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Quantitation of viable *Coxiella burnetii* in milk using an integrated cell culture-polymerase chain reaction (ICC-PCR) assay

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The obligate intracellular pathogen *Coxiella burnetii* has long been considered the most heat resistant pathogen in raw milk, making it the reference pathogen for determining pasteurisation conditions for milk products. New milk formulations and novel non-thermal processes require validation of effectiveness which requires a more practical method for analysis than using the currently used animal model for assessing *Coxiella* survival. Also, there is an interest in better characterising thermal inactivation of *Coxiella* in various milk formulations. To avoid the use of the guinea pig model for evaluating *Coxiella* survival, an Integrated Cell Culture-PCR (ICC-PCR) method was developed for determining *Coxiella* viability in milk. Vero cell cultures were directly infected from *Coxiella*-contaminated milk in duplicate 24-well plates. Viability of the *Coxiella* in milk was shown by a ≥0·5 log genome equivalent (ge)/ml increase in the quantity of IS111a gene from the baseline post-infection (day 0) level after 9–11 d propagation. *Coxiella* in skim, 2%, and whole milk, and half and half successfully infected Vero cells and increased in number by at least 2 logs using a 48-h infection period followed by 9-d propagation time. As few as 125 *Coxiella* ge/ml in whole milk was shown to infect and propagate at least 2 logs in the optimised ICC-PCR assay, though variable confirmation of propagation was shown for as low as 25 *Coxiella* ge/ml. Applicability of the ICC-PCR method was further proven in an MPN format to quantitate the number of viable *Coxiella* remaining in whole milk after 60 °C thermal treatment at 0, 20, 40, 60 and 90 min.

Keywords: *Coxiella burnetii*, ICC-PCR, quantitation, milk.

Coxiella burnetii, the causative agent for Q fever in humans, is an obligate intracellular pathogen found nearly worldwide (Hilbink et al. 1993). The organism is primarily found in ruminant animals including sheep, goats, and cattle. Farm and abattoir workers handling these animals are at the greatest risk for exposure, and disease is thought to occur primarily through aerosol exposure via contaminated excreta and birth products. Consumption of contaminated milk and dairy products has long been considered a potential means of transmission.

C. burnetii is the most heat resistant vegetative pathogen found in raw milk, which led to its use as the reference pathogen for establishing proper pasteurisation conditions. Pasteurisation conditions for raw fluid milk products were primarily based on studies conducted by Enright (1961) and Enright et al. (1957) where viability of the *Coxiella* in the heated samples was confirmed by their inoculation into guinea pigs and testing of second passage pigs for the presence of complement-fixing antibodies. The resulting data from these studies indicated that proper pasteurisation temperatures should be increased from those previously considered adequate, and that an additional margin of safety could be obtained for products having increased fats, solids or flavourings by the addition of 3 °C to the processing temperature. Unfortunately, this guinea pig model did not allow for thermal inactivation curves to be produced at each temperature because viable cells in the original milk sample could not be quantified. Questions regarding the experimental approach to Enright's work, including the inability to properly produce survival curves, have led researchers to question the need for such stringent pasteurisation conditions (Cerf & Condron, 2006; LeJune & Rajala-Schultz,

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2009). New formulations of milk having higher fats and sugars content, and added flavours, may need assessment for thermal inactivation kinetics of *Coxiella*. In addition, a rapid and sensitive method for quantitation of *Coxiella* in milk products would be useful for evaluating proper pasteurisation conditions for non-thermal techniques such as high pressure and cold plasma processing.

PCR has been used for detection of *Coxiella burnetii* in dairy products including cheese, yogurt, cream, margarine and raw milk (Loftis et al. 2010; Hirai et al. 2012; Eldin et al. 2013). Unfortunately, direct PCR is not able to differentiate viable cells from dead cells. In the aforementioned studies, viability was determined by culture in mice along with a secondary passage into mice or cell culture and final viability confirmation using a variety of methods including Gimenez staining, immunofluorescence, and PCR. Unfortunately, these procedures only provided a qualitative determination of viability. To study inactivation kinetics properly, the viable cells remaining after heat treatment need to be enumerated directly from the test product.

