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ORIGINAL ARTICLE

An easy-to-perform, culture-free *Campylobacter* point-of-management assay for processing plant applications

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Abstract

Aims: Current culture-based methods for detection and determination of *Campylobacter* levels on processed chickens takes at least 2 days. Here we sought to develop a new complete, low-cost and rapid (approximately 2·5 h) detection system requiring minimal operator input.
Methods and Results: We observed a strong correlation between culture-based cell counts and our ability to detect either *Campylobacter jejuni* or *Campylobacter coli* by loop-mediated isothermal amplification from the same samples. This knowledge was used to develop a rapid and simple five-step assay to quantify *Campylobacter*, which was subsequently assessed for its specificity, reproducibility and accuracy in quantifying *Campylobacter* levels from processed chickens. The assay was found to be highly specific for *C. jejuni* and *C. coli* and was capable of distinguishing between samples that are either within or exceeding the industry set target of 6000 *Campylobacter* colony forming units (CFU) per carcass (equivalent to 12 CFU per ml of chicken rinse) with >90% accuracy relative to culture-based methods.
Conclusions: Our method can reliably quantify *Campylobacter* counts of processed chickens with an accuracy comparable to culture-based assays but provides results within hours as opposed to days.
Significance and Impact of the Study: The research presented here will help improve food safety by providing fast *Campylobacter* detection that will enable the implementation of real-time risk management strategies in poultry processing plants to rapidly test processed chickens and identify effective intervention strategies. This technology is a powerful tool that can be easily adapted for other organisms and thus could be highly beneficial for a broad range of industries.

Introduction

Campylobacter infection is a significant worldwide public health concern affecting millions of people every year. In Australia, where this study was performed, *Campylobacter* infection is the most commonly notified foodborne infection (OzFoodNet 2011). In 2011, about 17 773 cases of *Campylobacter* infection were reported in Australia, although these numbers are estimated to represent only about 10% of the actual incidence of infections, since many individual cases are not reported (Hall *et al.* 2008). Consequently, *Campylobacter* infections result in significant costs to society in terms of the burden on the health care system and lost productivity. The two *Campylobacter* species responsible for the majority of *Campylobacter* enteritis cases are *Campylobacter jejuni* and *Campylobacter coli*, with symptoms including fever, headaches, dizziness, abdominal pain as well as acute diarrhoea of varying severity, which may last up to 7 days (Skarp *et al.* 2016). The prevalence of *Campylobacter* infections is in large part due to the low infective dose of approximately 500–800 cells (Robinson 1981; Black *et al.* 1988), which is

significantly lower than the approximately 10^5 infective dose of other common human pathogens such as *Escherichia coli* and *Salmonella enterica* serovars (Kothary and Babu 2001).

Campylobacter jejuni and *C. coli* form part of the normal gut flora of many warm-blooded animals, including food production animals and pets (Franco 1988; Newell and Fearnley 2003). hThese *Campylobacter* species are especially well adapted for growth in the low oxygen environment of the chicken gut as their optimum growth temperature of 42°C is the normal body temperature of chickens (Shane 1992; Manning *et al.* 2003). Poultry meat is a major source of human infection as it can easily become contaminated with *Campylobacter* if it comes in contact with the contents of the gut during processing (Hansson *et al.* 2018). Chicken meat production and consumption is increasing with an approximate doubling of worldwide chicken meat production observed between 2000 and 2014 (Skarp *et al.* 2016). Thus, the minimization of the *Campylobacter* load on processed poultry meat is a critical human health issue.

To mitigate the risk of human infection, a number of measures aiming to minimize the *Campylobacter* cell count on the fully processed chicken meat have been introduced. For example, the Australian poultry industry has self-imposed a target upper limit of 6000 *Campylobacter* CFU per carcass based on the significant reductions in *Campylobacter* infections observed in New Zealand after introducing this target (Lake and Cressey 2013). Despite the importance of this pathogen, *Campylobacter* enumeration of industry samples is still performed by culture-based methods, which takes at least 2 days due to the slow growth of the organism. This approach creates a disassociation between the data that is obtained with the current *Campylobacter* loads on processed chickens. Thus, there is a real need to develop a rapid *Campylobacter* quantification system that can provide up to date information to deploy same-day risk-management strategies. Through an iterative process, we have developed a robust methodology that requires very little hands-on-time by the user, but is able to efficiently purify low-concentrations of DNA from the chicken rinse solution and establish whether the processed chickens are exceeding the industry set target of 6000 *Campylobacter* per carcass.

Materials and methods

Microbial strains

The 21 *C. jejuni* and 18 *C. coli* isolates used in this study were all obtained from chicken caeca collected at slaughter in one national company across all six Australian states described previously (Templeton 2014). Type strains for *C. jeuni* (ATCC 35560^T), *C. coli* (ATCC 33559^T), *Campylobacter lari* (ATCC 35221^T), *Campylobacter upsaliensis* (ATCC 43954^T), *Campylobacter hepaticus* ($HV10^T$) and Australian field isolates of *Arcobacter butzleri* and *Helicobacter pullorum* (both obtained from chicken caecal droppings) were used. Genomic DNA was extracted using the DNeasy blood and tissue lysis kit per the manufacturer's instructions (Qiagen, Clifton Hill, Australia).

