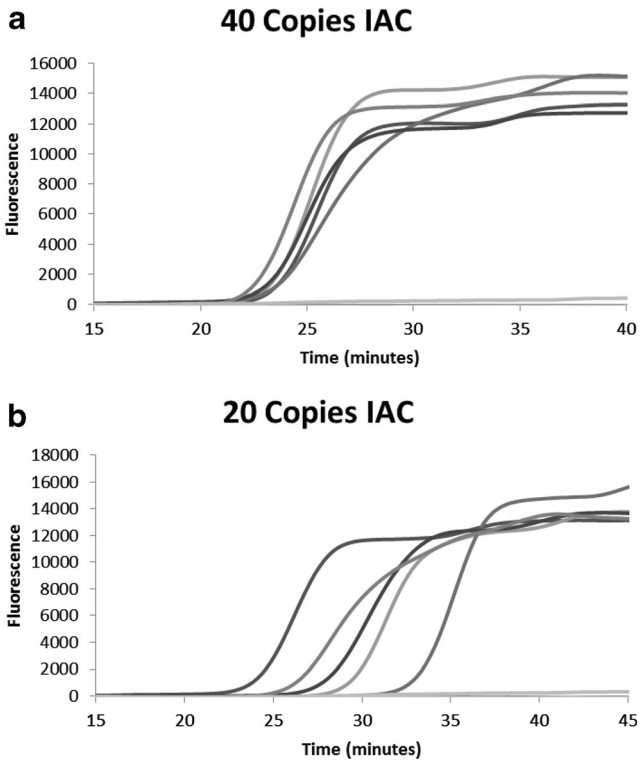

Fig. 3 **a** Plots obtained from amplification of 40 IAC copies (five replications) showing reproducible time to amplification. **b** Plots obtained from amplification of 20 IAC copies (five replications) showing varying times to amplification

Detection Limit of the LAMP Assay Amplicon peaks for 3.3×10^4 genome copies per reaction were consistently clearly visible. For lower numbers of genome copies per reaction, it was not always possible to see a clearly defined peak.

Survey of Animal Feeds for Salmonella spp. Contamination Thirteen out of 79 samples were presumptively positive for *Salmonella* spp. using the LAMP method, and all was confirmed as positive by ISO 6579:2002. Table 1 lists the positive samples and the strain of *Salmonella* where it was able to be identified.

Discussion

The LAMP-based method for analysis of animal feeding stuffs allows, through screening, the identification of uncontaminated samples in approximately 24 h less than the standard culture-based method alone. If the product is required to be certified as *Salmonella* free before it is released to the market, this would provide a significant cost saving. If a positive LAMP signal is obtained, the sample can be considered as presumptively positive for *Salmonella* contamination. Confirmation by following the standard method will satisfy any requirement for further identification of the *Salmonella* contaminant.

This is the first report of incorporation of a competitive IAC (using the same primers which amplify the target sequences) into a LAMP assay. The LAMP assay for *Salmonella* (Hara-Kudo et al. 2005) could detect ~30 genome copies per reaction when no IAC was present. Incorporation of an IAC resulted in a reduction in sensitivity; however, as