Integrated cell culture-PCR (ICC-PCR) has been used to determine the viability of obligate intracellular microorganisms such as enteric viruses in a variety of environmental samples (Lee & Jeong, 2004; Gallagher & Margolin, 2007; Rodriguez et al. 2013). This technique serves to confirm viability by allowing viruses to infect a tissue culture system with measurement of their propagation over time by using PCR to quantitate the increase. Although intact nucleic acids may be found in the system, viability is confirmed by a decrease in the cycle threshold (C_t) value obtained using real-time PCR specific for the organism. A similar approach has been used to determine antibiotic minimum inhibitory concentrations (MICs) for clinical use (Brennan & Samuel, 2003; Boulos et al. 2004) and to evaluate the chlorine exposure necessary to inactivate poliovirus (Blackmer et al. 2000). The goal of this work was to develop an ICC-PCR assay for detection of viable *Coxiella* in a variety of fluid milk products and evaluate its potential for characterising heat inactivation of *Coxiella burnetii* in whole milk.

Materials and methods

Organisms and reagents

Coxiella burnetii Nine Mile phase II RSA 439, a smooth-coated avirulent strain, and African green monkey kidney epithelial (Vero) cells were kindly provided by Dr. Robert Heinzen at Rocky Mountain Laboratories (National Institutes of Health, Hamilton, MT). Vero cells were routinely propagated using RPMI 1640 medium supplemented with 3% foetal bovine serum (FBS, Gibco, Grand Island, NY). Infected Vero cells were maintained using RPMI 1640 with 1% FBS. Ultra-high temperature (UHT) whole, skim and 2% milks and whipping cream were obtained from Gossner Farms (Logan UT, USA), and high-temperature short-time (HTST) half & half, cream, chocolate milk and eggnog were obtained from a local grocer and were refrigerated immediately after transport to the lab.

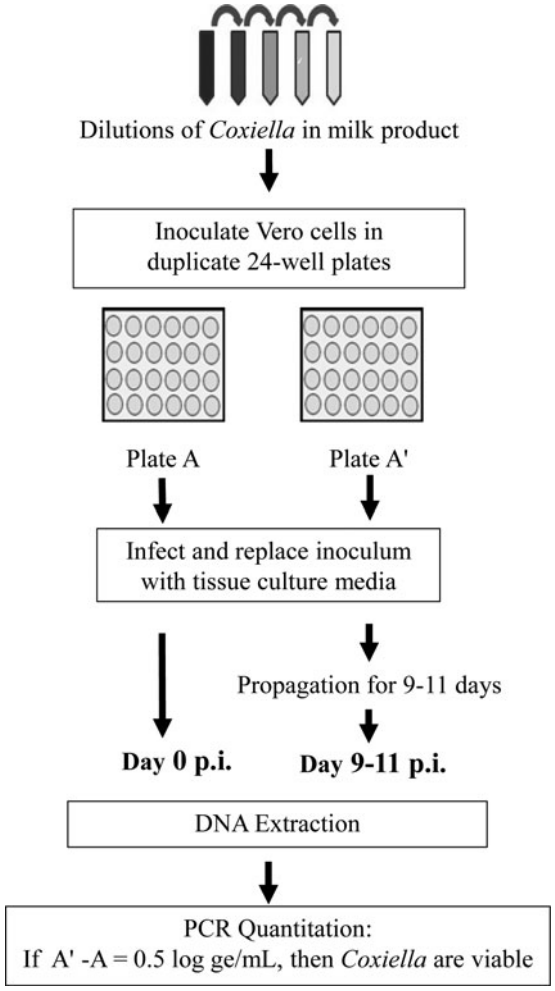

Fig. 1. Overview of Integrated Cell Culture-PCR (ICC-PCR) assay scheme. p.i.; post-infection.