Chicken rinse samples

Chicken rinse samples were collected from an Australian chicken processing plant on the same day they were processed. The chicken rinse solution was prepared as described in the Australian Standard AS.5013.6:2015 (Standards-Australia 2015). Briefly, a whole processed chicken was placed into a large plastic bag containing 500 ml of buffered peptone water. The chicken was rubbed for 2 min to facilitate the release of microbes from the chicken into the buffered peptone solution. A 500 μl aliquot of the chicken rinse solution was added to two plates each of different *Campylobacter* selective media, *Camylobacter* blood-free agar plates (modified CCDA) (Thermofisher Scientific, Sydney, Australia) and *Camylobacter* agar plates Preston (Thermofisher Scientific). The plates were incubated at 42°C in a microaerophilic atmosphere for 2 days before the *Campylobacter* were counted. The concentration of *Campylobacter* per ml of chicken rinse was calculated from the total colony count observed across the two plates.

Loop-mediated isothermal amplification Primer design

Seven primer sets for *C. jejuni* targeting the hippurate hydrolase (*hipO*) gene and the five loop-mediated isothermal amplification (LAMP) primer sets for *C. coli* targeting the *ceuE* locus were designed using PrimerExplorer v5 software (https://primerexplorer.jp/e/). All primer sequences were checked for self- and cross-priming using ThermoFisher Scientific's Multiple Primer Analyzer (https://www.thermofisher.com/au/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html). Only primer sets that did not show cross-reactivity at their 3′ ends were selected for further analysis.

LAMP DNA Amplification

LAMP amplifications reactions contained 0·5 mol l^{-1} betaine (Sigma-Aldrich), 1·2 mmol l^{-1} dNTP (New England Biolabs, Genesearch Pty Ltd, Arundel, Australia),

8 mmol l^{-1} $MgSO_4$, 20 mmol l^{-1} Tris (pH 8·8), 10 mmol l^{-1} $(NH_4)_2SO_4$, 10 mmol l^{-1} KCl, 1 mmol l^{-1} ATP, 0·1% Triton®-X-100, 1·6 μmol l^{-1} FIP primer, 1·6 μmol l^{-1} BIP primer, 0·2 μmol l^{-1} F3 primer, 0·2 μmol l^{-1} B3 primer and 0·32 U μl^{-1} *Bst* 2.0 warm start polymerase (New England Biolabs). In the early stages of this study, when extracting DNA from pure *Campylobacter* cultures, the LAMP reactions were performed at the standard incubation temperature of 63°C. However, the reaction temperature was increased to 65°C when working with the more complex chicken rinse samples to maximize primer specificity. Unless otherwise stated, reactions were performed at 65°C for 100 min.

Campylobacter quantification from culture

Frozen *C. jejuni* or *C. coli* stocks at known concentrations were thawed slowly on ice and diluted in buffered peptone water at concentrations of 0, 6, 12, 24 and 48 CFU per ml. A 1·5-ml aliquot of each dilution was added to a microcentrifuge tube containing a 5-mm disc of Whatman #1 filter paper, which helps to stabilize the *Campylobacter* pellet. The solution was centrifuged at 12 000 ***g*** for 5 min. The supernatant was discarded and another 1·5 ml of *Campylobacter* dilution was added to the tube. Again, the solution was centrifuged at 12 000 ***g*** for 5 min and the supernatant was discarded. The cells were washed in 1 ml TE buffer (10 mmol l^{-1} Tris (pH 8), 1 mmol l^{-1} EDTA) before the solution was centrifuged at 12 000 ***g*** for 5 min and the supernatant was discarded. The sample was incubated at 95°C for 10 min in a heat block to lyse the cells. The sample was cooled to room temperature and 23 μl of LAMP reagents were added directly into the tube before incubating at 63°C in a water bath for 60 min.

Campylobacter quantification from chicken rinse

A 2-ml aliquot of fresh chicken rinse that had been collected on the same day was added to a 2-ml tube containing two 3 mm diameter Whatman #1 filter discs and 105 μl of stabilizing buffer (5 mol l^{-1} NaCl, 1% Tween 20). The solution was centrifuged at 10 000 ***g*** for 5 min. The supernatant was discarded and 20 μl of 3 g μl^{-1} Proteinase-K in TE buffer (10 mmol l^{-1} Tris (pH 8), 1 mmol l^{-1} EDTA) was added and briefly vortexed to resuspend the pellet. The solution was incubated at 55°C for 30 min in a heat block followed by incubation at 95°C for 10 min. Two dipsticks with a 2 × 8 mm DNA binding area (Zou *et al.* 2017) were added to the tube and allowed to soak up all of the liquid in the tube. The dipsticks were dipped five times in wash buffer (10 mmol l^{-1} Tris (pH 8·8), 8 mmol l^{-1} $MgCl_2$) and then the DNA was eluted by dipping 15 times directly into the 50 μl LAMP reaction mix (Movie S1). One dipstick was used to purify the DNA for a LAMP reaction containing the *hipO-3* primer set and the other for a reaction containing the *ceuE-5* primer set. A 50-μl volume of mineral oil was placed on top of each reaction before placing the tubes in the 'Diagnostic Droid' that incubated the tubes at 65°C for 100 min while measuring the turbidity in each tube approximately once every 20 s. A positive amplification for either *C. jejuni* or *C. coli* indicates that the chicken rinse sample was above the industry set target of 6000 *Campylobacter* per carcass. The Diagnostic Droid is a small portable electronic device that was made in-house to incubate LAMP reactions and automatically interpret the results for the user. The Diagnostic Droid can analsze up to 12 reactions at once by monitoring their turbidity, which increases during DNA amplification due to the production of the insoluble byproduct, magnesium pyrophosphate.