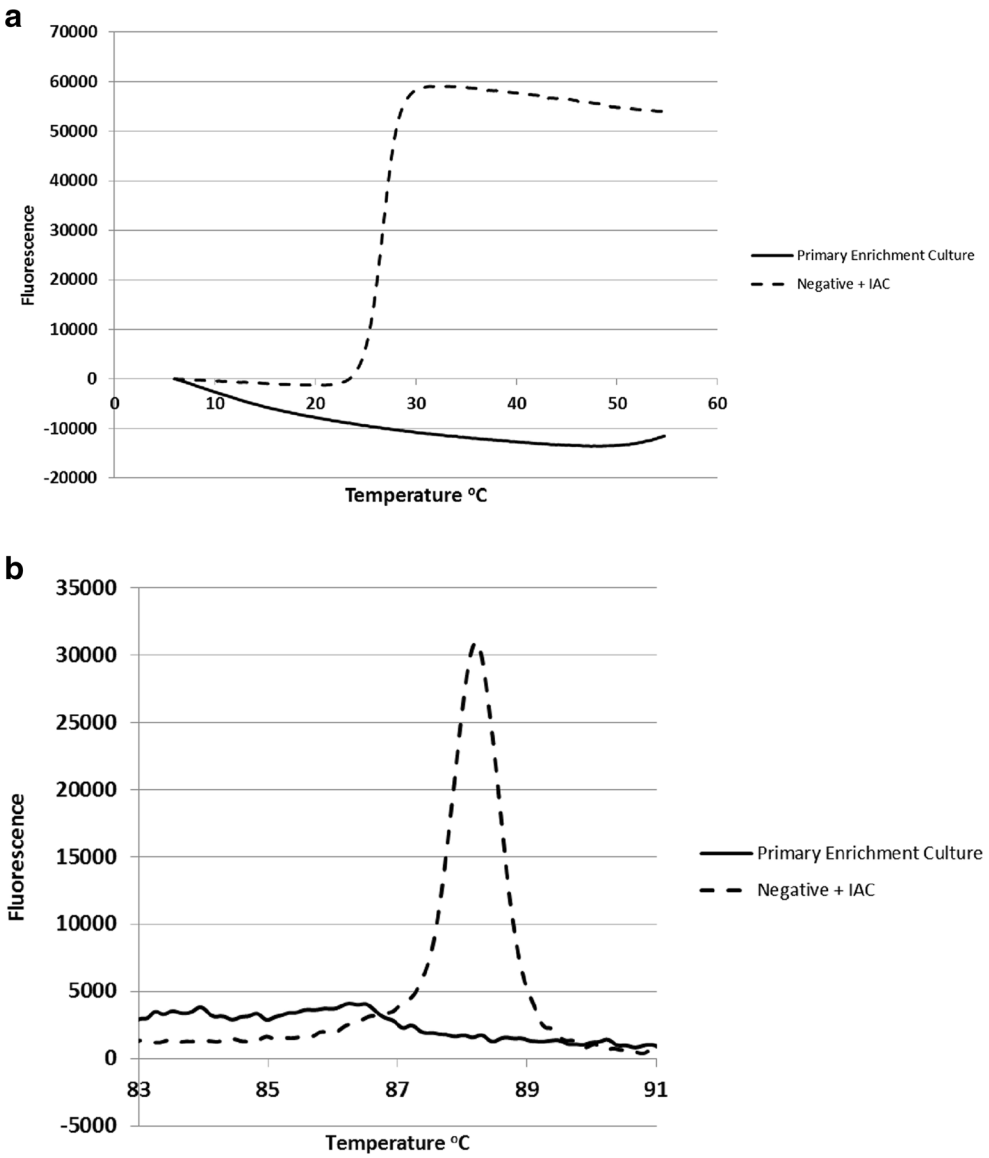

Fig. 4 **a** Failed reaction amplification plot of the primary culture used as a template in LAMP, producing no IAC signal. **b** Failed reaction annealing plot of the primary culture added to LAMP, producing no IAC signal. **c** Amplification plot of the 1:10 dilution of BPW added to LAMP, producing a restored target signal. **d** Annealing plot of the 1:10 dilution of BPW added to LAMP, producing a restored target signal

the LAMP is preceded by sample enrichment, it is not essential that the assay is highly sensitive.

The two-step enrichment procedure can be relied on to indicate the viability of a presumptive positive. It increases the number of viable cells originally in the sample to detectable amounts and will dilute out dead cells originally present. The likelihood that a LAMP signal will be derived from viable *Salmonella* can be demonstrated arithmetically. Approximately 10^4 genome copies and, hence, cells per LAMP are necessary to obtain a target signal. This means that 400 cells must be present in the 3 μl used as a template in the reaction. Consequently, it is necessary to have 1.33×10^7 viable cells in the 100 ml YPCE after 24 h of incubation. Assuming a doubling time of 30 min, to reach this number the LAMP-friendly enrichment would require only one viable cell at 0 h. Thus, the 1 l primary-enriched culture needs to contain at least 1×10^4 viable cells after 16 h of incubation in order to contain one viable cell in the 100 μl that is put into the 100 ml YPCE. One viable *Salmonella* cell in the original 100-g sample will reach this number in 16–20 h, even with a lag phase of ~8 h. The likelihood that a LAMP signal will not be derived from dead *Salmonella* can also be demonstrated arithmetically. The original sample is added to BPW to make the primary culture and then one out of 10,000th of that is added to the YPCE. Finally, less than one out of 30,000th of the secondary culture is incorporated into the LAMP. The LAMP assay can detect ~400 cells per reaction. Thus, at least 1×10^{11} dead *Salmonella* cells must be present in the original