Development and optimisation of ICC-PCR assay for detection of viable Coxiella in milk

Figure 1 illustrates the ICC-PCR assay scheme that was evaluated for determining the viability of *Coxiella* in milk products. This method involved inoculating duplicate 24-well plates containing Vero cells with a relatively small volume of diluted *Coxiella*-inoculated milk and allowing infection for a short period of time. The inocula were removed and tissue culture media was added to both plates. Immediately after infection, one plate was frozen and the duplicate plate incubated to allow propagation of the *Coxiella*. After the propagation period, DNA in both plates was extracted and the IS111a gene content of the baseline and propagated samples was compared using quantitation by real-time PCR. In this manner, viability was determined directly from the milk sample.

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For initial evaluation of the ICC-PCR scheme, a 2-h infection period was chosen. *Coxiella* stock was serially diluted in each matrix to result in a concentration of 7·18 log ge/ml and further diluted 1:1 with PBS. This mixture (200 µl) was layered on confluent Vero cells in duplicate wells in duplicate 24-well plates. No-matrix positive controls and uninoculated matrix controls were included for all experiments. A 2-h incubation at room temperature with slow rocking on a wrist-action platform shaker was used to allow infection of the Vero cells, after which 800 µl fresh RPMI 1640 + 1% FBS was added and mixed gently. An equal volume of inoculum/media mix was removed and replaced with 800 µl fresh media. This resulted in a substantial dilution of the milk matrix in order to remove growth inhibitors and provide fresh growth media for cell maintenance and propagation of the *Coxiella* in the Vero cells. One of the duplicate plates was immediately placed at −80 °C as a Day 0 post-infection (D0 p.i.) plate and the other was incubated for 11 d at 37 °C in a 5% CO_2 atmosphere to allow *Coxiella* propagation in the infected Vero cells. At Day 11 p.i., the duplicate plate was stored at −20 °C until the DNA was extracted, which was performed after three freeze-thaw cycles. PCR was completed on all samples from the D0 and D11 p.i. plates, and propagation of the *Coxiella* was evaluated by comparing the ge/ml estimated by the standard curve.

For extended infection time tests, 500 µl of 1:1 milk and PBS mixture was layered on confluent cells in duplicate 24-well plates as described previously. All plates were slowly mixed using the wrist-action shaker at room temperature for 2 h, after which one of the duplicate plates for the 2 h infection samples was frozen and the other plate was further incubated to allow propagation for an additional 9 d. The duplicate sets of plates for the 5-, 24- and 48-h infections were moved to 37 °C in 5% CO_2 for an additional 3, 22 or 46 h. No-matrix positive controls were prepared by dilution of *Coxiella* in PBS only. At the end of each infection period (2, 5, 24, or 48 h), the inoculum in each well was removed and 1 ml fresh RPMI 1640 + 1% FBS was added prior to freezing (for D0 p.i. plates) or incubation (for D9 p.i. plates) so that well volumes were equivalent for comparison by PCR.

ICC-PCR quantitation of the thermal inactivation of Coxiella burnetii in whole milk

Whole milk (1·5 ml) containing 7·2 log ge/ml *Coxiella* was aliquoted into 2 ml crimp-top glass vials (Fisher Scientific, Pittsburgh, PA), sealed, and placed into an ice bath for 5 min to cool. Vials were simultaneously placed into a vial holder, submerged in a shaking 60 °C water bath (Thermo Scientific, West Palm Beach, FL) for heat treatment and were removed at intervals (0, 20, 40, 60, and 90 min.) to an ice bath to stop inactivation prior to the ICC-PCR assay. Thermally treated milk samples were serially diluted using RPMI 1640 + 1% FBS prior to infection of the Vero monolayers as described earlier, testing 3–5 aliquots of each dilution in the tissue culture assay depending on the expected degree of inactivation. Quantitation of the remaining viable *Coxiella* in each treated sample was determined by MPN method using the number of D9 p.i. wells at each dilution level showing at least a 0·5 log increase over the highest of the D0 p.i. wells assayed for each dilution level. MPN's were calculated using an MPN Excel spreadsheet (Blodgett, 2010).