Results

Development of highly specific LAMP primers and establishment of detection limits

We designed and tested seven LAMP primer sets for *C. jejuni* targeting the hippurate hydrolase (*hipO*) gene (Hani and Chan 1995) and five primer sets for *C. coli* targeting the *ceuE* locus that encodes an iron-chelating protein (Richardson and Park 1995) (Table S1). Each primer set was tested for the ability to amplify a product in the presence of purified genomic DNA from *C. jejuni* (ATCC 33560^T) or *C. coli* (ATCC 33559^T) strains and the absence of self-amplification products in the absence of template DNA (data not shown). Of these, four *hipO* and three *ceuE* primer sets showed promising results and were thus further tested for cross-reactivity using purified genomic *C. jejuni* and *C. coli* DNA with three of the four *hipO* primer sets and all three *ceuE* primer sets showing strong species specificity (Fig. S1). The *hipO-3* and *ceuE-5* primer sets (Table 1) were then selected for additional specificity tests using purified genomic DNA from other closely related species including the type strains of *C. lari* (ATCC 35221^T), *C. upsaliensis* (ATCC 43954^T) and *C. hepaticus* ($HV10^T$); and Australian field isolates of *A. butzleri* and *H. pullorum*, with both primer sets producing an amplification product only in the presence of DNA from their target organism (Fig. 1). The ability of the *hipO-3* and *ceuE-5* primer sets to differentiate between *C. jejuni* and *C. coli* was further tested in amplification reactions in which the template DNA was purified from 21 *C. jejuni* and 18 *C. coli* isolates obtained from whole chicken caeca collected at processing plants across Australia (Templeton 2014). The amplification results revealed that the regions targeted

within the *hipO* and *ceuE* genes were highly conserved between isolates of the same species. The *hipO-3* primer set consistently produced strong amplifications for all 21 *C. jejuni* isolates but failed to generate a product for any of the *C. coli* isolates (Fig. 2a). Conversely, the *ceuE-5* primer set amplified products for all 18 *C. coli* isolates but not for the *C. jejuni* isolates (Fig. 2b).

To evaluate the ability of the LAMP assay to detect low concentrations of *Campylobacter* cells, buffered peptone water samples containing 0, 6, 12, 24 or 48 CFU per ml *C. jejuni* (ATCC 33560 ^{T}) or *C. coli* (ATCC 33559 ^{T}) were analysed. As detailed in Materials and methods, the bacterial suspensions were centrifuged with a small disc of Whatman no. 1 filter paper in the tube to stabilize the pellet and minimize the risk of losing the cells when aspirating the supernatant. Pellets were heat denatured and used directly for LAMP amplification reactions. Using this system, the *hipO-3* and the *ceuE-5* primer sets resulted in amplifications in all four dilutions containing *Campylobacter*, revealing that the system is capable of detecting the presence of at least 18 CFU of *Campylobacter* derived from 3 ml of a 6 CFU per ml culture (Fig. 3).

Development of an easy-to-perform *Campylobacter* detection system from chicken rinse

Chicken rinse samples were obtained from an Australian chicken processing plant and used to optimize our method. The previously developed method for extracting DNA from *Campylobacter* in buffered peptone water was found to be unsuitable for the industry samples due to the ability of chicken rinse extracts to completely inhibit DNA amplification. Over 180 different combinations of extraction buffers, wash solutions, filtration systems and materials were trialed for their efficacy in aiding the release and purification of *Campylobacter* DNA from the chicken rinse. An example of approaches trialed include size exclusion filtration, low speed centrifugation, stepped density gradient centrifugation, enzyme/chemical treatment (proteinase K, NaOH, detergent, and salt), *Campylobacter* capture by DNA aptamer bound paramagnetic beads and silica-based DNA binding matrices. Approaches that improved DNA purification away from inhibitory compounds were used in successive experiments aimed at further improving the method.

