APPENDIX 1

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Hazard Identification

Q fever is a zoonotic widespread disease caused by the bacterium *Coxiella burnetii* and is present in cattle, sheep and goats. *It* is an emerging disease that can cause considerable morbidity and serious long-term complications in humans. The main route of transmission to humans is via the inhalation of aerosols from the parturient (birth) products of infected animals. It is known that meat, milk and milk products can be contaminated, however, the link to clinical disease in humans is unclear (EFSA, 2010). Risk assessment is an important tool in assessing the risk posed by food borne pathogens and contaminants. Therefore within project FS101016, a risk assessment is being developed to assess the risk to human health from consumption of unpasteurised milk and milk products contaminated with *C. burnetii* in the UK.

As part of the risk assessment, a hazard identification has been undertaken. As part of this process a literature review has been undertaken to become familiar with the bacteria *C. burnetii* and to evaluate the presence of *C. burnetii* within the food chain of interest and the implications it has on human health.

1. The organism *C. burnetii*

C. burnetii has been considered a prototypical obligate intracellular bacterium that relies exclusively on a eukaryotic cell for growth. Intracellularly, the organism prospers in an acidified, phagolysosome-like vacuole. *C. burnetii* has evolved to replicate in this harsh environment by a mechanism involving acid activation of metabolism (Omsland and Heinzen 2011). However, neutral pH promotes prolonged pathogen viability in the environment while metabolic activation at acidic pH ensures metabolic activity only in a supportive intracellular replication niche. From the literature review, it can be stated that:

- *C. burnetii* does not grow outside the host.
- Acid activation of metabolism occurs
- Unlike other obligate intracellular bacteria, *C burnetii* has spore-like environmental stability

Oyston and Davies (2011) provide useful information on the bacteriology of Q fever. In particular, *Coxiella* has a unique intracellular lifestyle with two distinct morphological forms, the large cell variant (LCV) and the small cell variant (SCV). SCVs are the environmental extracellular form of the organism. They are 0.2 to 0.5 μm long, compact and typically rod-shaped with an electron-dense core bounded by cytoplasmic and outer membranes (McCaul and Williams, 1981). They are metabolically inactive, and show a high degree of resistance to chemical agents and physical conditions, such as osmotic

pressure and sonic disruption (McCaul and Williams, 1981), which confers the ability to survive for prolonged periods in the environment. *C. burnetii* is known to be resistant to physical stresses, such as elevated temperature, desiccation, osmotic shock and UV light, and to chemical stresses, such as disinfectants, which contributes to its stability in the environment. Although areas thought to be contaminated could be assessed for presence of *C. burnetii* DNA by PCR (Kersh *et al*., 2010; Fitzpatrick *et al*., 2010), determining viability would be difficult, and combined with the organism's resistance to chemical disinfectants, assessing decontamination efficacy would be difficult.

The genome of *C. burnetti* ranges from 1.5 to 2.4 Mb and consists of a linear chromosome with a facultative plasmid of between 36- and 42-kb (reviewed in Maurin and Raoult 1999). The genome of *C. burnetii* has been examined and classified by a number of different methods. Hendrix *et al* (1991) used restriction fragment length polymorphism (RFLP) to classify the genome into 6 genomic groups, labelled I to VI. Analysis of *C. burnetii* DNA using NotI and SfiI restriction fragments separated by pulsed-field gel electrophoresis (PFGE) identified four different fragment patterns representing isolates from genomic groups I, IV, V and VI (Heinzen *et al*, 1990). Thiele *et al* (1993) identified 16 additional restriction groups from 80 isolates collected worldwide using PFGE and NotI digestion. The wide range of restriction site distribution is thought to be due to significant chromosomal rearrangements, such as translocation, insertions and deletions (Maurin and Raoult 1999). *C. burnetii* contains three main plasmid types, QpH1 (36kb, one to three plasmids per cell), QpRS (39 kb) and QpDG (42 kb), which also contribute to the heterogeneity of the genome. The plasmids tend to be associated with different genomic groups. QpH1 is associated primarily with genomic groups I, II and III, QpRS with genomic group IV and QpDG with genomic group VI isolates (Maurin and Raoult 1999). QpRS plasmid has also been found to be inserted into the main chromosome in isolates from Q fever endocarditis patients (Savinelli and Mallavia, 1990). A recent microarray-based whole genome comparison method has identified a further two genomic groups (VII and VIII), to make 8 genomic groups, with 36 genotypes within the groups (Beare *et al* 2006). Glasunova *et al* (2005) used a multispacer sequence typing (MST) method to characterise 30 genotypes from 173 isolates from different locations and host species. They found that, despite genetic variability in isolates from the sample geographic regions, they could be grouped into the same monophylogenetic groups, of which 3 were identified, named Antiqua, Medievalis and Orientalis. It was hypothesised that *C. burnetii* emerged in the Old World and then spread to the New World.