PCR

Nucleic acid extracts for *Coxiella burnetii* stocks and infected Vero cell cultures were prepared by extracting 200 µl of sample using the Quickgene Mini80 system with the QuickGene DNA tissue Kit S (AutoGen, Holliston, MA) with final elution using 200 µl of the kit supplied buffer. Extracted DNA samples were stored at −20 °C prior to PCR amplification.

The real-time PCR primers and minor groove binder (MGB) probe sequences and concentrations used for amplification of the *Coxiella*-specific IS1111a gene were as per Howe et al. (2009) with forward primer 3′-AATTTCA TCGTTCCCGGCAG-5′, reverse primer 3′-GCGGCGTTT ACTAATCCCCA-5′ and MGB probe 3′-FAM-TGTCG GCGTTTATTGG-5′ manufactured by Applied Biosystems (Foster City, CA). Amplifications were run using the Quantitect PCR Probe mix (Qiagen, Valencia, CA) with 5 mM $MgCl_2$ and 5 µl template in 20 µl volumes. Reactions were run on a Roche LightCycler 480 (Indianapolis, IN) with a cycling profile of a denaturation cycle at 96 °C for 15 min. followed by amplification using 45 cycles of 96 °C for 10 s., and 60 °C for 30 s. A standard curve was produced over a 6-log range using extracted 10-fold serial dilutions of purified *Coxiella burnetii* with triplicate reactions giving a slope of −3·393, with an efficiency of 97·1% and R^2 of 0·952.

Evaluation of PCR matrix inhibition from various milk products

The initial ICC-PCR assay scheme used a 2-h infection with 1:1 diluted milk followed by inoculum removal and addition of fresh media. The final amount of milk matrix in each sample may have been slightly variable due to incomplete inoculum removal and dilution with fresh media. To alleviate this variability and better evaluate the real effect of the matrix on PCR detection of the *Coxiella*, a mix simulating the ratios of assay components in each well at the end of the infection period was prepared. Vero cells in 24-well plates were grown to confluence and rinsed with PBS followed by addition of 875 µl fresh RPMI 1640 + 1% FBS, 25 µl of milk product and 100 µl of *Coxiella burnetii* serially diluted in PBS to produce test wells containing log 3·7 *Coxiella* genome equivalents (ge)/ml. Negative controls were prepared by adding 100 µl of PBS instead of milk. The contents of the wells were lysed via three freeze-thaw cycles, scraped, and harvested to sterile 1·5 ml microtubes

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which were stored at −20 °C prior to DNA extraction and PCR analysis. The degree of inhibition caused by the presence of the milk product was evaluated by determining the cycle threshold shift between triplicate test and no-matrix control wells. A shift of more than 2 cycles from the no-matrix control was considered to be inhibitory to the PCR.

Results and discussion

Optimisation of Vero cell infection directly from inoculated milk

In our initial evaluation of a 2-h Vero cell infection time, *Coxiella* in matrices (whole and skim milk, cream, chocolate milk, and eggnog) were tested and compared to *Coxiella* in PBS (a positive control with no-matrix) in duplicate trials. This infection time is based on a report from Shannon & Heinzen (2008) indicating that pure cultures of *Coxiella* are able to infect and propagate at least 2 logs in Vero cell culture monolayers over a period of 6 d using the same 2 h infection period. The positive control samples increased 1·65 log ge/ml after 11 d propagation, which is close to the expected increase of 2 log ge/ml, however, propagation from the various milk samples was problematic. Variable growth of *Coxiella* was shown in skim and chocolate milk and no growth was seen in eggnog and whole milk (data not shown). Inhibition analysis indicated that the PCR was not influenced by the presence of the skim and whole milk. Therefore, it was assumed that the inability to see propagation in these samples was more likely due to reduced infection of the Vero cells and/or reduced propagation of the *Coxiella* once they have infected Vero cell monolayers for these samples.