Through the iterative method development process, we observed that samples containing less than 12 *Campylobacter* cells would rarely give a positive LAMP amplification for either the *hipO-3* or *ceuE-5* primer sets. Thus, we determined that the centrifugation of 2 ml of chicken rinse in the presence of small (3 mm) Whatman filter disks would be required to collect enough bacteria to assay for both *C. jejuni* and *C. coli*. We also observed that the addition of our stabilization buffer (final concentration of 250 mmol l^{-1} NaCl and 0·05% (v/v) Tween 20) to the chicken rinse solution helped to form a tight pellet after centrifugation that minimized losses of *Campylobacter* cells during supernatant removal. Similarly, we found that incubation of the resuspended pellet with proteinase-K, at a final concentration of 3 mg ml^{-1}, proved to be critical for the release of *Campylobacter* DNA and the removal or inactivation of inhibitory compounds from the extract. Samples treated with proteinase-K and subsequently used in an amplification reaction were able to produce an amplicon (Fig. S2). In contrast, without proteinase-K treatment, extracts from the same chicken rinse sample failed to produce an amplicon despite the presence of the purified template in the LAMP reaction. As a final step, *Campylobacter* DNA was purified from the crude proteinase-K treated extract using the newly reported DNA dipstick technology (Movie S1; Zou *et al.* 2017). After significant testing and optimization, we had developed an easy-to-perform method for purifying *Campylobacter* DNA from chicken rinse with four major steps: (i) centrifugation of the chicken rinse, (ii) proteinase K treatment of the pellet, (iii) heat denaturation and (iv) dipstick purification.

Validation of the *Campylobacter* detection system

To validate the newly developed system, chicken rinse samples from an Australian processing plant were

Table 1 Oligonucleotide primer sequences used for *Campylobacter* detection

Name	Sequence (5′–3′)
hipO-3-F3	GCAAAGAAGCAGCATAAATAGGAT
hipO-3-B3	CTTTATTTTCAACCTGCTGAAGAGG
hipO-3-FIP	GCGATGATGGCTTCTTCGGATTTCCATGACCACCCCTTCCAATAACTTC
hipO-3-BIP	TACCAAAAGGCATATTGTGCCAGCTAAGGCAATGATAGAAGATGGATTG
ceuE-5-F3	GCTTTTTAGTAAAAGATAGCCTAGGTGA
ceuE-5-B3	GTCCTACAAACATAGTTGGAGCAATT
ceuE-5-FIP	TTGGCAAGTTTTTAGCTGGAACTATACTTTCCATGCCCTAAGACTTAACG
ceuE-5-BIP	AAGACAAGCCTAGTATAGGTGGAGTTTGGCGTCCGGAAATGATAATAAGATCAG

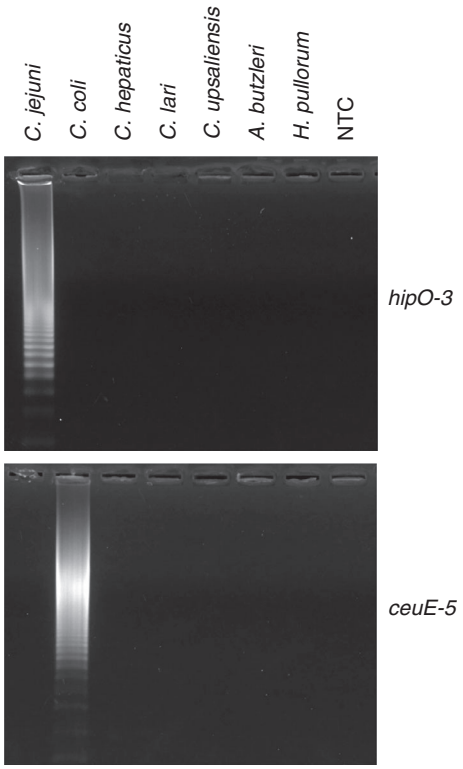

Figure 1 The *hipO-3* and *ceuE-5* primer sets are specific for their target organisms. Primers developed to detect *Campylobacter jejuni* (*hipO-3*) and *Campylobacter coli* (*ceuE-5*) were tested in LAMP amplification reactions using 5 ng of purified DNA from *Campylobacter jeuni* (ATCC 35560^T), *C. coli* (ATCC 33559^T), *Campylobacter lari* (ATCC 35221^T), *Campylobacter upsaliensis* (ATCC 43954^T), *Campylobacter hepaticus* (HV10^T) and Australian field isolates of *Arcobacter butzleri* and, *Helicobacter pullorum and Campylobacter hepaticus*. LAMP reactions were performed at 65°C for 1 h and then separated by gel electrophoresis and viewed under a UV transilluminator. NTC, no template control.

obtained and analysed on the same day the chickens were processed. The samples were processed with the newly developed method and analysed using LAMP amplification for the presence of *C. jejuni* and *C. coli*. *Campylobacter* cell counts were performed for all samples at the processing plant facilities as well as our laboratory and the highest value recorded. *Campylobacter* cell counts ranged from 0 to 955 CFU per ml (Table 2) with a similar number of samples testing positive for *C. jejuni* and *C. coli* (23 and 21 samples, respectively). As expected, there was a positive correlation between *Campylobacter* cell counts and detection of *Campylobacter* DNA by LAMP amplification. Analysis of the results revealed that 86·5% of the samples containing counts above the industry target of 6000 CFU per carcass (equivalent to 12 CFU per ml of chicken rinse) resulted in a positive amplification for *C. jejuni*, while for *C. coli* the correlation between cell count (>12 CFU per ml) and amplification results was 82·7%. However, by assigning a 'high' call (i.e. above 6000 CFU/carcass) to any sample in which either *C. jejuni* or *C. coli* is detected (Table 3), a high correlation (>92%) is observed between the traditional plate-based culture results and the DNA amplification data, resulting in a single (<2%) false negative result in our survey.