2. Q fever infection in humans

There are two forms of Q fever in humans, acute and chronic. Acute Q fever usually manifests as an asymptomatic or mild flu-like disease with spontaneous recovery (Maurin and Raoult, 1999). However, a small minority of patients present with more serious disease which can lead to serious complications and death. These symptoms include pneumonia, hepatitis, meningo-encephalitis and myocarditis. In some people, the disease can lead

to a chronic infection that can manifest years later, even in the absence of primary, acute Q fever symptoms. In these patients, the disease generally manifests as endocarditis, although chronic osteomyelitis, chronic infection of vascular aneurysms or prostheses, lung tumours, pneumonic fibrosis and chronic hepatitis have also been reported (Raoult *et al* 2000). Risk factors for chronic disease include cardiac valve defects and vascular disease, immunocompromised hosts and pregnancy. It is generally believed that the incidence of Q fever is under-reported, mainly due to asymptomatic and mild disease and also because the range of symptoms are variable that a degree of clinical suspicion is required for a diagnosis to be made (Maurin and Raoult 1999). An epidemiological study of Q fever in the UK estimates that only 50 of approx. 700 cases of Q fever pneumonia are reported to the reference centres each year (Pebody *et al* 1996). Of the recorded Q fever cases in the UK and in France (Pebody *et al* 1996, Raoult *et al* 2000), males of working age (18-65) tend to be more frequently diagnosed, most probably due to occupational exposure on farms or in rural and agricultural settings. The main route of exposure to Q fever is by aerosol; ingestion of high doses of *C. burnetii* through consumption of contaminated dairy products is considered to be a rare alternative for acquiring the disease (Fishbein and Raoult 1992).

3. Epidemiological information relating to unpasteurised milk/milk products causing human infection

A literature review was undertaken in order to try and ascertain the importance of unpasteurised milk/milk products as a foodborne route for human Q fever infection. The relevant studies are summarised below:

3.1 Epidemiological investigations of outbreaks

Signs *et al.* (2012) report on evidence of infection with *C. burnetii* in a small group of regular consumers of raw (unpasteurised) milk from the same dairy in Michigan (USA). Between 9 May 2011 and 22 August 2011, five individuals were reported with serologic testing suggesting acute Q fever. All five regularly consumed raw milk from the same dairy for at least one year. Case 1 had not visited the farm in 9 months, and case 5 had never visited the farm, making raw milk consumption the only plausible route of exposure in these two cases.

Fishbein and Raoult (1992) reported on an outbreak of Q fever among patients and staff of a psychiatric institution in southern France. Some of the patients and staff left the institution daily to work on a farm where goats were raised for raw milk and cheese production. Seropositive persons were no more likely to have worked on the farm than seronegative ones. Similarly seropositive persons were no more likely to have reported that they consumed unpasteurised dairy products than seronegative ones. However, seropositive persons were significantly more likely ($P = 0.003$) than seronegative persons to have worked on the farm or consumed unpasteurised milk products or both. According to Fishbein and Raoult (1992) some of the seropositive persons had close contact with goats, although the only identifiable exposure of others was the consumption of unpasteurised dairy

products. However, the association of seropositivity with exposure to the goats and raw milk products could be confirmed statistically only when these two factors were combined. It is interesting to note that the goats had been vaccinated annually against a *C. burnetii* antigen. Shedding of *C. burnetii* was subsequently documented in the milk of two of the three goats examined. Furthermore, although the study of Fishbein and Raoult (1992) incriminated unpasteurised dairy products, persons exposed to infected raw milk were less likely to report an associated illness than those who worked with the infected animals. It should be noted that vaccination of that herd (in 1986/87) did not prevent the outbreak and might have increased shedding of *C. burnetii* in the dairy products.