Since it was seen that the residual milk had little toxicity to the Vero cells, it was thought that extending the infection time might improve *Coxiella* entry from the whole milk samples into Vero cells, thus improving propagation of the *Coxiella*. Extended infection times of up to 48 h were tested for *Coxiella* in UHT whole milk and PBS (as a positive control) in duplicate trials (Fig. 2). Increased infection times for the positive control samples resulted in both slightly increased D0 p.i. infection levels and increased propagation after 9 d as quantitated by PCR, although the *Coxiella* did not propagate after a 48-h infection period. Microscopic inspection of these wells showed extensive cell death, presumably due to cell damage from lack of nutrients for the cells during the extended infection period. Propagation in the positive control samples also increased slightly with increased infection times, but reached a maximum of 8 log ge/ml regardless of the initial infection time tested.

For the whole milk samples, there was no clear increase in quantitated infection level with extended infection time, but there was a substantial increase in growth when a 48 h infection period was used, with the D9 p.i. samples reaching 8 log ge/ml, the maximum level seen in the positive control samples. This indicates that extending the infection time to 48 h overcomes the issues causing reduced propagation in the UHT whole milk samples. Additionally, the more extensive inoculum removal using the extended infection time assay scheme may have contributed slightly to the improvement in the infection and propagation from the milk, though the D0 and D9 p.i. levels were similar for both the initial and modified 2 h infection wells.

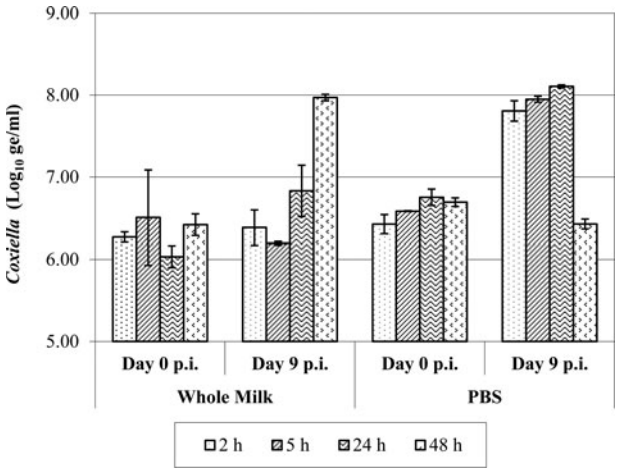

Fig. 2. Effect of increased Vero cell infection time for whole milk and control samples. Results represent averages and standard deviations *Coxiella* ge/ml in 6 replicate wells from two trials. No-matrix controls were completed using phosphate buffered saline (PBS). Total assay times included infection times of 2, 5, 24 and 48 h followed by an additional 9 d of incubation to allow propagation of the *Coxiella*.

Effect of 48-h infection on propagation and viability determination of Coxiella burnetii in various milk products

Infection and propagation of *Coxiella* in UHT whole milk was made possible by extending the infection time to 48 h. However, the effect of the extended infection time on detection of *Coxiella* in other dairy products was not known. To this end, the modified infection assay scheme was tested on an extended array of dairy products using a lower initial inoculum of *Coxiella* (~10^4 ge/ml). Inoculated RPMI + 1% FBS was used as a control because of the previously mentioned deleterious effect of PBS on the Vero cell monolayers using 48 h infection. The improved assay was tested on a variety of milk products in duplicate trials with duplicate test wells. The level of *Coxiella* propagation for these milk products using the 48-h infection scheme is shown in Fig. 3. *Coxiella* levels in the UHT whole, UHT skim, UHT 2% milk, and UHT whipping cream samples all increased at least 2 log ge/ml after 9 d of propagation. Slightly less propagation (~1·8 log ge/ml) was seen for the HTST half & half and HTST eggnog samples. Propagation from HTST chocolate milk was highly variable, with 2 of 4 trials showing no propagation. Overall, the modified 48-h Vero cell infection scheme allowed a ≥2 log increase in *Coxiella*