To make the assay more suitable for deployment to chicken processing plants, we used a simple electronic device, named 'Diagnostic Droid', which was previously developed by our group to perform the DNA amplification incubation and monitor the reaction in real time (Fig. 4). The 'Diagnostic Droid' takes advantage of the increase in turbidity observed in LAMP reactions during amplification by illuminating the amplification tubes with an LED light and measuring the amount of scattered light using a phototransistor (light sensor) mounted perpendicular to the LED light source. Using this system, samples that do not produce an amplicon show relatively constant turbidity levels, whereas those that produce an amplicon show a sudden increase in turbidity during the incubation period (Fig. S3a,b,c). Custom-made software incorporated into the 'Diagnostic Droid' continuously monitors turbidity and automatically detects the logarithmic increase in turbidity observed during amplification, distinguishing it from background noise, to provide a detection call. The 'Diagnostic Droid' circumvents the need for relatively time-consuming and laborious DNA detection procedures such as agarose electrophoresis (Fig. S3c) and analyses the data to provide a positive or negative result, eliminating the need for human interpretation of the results.

By combining the chicken rinse DNA purification method with the 'Diagnostic Droid' we have created a complete *Campylobacter* detection system (Fig. 4). To validate the system, we analszed 29 fresh chicken rinse samples obtained from chicken processing plants and compared with traditional culture results. DNA purification was achieved as described above while LAMP reactions and result interpretation was performed using the 'Diagnostic Droid' and primer sets for *C. jejuni* or *C. coli*. Final sample categorization was performed using the criteria outlined in Table 3. The complete detection system correctly categorized 26 out of the 29 samples (89·7%) as being above or below 6000 CFU per carcass. Among the three samples that were incorrectly categorized, two samples had relatively low cell counts of 23 and 37 CFU per ml but did not produce the expected amplicon while one sample produced a positive amplification in a sample in which no *Campylobacter* colonies were observed on the culture plates.

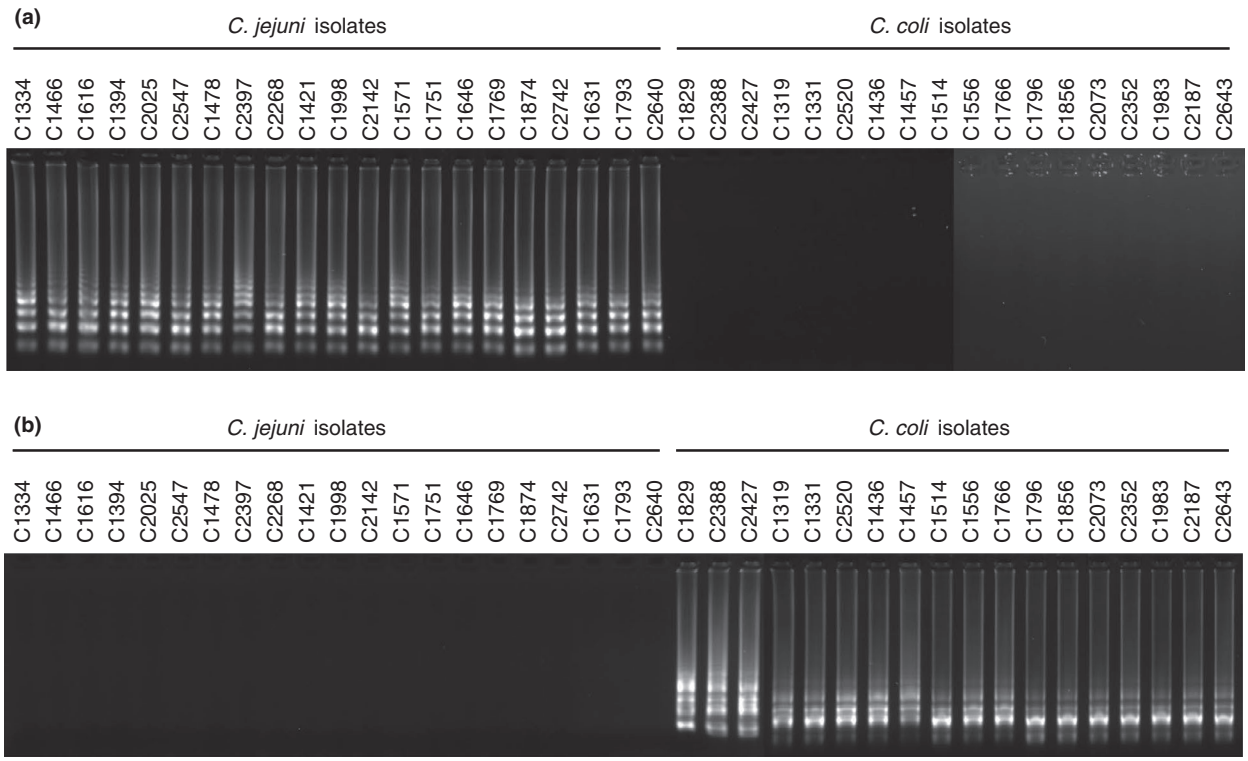

Figure 2 The *hipO-3* and *ceuE-5* primer sets show high specificity for their targets. LAMP amplification reactions using purified DNA from 21 *C. jejuni* and 18 *Campylobacter coli* isolates obtained from whole chicken caeca collected at processing plants across Australia. Reactions were performed with either the *hipO-3* (a) or *ceuE-5* (b) primer sets at 65°C for 1 h. The reactions were subsequently separated using gel electrophoresis and viewed under a UV transilluminator. The gel images are a composite of multiple images of the agarose gel used which was too large to fit within a single image.