Brown *et al* (1968) reported on an outbreak of Q fever in a boys' detention centre in Staffordshire in April 1967. In total, 24 boys and 5 staff were infected. The clinical symptoms included high temperature associated with shivering, sweating, backache, sore throat, headache, chest pains and neck stiffness. One boy displayed symptoms of pneumonia. Serological evidence of Q fever was gained from 19 boys and 5 staff. The centre housed a farm with cattle. Whilst some boys at the centre were involved in agriculture and animal husbandry practices at the farm, none of the infected boys or staff had any contact with the farm. Raw milk from the farm was consumed with cornflakes once or twice a week and the staff took raw milk in their tea. Epidemiological investigations at the farm showed that two out of 20 cows were actively shedding *C. burnetii* and three other cows had antibody titres of >1/10 in their serum. Neither the farmer, his wife nor two farm hands had antibodies or drank raw milk. In this instance, it was concluded that infection from the raw milk was responsible for the outbreak.

3.2 Other epidemiological investigations

Raoult *et al* (2000) have studied 1,383 confirmed Q fever cases in France from 1985-1998. In 477 cases of acute Q fever, risk factors were analysed and 23% (85/366) of these cases were found to have consumed unpasteurised farm goat's cheese. Of these cases, 16 patients had an isolated fever, 40 had hepatitis, 15 had pneumonia and 11 had hepatitis and pneumonia. 26 patients, who had reported consuming raw milk cheese, were diagnosed with endocarditis. Another epidemiological study of Q fever cases in the UK from 1984-1994 has reported that, out of 1117 cases of Q fever investigated in the study, three were reported to have drunk unpasteurised milk (Pebody *et al* 1996). Differences in clinical presentation of disease are thought to be due to the route of infection. Hepatitis manifestations are reported more frequently from Ontario, in Andalusia, Spain and in California, US and it has been speculated that this is due to ingestion of contaminated food, especially raw milk (Raoult *et al*, 2000, Marrie *et al*, 1996).

Marmion and Harvey (1956) conducted an epidemiological study of two towns in Northern Kent following an outbreak of Q fever in a London Hospital in 1950. Milk from one patient, who resided in one of the towns, was shown to be contaminated with *C. burnetii* and was thought to be the cause of the initial outbreak. The epidemiological study of the two towns looked at residents who had reported symptoms of pneumonia or undiagnosed fever between 1948

and 1954. A total of 22 patients from a group of 160 patients identified from medical records had an antibody titre of 1/40 or more to *C. burnetii* by complement fixation (CF) assay. A study of healthy blood donors in the area recruited through the local National Blood Transfusion Service identified two of 240 cases with CF antibody titres of 1/40. Data from the length of residence in the two towns on the liability of infection suggested that Q fever infection was more common amongst new arrivals to the town (>10 years) than in older residents. Of the cases with serological evidence of Q fever infection, one had occupational contact with potential sources of infection, none had residence within 200 yards of a potential source of infection, 5 had visited a potential source of infection (local farm) and 21 (91%) had consumed raw milk. Evidence of Q fever infections was found in the herds at 2 farms in the nearby vicinity of the towns. From 16 dairies supplying raw and pasteurised milk to the towns, five supplied milk to all those infected with Q fever. Of these, two dairies supplied milk to 13 of the 23 overall cases and were supplied milk from farms known to contain *C. burnetti* infected herds.