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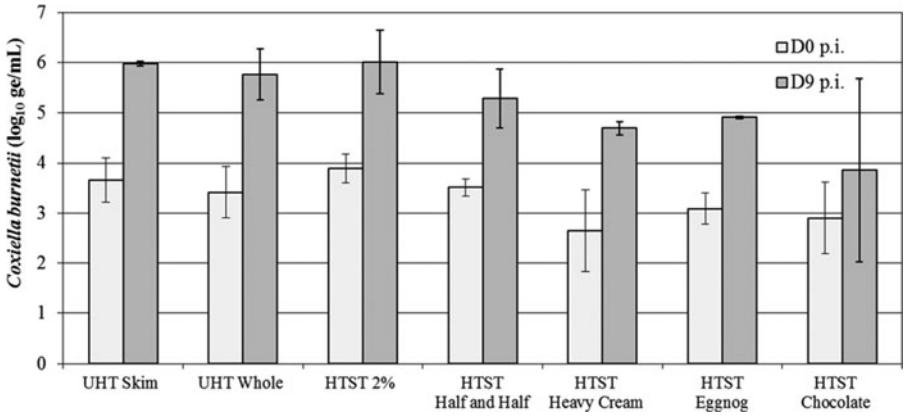

Fig. 3. Comparison of *Coxiella* burnetii infection and propagation from various milks using 48-h tissue culture infection. Results indicate average and standard deviation for *Coxiella* ge/ml for 6 replicate tissue culture wells in two trials at Day 0 (D0) and Day 9 (D9) post infection (p.i.).

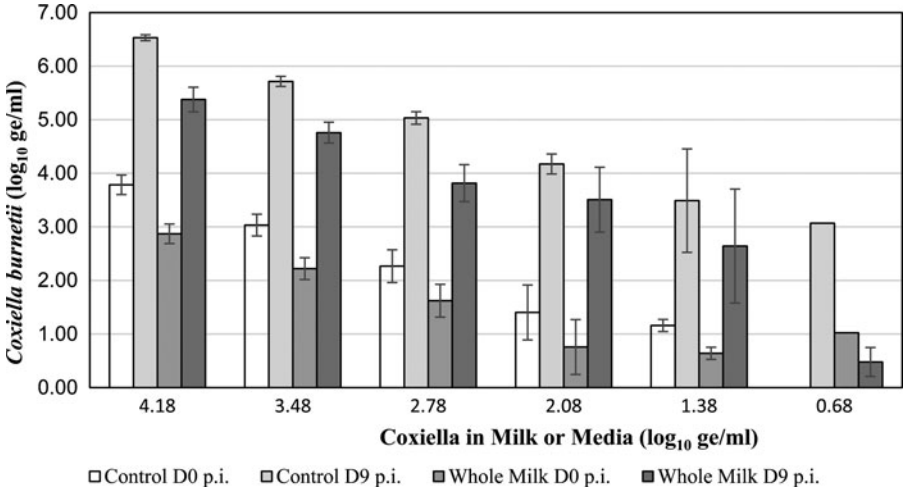

Fig. 4. Limit of PCR detection for *Coxiella* infection and propagation from whole milk and media control samples using 48-h infection and 9-d propagation. Milk and tissue culture media (control) inoculated at 5-fold dilutions from 4·18 log ge/ml were assayed in two trials with triplicate wells. Data represent the average and standard deviation of 6 replicate wells at each initial inoculation level per ml of milk.

ge/ml indicating that infection and propagation were possible from *Coxiella* present in a wide range of milk products.

PCR limits for detection of infection and propagation of Coxiella burnetii in UHT whole milk

After optimising the assay infection time we aimed to determine the lowest level at which *Coxiella* can infect and propagate in cell culture with and without the influence of the milk. This was accomplished by infecting Vero cell monolayers with serial 5-fold dilutions of *Coxiella* made in either RPMI + 1% FBS (as a positive control) or whole milk at concentrations of 4·18–0·68 log ge/ml. To reduce evaporation in the test wells with the extended infection time (48 h), the volume of inoculum was increased from 200 to 500 µl. Triplicate test wells for the D0 and D9 p.i. samples for each inoculum level were averaged over two trials.