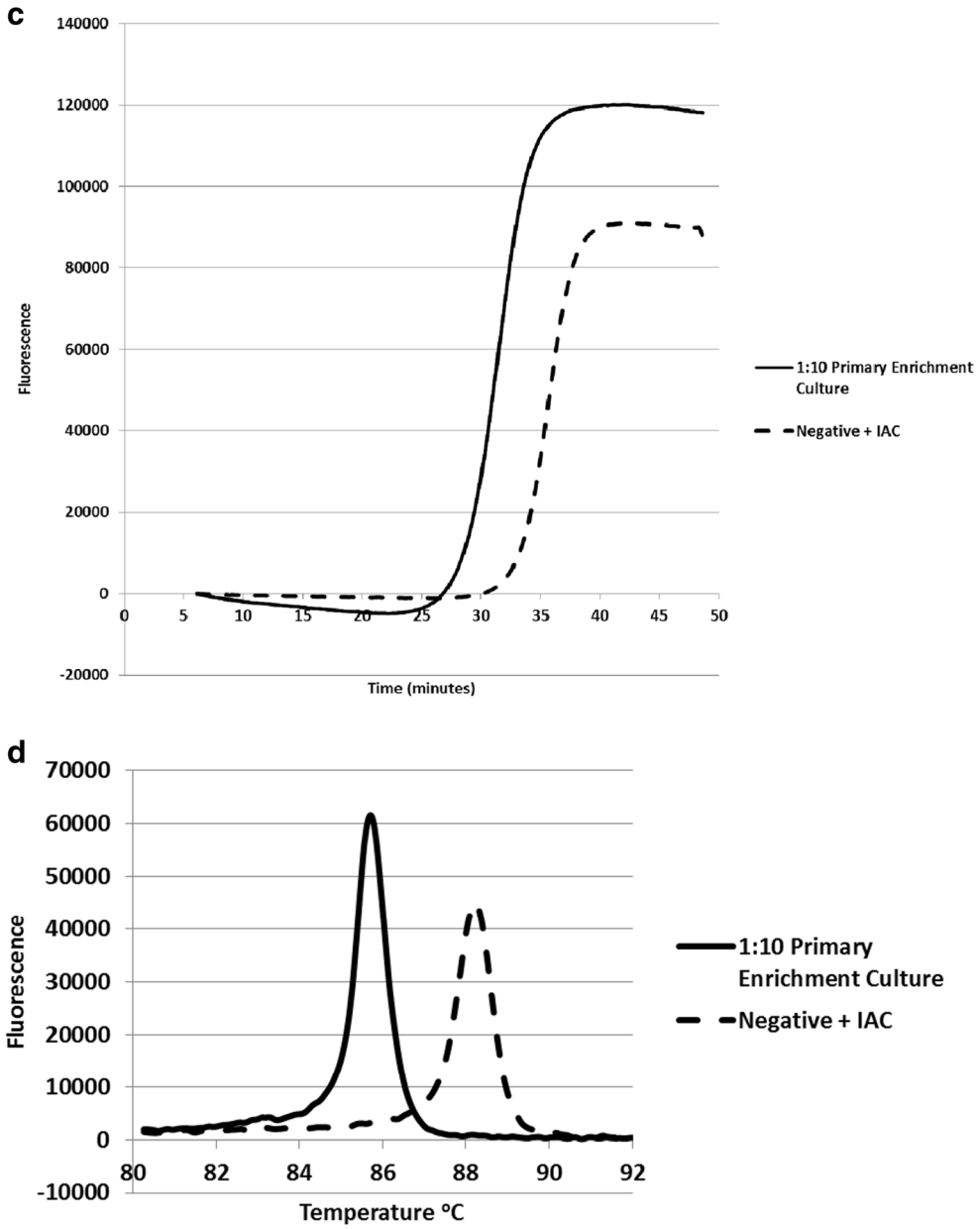

Fig. 4 (continued)