3.3 Human trials and animal model studies

A number of trials with human volunteers have been conducted to determine the link between infected milk and food products with Q fever infection have been conducted. Krumbieoel and Wisniewski (1970) gave 34 volunteers raw milk that was naturally infected with Q fever over a period of 1 month. The volunteers consumed an average of 4.5 litres of milk under supervision during the month of the trial. The volunteers were followed up for evidence of seroconversion and clinical symptoms. None of the volunteers developed any clinical symptoms even after 12 years. Serum samples were taken 1 month and 2 months after initial ingestion for signs of seroconversion. No detectable antibodies were found in the volunteers by complement fixation test, capillary agglutination test or radioactive precipitin test. The authors concluded that either the milk may have contained a strain that is not infectious to humans or that an inapparent infection without serological response had occurred, which is a phenomenon seen in animals. Another reported trial in Portugal (Fonseca *et al*, 1949) gave Q fever infected food to 11 volunteers. Of these volunteers, only 2 showed signs of seroconversion by complement fixation assay and none developed clinical symptoms. In contrast all 29 persons infected intradermally developed signs of disease. However, only two of 10 volunteers inoculated intranasally were infected, although it is not clear whether volunteers received the same doses as those exposed through the oral route. The authors concluded that Q fever is not readily transmitted through infected food products, except when the strain is very virulent.

Animal model studies of the virulence of different genomic groups in immunocompetent and immunocompromised animals have shown that all genomic groups tested can produce disease in immunocompromised animals, whilst producing either asymptomatic or mild disease in immunocompetent animals (Russell-Lodrigue *et al* 2009, Stein *et al* 2005). This suggests that host factors are an important indicator of disease severity. Both groups noticed genomic group-consistent trends in disease. Genomic group I led to severe acute disease in a guinea pig model, mild to moderate disease with genomic group V isolates and no acute disease with genomic groups IV and

VI after aerosol challenge (Russell-Lodrigue *et al* 2009). All the genomic groups led to disease in a SCID mouse model and led to genomic group-consistent changes in cytokine levels in Balb/c mice, although none of these mice displayed clinical symptoms. Stein *et al* (2005) found that the Nine Mile strain, a genomic group I isolate with a QpH1 plasmid was more virulent in SCID mice than strain Q212, containing QpRS plasmid integrated into the chromosome (associated with genomic groups IV and V, and isolated from a case of chronic Q fever). Differences in the histopathology and dissemination of the organism were found between the two strains, suggesting that strain Q212 was much less virulent than Nine Mile strain. The authors hypothesised that there are unique pathogenic factors present in different strains of *C. burnetii* that may be involved in the various manifestations of acute Q fever in humans.

3.4 Information on genotypes

It is generally reported that the clinical course of infection may be strain related, with some genotypes giving rise to more serious infections than others (Maurin and Raoult, 1999, Samuel *et al* 1985, Stein *et al* 2005). A study of genotype variability in Spain was conducted by Jado *et al* (2012). They developed a PCR based method to amplify 8 targets to identify 8 genomic groups and 16 genotypes within these groups. This method was used to classify isolates from cases of acute and chronic Q fever, and domestic and wild animals and ticks. The study found that strains containing the gene adaA were most likely to cause acute disease with pneumonia being the most common manifestation. Strains isolated from chronic cases and cases of Q fever hepatitis, which were more common in the south of the country and in the Canary Islands were more likely to be adaA negative, indicating that the clinical outcome of infection is dependent on the genotype involved in infection. The genotypes isolated from chronic disease in humans were also more likely to belong to genome group IV, with other isolates in this genotype being detected in goats, sheep, rats, wild boar and ticks. Isolates of genomic group VII were found in ticks and three cases out of 10 with fever with hepatitis in humans, suggesting that ticks could be a source of transmission of Q fever. None of the isolates from domestic animals in this study were of genomic group VII. Genomic group III was only detected in isolates from cattle collected from 7 samples in 4 different regions, whilst no human samples were of this genomic group. However, a number of groups have reported that genomic groups I and III are phylogenetically similar and they are grouped together in phylogenetic trees (Beare *et al* 2006, Arricau-Bouvery *et al* 2006).