As seen in Fig. 4, the Day 0 infection levels for the positive control samples were as expected for each level down to the 0·68 ge/ml milk level, where neither sample contained detectable *Coxiella*. Although initial infection level could not be quantitated for the 0·68 ge/ml inoculum level, the D9 p.i. samples contained ~3 log ge/ml, indicating that the D0 p.i. levels for the 0·68 ge/ml inoculum were at the PCR limit of detection. The control samples consistently showed *Coxiella* propagation of greater than 2·5 logs at each inoculation level.

The presence of the whole milk in the modified ICC-PCR reduced the ability of the *Coxiella* to infect the Vero cell monolayers by ~0·5 log at each inoculation level vs. the samples prepared in media alone. It is believed that the presence of the milk and the increased volume used for the 48-h infection may have prevented complete infection of the monolayers. The lowest

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Table 1. Inactivation of *Coxiella burnetii* in whole milk at various treatment times

	Heating time (min)				
Milk dilution	0	20	40	60	90
10^{-1}	ND	ND	3/3†	0/3	0/3
10^{-2}	ND	ND	3/3	0/3	0/3
10^{-3}	ND	3/3	5/5	0/3	0/3
10^{-4}	ND	3/3	1/5	ND	ND
10^{-5}	3/3	3/3	1/5	ND	ND
10^{-6}	3/3	3/3	0/5	ND	ND
log MPN/ml milk	>6·7	>6·7	4·26	<1·15	<1·15
95% confidence limits (log MPN/ml)			3·78–4·74		

ND, not determined
Initial inoculation level = log 7·2 ge/ml
†Number positive wells/number test wells

inoculum level at which propagation could still be detected from UHT whole milk was log 1·38 ge/ml, though propagation increases were more variable at this level. *Coxiella* were detected at D0 p.i. for the 0·68 ge/ml inoculation level samples, however there was no propagation seen at D9 p.i. A possible explanation for the PCR signal is the presence of non-infectious background *Coxiella* or *Coxiella* DNA fragments in the milk. Kim et al. (2005) indicated that *Coxiella* DNA is present in >90% of bulk tank milk in the U.S. In addition, we have sporadically seen false positive PCR results at C_ts of ≥38 cycles where we do not observe an increase in the concentration of *Coxiella* over the 9 d propagation period. Overall, the results from this trial indicate that whole milk containing as few as log 2·08 *Coxiella* ge/ml will consistently infect Vero cell monolayers and propagate at least 2 log ge/ml in 9 d, though milk containing fewer cells (down to 1·38 ge/ml) will infect and propagate less reliably.

PCR inhibition analysis

PCR inhibition due to the presence of residual milk products in the cell culture assay was evaluated by extracting and amplifying *Coxiella* DNA from a mixture simulating assay well contents that are present at the end of the 11 d propagation period. The impact of the milk matrix on detection was determined by comparing the C_t of the milk product samples to that of a no-matrix control. Differences between the no-matrix and test samples for each product were 0, 0·21, 0·84, 2·17, and 2·84 cycles for skim, whole, heavy cream, eggnog and chocolate milks, respectively, indicating that the only milk products which would cause significant (>2 cycle) PCR inhibition were the eggnog and chocolate milk. This is consistent with the presence of inhibitory compounds such as egg components (Woodward & Kirwan, 1996), spices, and various stabilisers in the eggnog, and the polyphenols from the chocolate in the chocolate milk (Margot et al. 2013).

Quantitation of Coxiella burnetii heat inactivation in whole milk

The potential of the ICC-PCR assay for quantitation of viable *Coxiella* present in milk after heat inactivation was evaluated by batch-heating log 7·2 *Coxiella* ge/ml UHT whole milk in submerged glass vials at 60 °C for 0, 20, 40, 60, and 90 min. The equation derived by Enright et al. (1957) for calculation of thermal death time (TCDT) for inactivation of 10^5 guinea pig infective doses of *Coxiella* in 2 ml raw milk is as follows: Log time (min) = 19·78711 – 0·128105 Temp (° F). For a 60 °C (140 °F) heat treatment, this equation gives a median thermal death time of 71·2 min. Regression lines were also produced for the heating conditions whereby surviving *Coxiella* would be expected. For this study, since the 60-min treatment had the potential to have viable cells, a 90-min heat treatment was included as a control for total inactivation.