100 g of animal feed to obtain a positive LAMP signal. Confirmation of the viability of presumptive positives is anyway conferred using the standard culture-based method.

If monitoring of animal feed for pathogens is to be effectively performed, then it is vitally necessary that the reliability of the analytical results can be verified (Rodríguez-Lázaro et al. 2007). In NAA-based diagnostics, IACs are essential to identify failed reactions, through e.g. inhibition or operator error (Rodríguez-Lázaro et al. 2007; D'Agostino et al. 2011). To date, however, no LAMP assay has been published which contains an IAC which uses the same primers as for the amplification of the target to ensure complete control of the assay. LAMP assays for plant-related diagnostics have been published which have contained controls based on amplification of non-target genes found within the plant material, but this approach is not universally valid. We modified the inter-primer region to ensure that the IAC amplicons can be distinguished from the target amplicons by a difference in melting and annealing temperature (see e.g. Fig. 1).[1] Optimization of IAC concentration ensures consistent detection of IAC in the absence of a target (Rodríguez-Lázaro et al. 2004) (see Fig. 2).

[1] This IAC approach will not be applicable if using turbidity as an end-point detection system (Chen et al. 2011; Soli et al. 2013). However, users of turbidity-based detection should recognise its limitations and the potential ambiguity of its results; in that, false-negative result interpretation cannot be totally avoided.

Table 1 Detection of *Salmonella* spp. in animal feeds

Feed type	*Salmonella* contaminant
EU corn distillers (bioethanol)	*Salmonella* spp.
Rapemeal	*Salmonella* spp.
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Corn gluten	*Salmonella* spp.
Whole rape	*Salmonella* spp.
Organic full fat soya	*Salmonella choleraesuis* subsp. *arizonae*
GM distillers grains	*Salmonella* spp.
Soya bean meal	*Salmonella* spp.
Soya bean meal	*Salmonella* spp.
Microalgae biomass powder	*Salmonella* spp.
Containing DHA omega-3 oil	*Salmonella* spp.
Soya bean meal	*Salmonella* spp.
Fish meal	*Salmonella* spp.

An example of a failed LAMP assay is shown in Fig. 3a, b. Upon performing a 1:10 dilution of the sample, the target signal has been restored (Fig. 3c, d), demonstrating that the false-negative result was due to the presence of inhibitors in the culture. Another interesting aspect of the LAMP assay used here is the use of the thermostable enzyme *Thermodesulfotator indicus* (*Tin*) DNA polymerase enzyme. This allows the direct addition of whole bacterial cells to the LAMP reaction as when the temperature rises past 90 °C, they are lysed and release their nucleic acids directly into the reaction. This removes the necessity for prior nucleic acid extraction and is the most useful feature. YPCE secondary enrichment medium has been shown to be effective in a polymerase chain reaction-based method for the detection of *Listeria monocytogenes* (D'Agostino et al. 2004); in the current study, it was equally effective and, therefore, no other secondary enrichment broths were evaluated.

The utility of the LAMP-based method was demonstrated by its use in the analysis of a variety of actual feed samples. Several naturally *Salmonella*-contaminated feeding stuffs were identified using the LAMP-based method. The method has been verified as equivalent to ISO 6579:2002 through intra- and inter-laboratory validation, as will be described in a future publication.

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Conflict of Interest Marta Diez-Valcarce has no conflict of interest. Nigel Cook has no conflict of interest. Martin D'Agostino has no conflict of interest. Susana Robles has no conflict of interest. Begoña Losilla-Garcia has no conflict of interest. This article does not contain any studies with human or animal subjects.

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