Genotyping of isolates from goats and from acute and chronic cases in humans from the Dutch Q fever outbreak 2007-2010 by MVLA showed that all the isolates were descended from one predominant MVLA genotype, genotype G, indicating a clonal spread of *C. burnetii* across the country (Tilberg *et al* 2012). The sudden increase in human cases was hypothesised to be due either to the expansion of goat farming in the Netherlands in the previous two decades or to a hypervirulent lineage that was disseminating much more rapidly than other genotypes.

Genotyping of the *C. burnetii* DNA in commercial semi-skimmed milk from cows shows this is the result of the presence of a predominant genotype in the dairy cattle population (Tilburg *et al* 2012). Tilburg *et al* (2012) suggest a possible clonal spread of *C. burnetii* among the European dairy cattle population. This dominant genotype is only incidentally found in humans so risk to humans from exposure to infected cattle may be much lower than via exposure to infected small ruminants. It is concluded that the risk assessment needs to consider which genotype of *C. burnetii,* although there may be other minority genotypes in cattle that may be relevant to humans after all (Tilburg *et al*. 2012*).*

Reichel *et al* (2012) used multilocus variable-number trandem-repeat analysis (MLVA) for genotyping the C. *burnetii* in goat samples in the UK. The genotypes were different from the major genotype involved in the recent outbreak in the Netherlands of 2007-2011. The implications of this for risks to humans are not clear.

4. Occurrence of the infection in livestock

Accurate surveillance data on prevalence of Q fever in livestock in the UK is lacking, although it is considered endemic in domestic animal populations (Defra 2008; Paiba *et al*. 1999). According to ACDP (2007), "Although *C. burnetii* is endemic in the UK, the prevalence in UK sheep and cattle herds is not accurately known but has been estimated in England, to range from ~20% in dairy herds and between 1.6%-6.3% in sheep".

Rodolakis *et al*. (2007) compared the shedding of *C. burnetii* in milk of dairy bovine, caprine and ovine herds. Cows and goats excreted the bacteria mainly in milk while ewes shed mostly in faeces and vaginal mucus. The shedding in milk was sometimes intermittent and several animals shed the bacteria but were negative by ELISA. Shedding of *C. burnetii* differs among ruminant species, milk being the primary route in cattle (Rodolakis *et al*. 2007). Sheep shed mainly in the faeces and vaginal mucus and to a lesser extent in milk.

4.1 Cattle

There have been few studies of *C. burnetii* prevalence in livestock in the UK (Valergakis *et al*. 2012). Paiba *et al*. (1999) investigating cows' BMT (bulk milk tank) from England and Wales reported a herd prevalence of 21%. McCaughey *et al*. (2009) reported a Northern Ireland cattle herd seroprevalence of 48.4%. Of 5182 cattle tested by ELISA from 273 herds in Northern Ireland, 6.2% of animals and 48.4% of herds were seropositive (McCaughey *et al*. 2010). Dairy cattle in south-west England at least have very high overall herd prevalence (69.7% Valergakis *et al*. 2012) by PCR.

In cattle, *C. burnetii* colonises the mammary glands and can be excreted in milk for months after infection (see Loftis *et al*. 2010). Ryan *et al*. (2011) reported that 37.9% of BMT (cattle) herds (n = 290) in the Republic of Ireland were antibody positive. Herds with positive milk samples were randomly

distributed in Ireland and no clustering was observed. Modelling of Q fever spread within dairy cattle herds has shown that antibody status seems to be a key point in the bacterial spread; thus shedders with antibodies shed for a longer period of time than shedders without antibodies (Courcoul *et al*. 2010).

Astobiza *et al*. (2012) studied BMT from dairy cattle herds from a Q fever-endemic region in northern Spain. BMT from 67% of herds was positive as determined by the presence of anti-*C burnetii* antibodies. Animals from herds with positive BMT samples had a mean seroprevalence of 8.9% compared to 2.5% in negative BMT samples. The proportion of herds positive by BMT PCR was 51.7%.