In our thermal inactivation trial, heat-treated milk was serially diluted and used to infect Vero cells in 3–5 culture wells with the intent to use the qualitative propagation results to calculate a viable *Coxiella* MPN. To define which samples showed propagation of the *Coxiella*, the PCR-determined quantities of *Coxiella* in the D9 p.i. wells were compared to the highest ge/ml level found in the D0 p.i. test wells for each time point assayed. Although untreated *Coxiella* were generally able to propagate by more than 2 log ge/ml, it was apparent that the injury caused by the heat treatment necessitated redefining the increase in ge/ml which could be considered as positive for propagation (data not shown). To that end, a 0·5 log ge/ml increase from D0 p.i. to D9 p.i. (equivalent to ~1·6 doublings) was chosen as a more conservative measure of propagation than a doubling. If the *Coxiella* ge/ml in the D9 p.i. wells increased by ≥0·5 log ge/ml the result was scored as positive for growth. This cutoff also reduced potential error due to run-to-run amplification variation and error in the PCR standard curve.

Table 1 details the MPN results of the 60 °C thermal inactivation trial. The 0 and 20 min samples showed little inactivation with calculated MPN's greater than the upper assay

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detection limit of 6·7 log MPN/ml. The samples treated for 60 and 90 min had no wells showing propagation in the ICC-PCR assay, resulting in an MPN quantitation of less than 1·15 log MPN/ml, and at least a 6-log reduction in viable *Coxiella*. The MPN for the sample treated for 40 min was 4·26 MPN/ml with a 95% confidence range of 3·78–4·74 log MPN/ml, indicating an ~3-log reduction in viable *Coxiella* from the initial inoculation level. Overall the data in this study match those from the thermal death time data of Enright et al. (1957), and they indicate that the ICC-PCR assay may be more sensitive than the guinea pig model.

An interesting result of this thermal inactivation trial was that there was no measurable decrease in viability after the 20-min thermal treatment, which would indicate that the thermal inactivation of *Coxiella* in milk is possibly biphasic instead of linear. Unlike the guinea pig model used by Enright et al. (1957), the ICC-PCR method allows production of survival curves which should allow better characterisation of thermal inactivation. This method also has a distinct advantage in that it can be used to confirm viability of the *Coxiella* directly from the test sample. This method can also be implemented in a format which can allow quantitation of the remaining viable cells in the sample using a statistical probability method. Further experiments with thermal treatment times are necessary to produce survival curves for characterisation of the thermal inactivation of *Coxiella* in milks.

Conclusions

To our knowledge, the optimised method used in this study is the first method to allow a determination of *Coxiella* viability directly from inoculated milk using growth in tissue culture and quantitation via real-time PCR. Although eggnog and chocolate milk were problematic for infection, most other milk types allowed infection and propagation which suggest that the method could be used in future studies to compare inactivation in different milk products. By confirming that there was an increase in PCR ge/ml from the Day 0 to Day 9 post-infection samples, we were able to consistently prove viability of *Coxiella* in samples containing as few as ~25 (log 1·38) *Coxiella* ge/ml in UHT whole milk. In addition, the ICC-PCR assay was applied in a multiple-well format which allowed MPN calculation of the viable numbers of *Coxiella* in milk samples thermally treated at 60 °C for up to 90 min, and which resulted in a 6-log decrease in viable cells. Results also indicate a 3 log MPN/ml inactivation with a 40 min treatment at 60 °C. However, there are also indications that inactivation may not be linear due to a lack of measureable inactivation in samples subjected to a 20 min heat treatment. Overall, the utility of the ICC-PCR method for characterising thermal inactivation of *Coxiella* in milk products will need to be investigated further.

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