Discussion

The 2–3 day incubation period required by the current culture-based methods for the detection and quantification of *Campylobacter* in chicken rinse is clearly inadequate for surveillance and quick response purposes. Molecular methods, such as quantitative PCR, have been developed to provide accurate and fast quantification data (Hong *et al.* 2007; Ronner and Lindmark 2007; Josefsen *et al.* 2010; Schnider *et al.* 2010), but require complicated nucleic acid purification processes, expensive thermocyclers and a high level of molecular biology expertise, which is beyond the capacity of most staff at processing plants. In this study, we have developed a method with the end-user in mind, in which accuracy, technical simplicity and low-cost were the major guiding factors. Our protocol requires only a few minutes of hands-on processing time by an operator to perform an assay consisting of five major steps. While this study focused on simplicity rather than speed, reductions in centrifugation and incubation times could further shorten processing time while the rate of LAMP amplification could potentially be increased by including additional loop primers (Nagamine *et al.* 2002).

Current practices to detect *Campylobacter* by culture-based methods include the rinsing of whole processed chickens in a plastic bag containing buffered peptone water before plating on *Campylobacter* selective growth media (Standards-Australia 2015). To produce a testing method with minimal disruption of the current adopted practices, we needed to develop a DNA extraction and purification procedure from industry-supplied chicken rinses. However, many research groups have previously reported the strong inhibitory effect on DNA amplification caused by the chicken rinse including the buffered peptone and biological contaminants such as blood, skin and fats (Wolffs *et al.* 2005; Hong *et al.* 2007; Botteldoorn *et al.* 2008; Josefsen *et al.* 2010; Schnider *et al.* 2010). Although dilution strategies have sometimes been able to overcome this problem, it results in a concomitant loss of sensitivity to the already low threshold required by the industry. Our findings are consistent with this research in that our initial analysis of chicken rinse sample extracts spiked with purified *Campylobacter* DNA

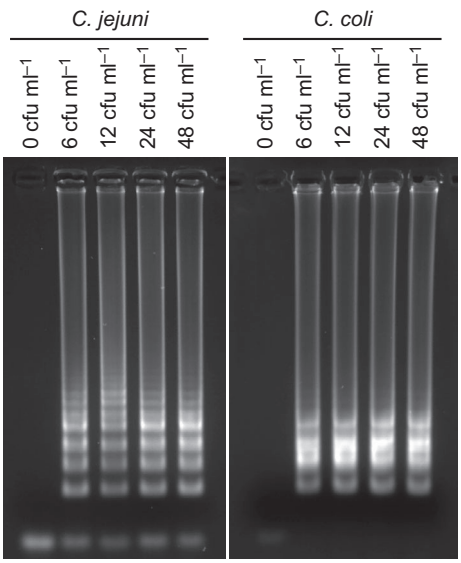

Figure 3 LAMP primers capable of detecting at least 18 CFU *Campylobacter*. A 3 ml aliquot of *Campylobacter jejuni* (ATCC 35560^T) or *Campylobacter coli* (ATCC 33559^T) at 0, 6, 12, 24, or 48 CFU per ml was centrifuged and the pellets were boiled and added directly into LAMP amplification reactions using *hipO-3* or *ceuE-5* primer sets for *C. jejuni* and *C. coli* cultures respectively. LAMP reactions were performed at 63°C for 50 min.

failed to produce any amplification products. Thus, a number of innovative strategies needed to be developed to both overcome the inhibitory compounds in the chicken rinse whilst, at the same time, creating an easy-to-perform and interpret assay with only a few major steps.

There are a number of key innovations that have contributed to the robustness and reliability of our method. First, the addition of small cellulose filter discs and a stabilizing buffer to the chicken rinse solutions improved the stability of the *Campylobacter* containing pellet post-centrifugation and reduced the chance of accidental loss of *Campylobacter* cells during pipetting steps. This is the first report of using cellulose discs to stabilize the microbial pellet and we have found that it is critically important for the reliability of the assay. Samples at 6000 CFU per carcass contain only 24 *Campylobacter* cells in the 2 ml aliquot used for extraction and thus the loss of just a few cells can have a significant influence on the results. Early in the method development process it was observed that 3 mm diameter filter discs conformed better to the curved inner walls of the centrifuge tubes compared to the original 5 mm diameter discs, and thus the 3-mm discs were adopted into the method. Second, the combination of the proteinase-K treatment with the 95°C denaturation step and DNA purification using the recently developed nucleic acid purification dipsticks

Table 2 Comparison between industry cell counts and LAMP results during assay development