Guatteo *et al*. (2012) reported concomitant shedding via both milk and vaginal mucus in 46% of detected shedder cows 14 and 28 days after abortion. In the study of Guatteo *et al*. (2012) 21% of the cows were persistent shedders (regardless of shedding route) from day of abortion to day 28. Those authors hypothesised that persistent shedding in vaginal mucus is related to abortion when bacterial loads are high. Similarly, cows exhibiting a high bacterial load have been demonstrated to be at risk of persistent shedding in milk (Guatteo *et al*. 2007a). An increased estimated titre in *C. burnetii* in positive BMT reflected a higher proportion of heavy milk-shedder cows among the milk-shedder cows (Guatteo *et al*. 2007b). Guatteo *et al*. (2007a) give titre estimates for milk and mucus in shedding cows. Titres (determined by PCR and comparison with solutions with a known *C. burnetii* concentration by serial dilution) vary according to the shedding pattern (e.g. persistent shedding, intermittent shedding) with median values of 3.34 $\log_{10}$/ml of milk for persistent shedding cattle and a maximum of 5.33 $\log_{10}$/ml of milk (n = 98 cows). The viability of the titres is not known.

4.2 Sheep

A super-spreading ewe was linked to 299 reported Q fever cases in humans in Germany (Porten *et al*. 2006). Proximity to and stopping for at least a few seconds at the sheep's pen were among the risk factors. The ewe as well as 25% of its flock were seropositive for *C. burnetii*.

McCaughey *et al* (2010) tested 1,022 sheep from 58 flocks in Northern Ireland and found that 12.3% of animals and 62.1% of flocks were seropositive by ELISA.

Lambton *et al*. (unpublished) tested sheep sera collected in 2008 and reported 53 sheep in 37 flocks tested positive for Q fever by ELISA. Estimates of animal, between-flock and within flock prevalence were 1.04%, 10.2% and 10.2% respectively for sheep. Likelihood of an animal testing positive for Q fever antibodies increased with total number of sheep in the flock and number of breeding ewes in the flock.

4.3 Goat

The seroprevalence in goat flocks in Northern Ireland by ELISA was 42.9% (McCaughey *et al*. 2010).

Lambton *et al*. (unpublished) tested goat sera collected in 2008 and reported four goats in four herds, tested positive for Q fever by ELISA. Estimates of animal, between-flock and within flock prevalence were 0.887%, 2.97% and 29.9% respectively for goats. There was a tendency for the likelihood of an animal testing positive to increase with goat density.

Farm prevalence in goats in the Netherlands was 43.1% and overall goat seroprevalence was 21.4% (Schimmer *et al* 2011). Van den Brom *et al*. (2012) report Q fever prevalences in Dutch dairy goat and dairy sheep bulk milk tank (BMT) samples. In 2008, 32.9% of BMT samples tested from farms with more than 200 dairy goats were positive by PCR and 29.8% were positive by ELISA. BMT -positive farms had significantly higher within heard seroprevalences than BMT-negative farms. The higher percentage of BMT-positive farms in the area where the human Q fever outbreak started in the Netherlands in 2007 is consistent with the suspected relation between human cases and infected dairy goat farms.

Rousset *et al*. (2009) reported that *C. burnetii* was shed into vaginal mucus, faeces and milk of 44%, 21% and 38%, respectively, of goats that aborted and 27%, 20% and 31%, respectively of goats that delivered normally, although the differences between the two groups were not statistically significant. Rousset *et al*. (2009) also reported two opposite shedding trends for milk and vaginal-faecal routes. Thus unlike positive milk shedding, positive vaginal and faecal shedding were observed more often in animals that were weakly positive or negative by ELISA or indirect immunofluorescence assay.

4.4 Microbial load of *C. burnetii* in milk

In a microbiological risk assessment, both the probability of the hazard being present and the microbial load are considered. To date, no published paper has been found giving counts of viable *C. burnetii* per ml of milk or cheese. The PCR method has been used to estimate titres in cows' milk by Guatteo *et al* (2007a). Titres were determined by comparison of PCR results with those from solutions with a known *C. burnetii* concentration obtained by serial dilution of an external positive control. Guatteo *et al*. (2007a) give titre estimates for milk and mucus in shedding cows. Titres vary according to the shedding pattern (e.g. persistent shedding, intermittent shedding) with median values of 3.34 $\log_{10}$/ml of milk for persistent shedding cattle and a maximum of 5.33 $\log_{10}$/ml of milk (n = 98 cows). The viability of the titres is not known. Viability (as yes or no) for some PCR positive raw cheese samples (Hirai *et al*. 2012) and raw milk samples (Loftis *et al.* 2010) has been tested in mice, but this is not enough to give quantitative estimates of viable titres.

5. Contamination of unpasteurised milk/milk products

DNA of *C. burnetii* was detected in >90% of bulk milk tanks in a study in the USA, demonstrating the ubiquity of this pathogen in US dairy herds (Kim *et al*. 2005; Loftis *et al*. 2010). Much of that bulk tank milk is pasteurised and the DNA may not represent viable pathogen. Loftis *et al*. (2010) therefore looked

at the raw milk in the USA, and found that 42.9% of purchased raw milk samples were PCR positive for *C. burnetii*, and 2 of the 6 samples infected live mice confirming the pathogenic potential. Specifically Loftis *et al*. (2010) purchased 21 unpasteurised milk samples from 20 different dairies in 12 US states. These included 7 goat milk dairies and 13 cattle milk dairies. Of the 21 raw milk samples, 9 (42.9%) contained DNA from *C. burnetii*, including 2 samples of cow milk purchased from the same dairy about one month apart. One of 7 samples of goat milk (14.3%) and 8 of 14 (57.1%) samples of cow milk tested positive. Isolation of the pathogen in mice was attempted for six positive samples. Two of four mice inoculated with one sample and four of five mice inoculated with another sample became infected with *C. burnetii* which was successfully passed onto naïve mice. Two other milk samples may have caused infection in live mice as small amounts of *C. burnetii* DNA were found in the spleen. Other bacterial infections may have prevented isolation. These results confirm the viability of *C. burnetii* in commercially sold milk.

Hirai *et al*. (2012) developed a method for *C. burnetii* in cheese with a detection limit of 600 bacteria per gram. 28 of 147 cheese samples from commercial markets in Japan were positive. However, samples from the seven unpasteurised milk cheeses which were PCR-positive were non-viable in mice.

Tilburg *et al*. (2012) report that real time PCR shows the widespread presence of *C burnetii* DNA in a broad range of commercially available milk and milk products. Indeed Tilburg *et al*. (2012) reported that 88 of 116 (76%) of commercially available milk samples (typically semi-skimmed milk from cows obtained from large supermarket chains) contained significant amounts of *C. burnetii* DNA. The study sampled milks for 28 countries including the UK for which one sample of semi-skimmed milk was PCR-positive. The study also found *C. burnetii* DNA in coffee creamers and milk powder.

6. Survival and growth of *Coxiella burnetii* in the manufacture, processing and storage or milk products

As described in Section 1, *Coxiella* has a unique intracellular lifestyle with two distinct morphological forms, the large cell variant (LCV) and the small cell variant (SCV). It is not known which form (LCV or SCV) is mainly present in milk and processed milk products (Brandwagt 2012). Also , the total number of of *C. burnetii* in unprocessed milk is unknown (Brandwagt 2012).

ACDP (2007) write that the small cell variants (they refer to them as small dense cells, SDC) are believed to survive extracellularly as infectious particles for ~150 days although they don't give a reference.

Hirai *et al*. (2012) note that the low detection of *C. burnetii* in cheese samples compared to milk are consistent with the reduced or no growth of bacteria in cheeses.

C. burnetii is inactivated by pasteurisation of commercial milk (Tsurumi 2003; Cerf and Condron 2006). Indeed, pasteurisation standards today are based on the destruction of *C. burnetii* (Holsinger *et al*. 1997).