CFU per ml	cpcc	*hipO-3*	*ceuE-5*	CFU per ml	cpcc	*hipO-3*	*ceuE-5*
0	0	–	–	20	10 000	–	+
0	0	–	–	30	15 000	+	+
0	0	–	+	30	15 000	+	–
0	0	–	–	32	16 000	+	–
0	0	–	–	32	16 000	+	+
0	0	–	–	40	20 000	+	–
0	0	–	–	40	20 000	+	–
0	0	–	–	86	43 000	+	+
0	0	–	–	92	46 000	–	+
1	500	–	–	92	46 000	–	+
1	500	–	–	97	48 500	+	+
2	1000	–	–	97	48 500	–	+
2	1000	–	–	138	69 000	–	–
3	1500	–	–	138	69 000	+	+
3	1500	–	–	233	116 500	+	–
3	1500	–	–	233	116 500	+	+
3	1500	–	–	233	116 500	+	+
4	2000	–	–	416	208 000	+	+
4	2000	–	–	725	362 500	+	+
5	2500	–	–	725	362 500	+	+
5	2500	+	–	825	412 500	+	+
5	2500	+	–	825	412 500	+	+
7	3500	–	–	840	420 000	+	+
7	3500	–	–	840	420 000	+	+
16	8000	–	+	955	477 500	+	–
20	10 000	–	+	955	477 500	+	–

DNA was extracted from industry obtained chicken rinse samples and used as template DNA in DNA amplification reactions using the *ceuE-5* and *hipO-3* primer sets. The presence (+) or absence (–) of a DNA amplicon as well as the *Campylobacter* cell counts, obtained by traditional culture methods, and the total cfu per chicken carcass (cpcc), are listed for each sample tested.

Table 3 Simple *Campylobacter* quantification system

Call	*hipO-3*	*ceuE-5*
Low	Negative	Negative
High	Negative	Positive
High	Positive	Negative
High	Positive	Positive

The calling system developed to rapidly predict whether chicken rinse samples are above or below the industry set benchmark of 6000 *Campylobacter* cells per carcass based on a correlation with LAMP amplification data.

(Zou *et al.* 2017) effectively neutralizes and/or removes the numerous inhibitors present in the chicken rinse solution to allow DNA amplification. The dipstick purification system (Movie S1) also significantly contributed to the simplicity of the assay as purification and elution of *Campylobacter* DNA into the two LAMP reactions can be

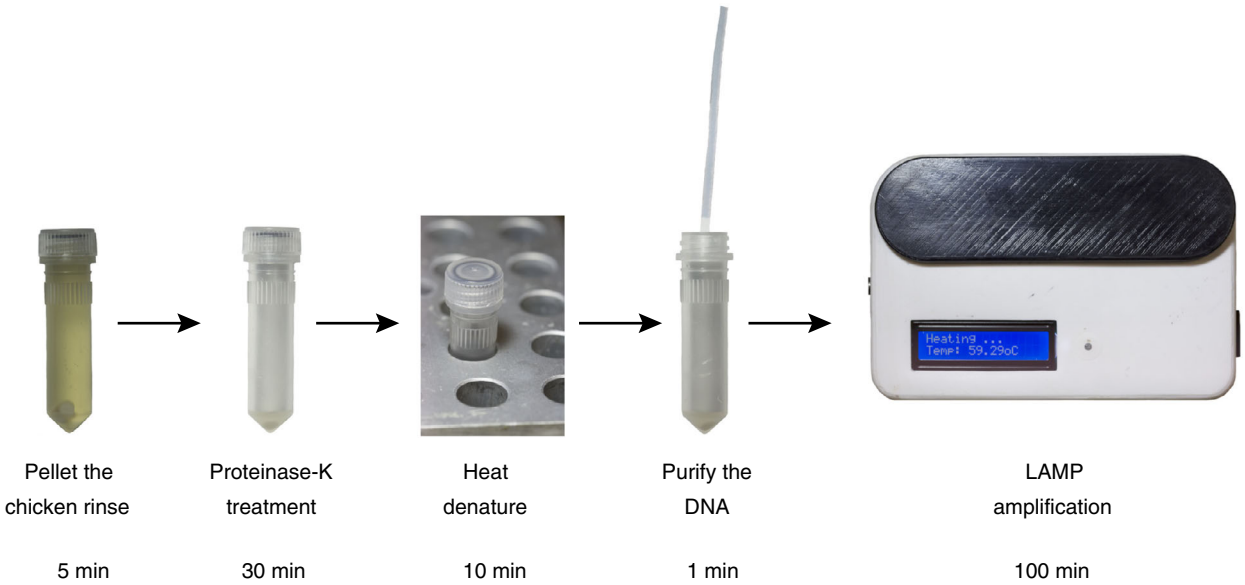

Figure 4 Overview of the *Campylobacter* quantification assay. A 2-ml aliquot of chicken rinse is pelleted by centrifugation for 5 min and the pellet is subsequently treated with proteinase-K and then heat denatured to lyse the cells and release DNA. Two cellulose dipsticks are added to the crude lysate to bind the DNA and purify it away from the contaminants using a single wash step before eluting the DNA into LAMP reactions for *Campylobacter jejuni* or *Campylobacter coli*. LAMP reactions were performed for 100 min at 65°C using our custom designed 'Diagnostic Droid' device that monitors the reactions and interprets the results for the user.

performed rapidly (less than 1 min) without need for any pipetting.

The adoption of a 'high/low' criteria for decision-making of whether samples were above or below the industry 6000 CFU/carcass threshold (Table 3) provided increased accuracy to our method with a high correlation (89·7%) between our data and the culture-based assays in industry supplied samples (Table 4). It is difficult to draw conclusions about the three samples in our survey showing conflicting results between our assay and the cell culture due to their low cell counts. A limitation of plate counts is that they have a relatively narrow reliable countable range between 25 and 250 CFU (Breed and Dotterrer 1916; Tomasiewicz *et al.* 1980). Thus, all of the processed chicken rinse samples with cell counts below 50 CFU per ml (25 000 CFU per carcass) fall outside this reliable count range. Thus, the reduced accuracy of the plate count data in this range makes it impossible to determine which of the two methods, our molecular system or the cell culture data, was correct. However, the high correlation of results from the two methods suggest that they both have similar accuracy within the range of *Campylobacter* concentrations found in chicken rinse.

The simple electronic device (Diagnostic Droid), we used to heat and monitor the LAMP reactions was initially built as a proof-of-concept and effectively

Table 4 Validation of the developed assay using industry obtained chicken rinse samples

CFU per ml	cpcc	*hipO-3*	*ceuE-5*	CFU per ml	cpcc	*hipO-3*	*ceuE-5*
0	0	+	–	58	29 000	+	–
0	0	–	–	62	31 000	+	+
0	0	–	–	62	31 000	+	–
0	0	–	–	152	76 000	+	–
0	0	–	–	180	90 000	+	–
0	0	–	–	279	139 500	+	+
0	0	–	–	315	157 000	+	–
0	0	–	–	561	280 500	+	+
2	1000	–	–	575	287 500	+	–
3	1500	–	–	600	300 000	+	+
6	3000	–	–	647	323 500	+	–
19	9500	+	–	685	342 500	+	–
23	11 500	–	–	770	385 000	+	–
37	18 500	–	–	1760	880 000	+	–
45	22 500	+	–				

Industry obtained chicken rinse samples were collected and tested on the same day as the chickens were processed. The samples were assayed using the complete *Campylobacter* diagnostic assay including our electronic amplification and readout device. The presence (+) or absence (–) of a DNA amplicon using either the *hipO-3* or *ceuE-5* primer sets as well as the *Campylobacter* cell counts, obtained by traditional cell culture methods, and the total cfu per chicken carcass (cpcc), are listed for each sample tested.

eliminates the need for expensive equipment such as a quantitative real-time PCR machines and fluorescent probe-based LAMP master mixes. Similar simplified devices capable of running and monitoring LAMP reactions have been published previously (Mori *et al.* 2004; Myers *et al.* 2013). The 'Diagnostic Droid' is not essential to the overall assay and could be replaced by incubation in a standard water bath or heat block followed by naked eye observation of the increase in turbidity although this skill would need to be mastered by operators (Mori *et al.* 2001). In addition, there are a number of additional colorimetric readouts for LAMP reactions such as the metal ion-sensitive indicator hydroxy naphthol blue (Goto *et al.* 2009; Tomlinson *et al.* 2010; Gosch *et al.* 2012). However, the automatic data interpretation from the 'Diagnostic Droid' provides a significant advantage over naked eye readouts eliminating user bias in the interpretation of the results.

In conclusion, we have developed a simple, five-step method to identify naturally contaminated chicken meat samples that exceed the Australian poultry industry set target of 6000 *Campylobacter* CFU per carcass. The development of a rapid, low-cost and easy-to-perform technique for detection and quantitation of *Campylobacter* on processed chickens will provide significant benefits to the poultry industry and other industries interested in food safety by enabling them to implement same-day risk management strategies to limit the number of *Campylobacter* infections. The simplicity and speed of the assay also makes it well suited for other applications such as investigations to identify the source of *Campylobacter* contamination after food poisoning events providing the investigators with information on bacterial loads in hours rather than days.

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Conflict of Interest

The DNA purification dipsticks used in this manuscript are protected by a current Australian Patent Cooperation Treaty (PCT); Application # W02018195594; Title: Simple nucleic acid extraction; Applicant: MG Mason and JR Botella (The University of Queensland). The authors have neither licensed or sold their technology to any company nor has it been commercialized by them.

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Supporting Information

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

Figure S1. Identification of reliable primer sets to detect *Campylobacter coli* (ATCC 33559^T) and *Campylobacter jejuni* (ATCC 35560^T).

Figure S2. Proteinase-K is important for reliable extraction of *Campylobacter* DNA from chicken rinse. An example optimization result in which the addition of proteinase-K (PK) and different buffers were tested for their ability to remove/neutralize inhibitors from the chicken rinse extracts.

Figure S3. DNA amplification using the 'Diagnostic Droid'.

Table S1. Oligonucleotide primer sequences assessed for detection of *Campylobacter jejuni and Campylobacter coli.*

Movie S1. Rapid *Campylobacter* DNA purification from treated chicken rinse lysate. After the chicken rinse has been pelleted, proteinase-K treated and heat denatured, two cellulose dipsticks are added to the lysate to bind the DNA. One at a time the dipsticks are briefly washed to remove contaminants before eluting the DNA directly into the LAMP reaction containing either the *hipO-3* or *ceuE-5* primer sets.