There are good data for the effect of pasteurisation of milk (61.7°C to 72.2°C) on inactivation of *C. burnetii* as detected by infection of guinea pigs (Enright *et al*. 1957). However, there is evidence that the fats and sugars in milk products like cream, chocolate and ice cream mixes may promote survival, such that the pasteurisation conditions defined for whole raw milk are inadequate (Enright 1961). Indeed Enright (1961) recommended that the pasteurisation temperature be increased to kill *C. burnetii* in these products. Indeed if the fat content of the milk product is >10%, or the total solids >18%, or if sweetners are added, then the specified temperature should be increased by 3°C (Grade A Pasteurised Milk Ordinance, Revision 2009). Kukreja (2011) notes that the composition of the milk products have changed significantly since the 1950s and new products have been launched underpinning the need for development of better detection methods for viable organism in milk products. Data on inactivation by heat treatment of *C. burnetii* in cheese making are lacking (EFSA 2010). However, pasteurisation at 161°F for 15 seconds in cream and chocolate milk is inadequate (EFSA 2010). According to Robert Heinzen (pers comm), much of the data on *Coxiella* survival under various conditions is quite old. The table on resistance from Williams (1991) is about the extent of our current knowledge (see Table 1). RIVM in the Netherlands have no information on survival of *Coxiella* in milk or cheese (Wim van der Hoek, pers comms. 28 Jan 2013). Robert Heinzen's guess is that *Coxiella* retains better viability in cheese at neutral pH than at pH 5.0. This is based on their experience of freezing *Coxiella* in acidic media, and also the information on the table showing poor viability in soured milk but viability in cheese for 30-40 days. Robert Heinzen's recommendation was, given the lack of knowledge, we do our own testing.

Jellison *et al*. (1948) reported the presence and persistence of infectious *C. burnetii* in butter made from naturally infected milk. The method was seroconversion of guinea pigs injected intraperitoneally with the butter or milk. Refrigerated butter stored at below freezing temperature was still infectious to guinea pigs 41 days after preparation. Sera from four guinea pigs injected with freshly churned butter made from naturally infected raw milk were positive at high titre. These survival experiments give no indication of the decay rate.

Data from Hirai *et al*. (2012) using mice studies suggest that viability of *C. burnetii* was lost in PCR-positive unpasteurised cheese samples.

Sipka (1958) used cow's milk infected with *Coxiella burnetii* which in dilutions of 1:100 was still pathogenic for guinea pigs, for the preparation of a cottage-type cheese, which retained its infectivity for guinea pigs for a period of observation of 42 days. Survival of *C. burnetii* in milk and cheese was demonstrated by complement fixation with guineapig blood.

TABLE 2
Resistance of *Coxiella burnetii* Under Various Conditions

Year	Experimental conditions	Temperature	Survival period or result	Ref.
1938	In leptospira media (cell-free)	28°C	109 d or >	38
1940	"Lyophilized" guinea pig spleen	—	5 months or >	39
1943	In the tick, *Ornithodorus hermsi*	—	979 d	40
1948	Tick feces	Room temp.	586 d or >	41
1949	Dried guinea pig blood	Room temp.	182 d or >	42
	Dried guinea pig urine	Room temp.	49 d	
1948	Butter made from unpasteurized cream	—	41 d	43
1949	Infected milk: 30 min at	71.5°C	Killed	44
	15 min at	61.5°C	Some survive	
1952	Infected milk: 30 min at	61.5°C	Some survive	45
	17 min at	74°C	Some survive	
1952	Infected milk: 30 min at	63°C	Some survive	46
	1 min at	80°C	Killed	
1956	Infected milk: 30 min at	61.7°C	Some survived	47
	30 min at	62.8°C	Killed	
	15 s at	71.7°C	Killed	
1951	Infected milk: In constant temperature	63°C	30 min	48
	water bath	65°C	<30 min	
1953	Infected milk stored aseptically	—	45 d or >	49
	Infected milk allowed to sour	—	24 h	
1953	In dried cheese made from infected milk	—	30 to 40 d	50
1956	*Coxiella burnetii* in water	20—22°C	160 d or >	51
1957	*Coxiella burnetii* in 10% salt solution	Room temp.	180 d	52
		4°C	270 d	
	Tissues from guinea pig sacrificed			
	at height of fever: Unsalted in glass	4°C	30 d or >	
	In 10% salt solution	4°C	150 d or >	

Table 1: *C. burnetii* resistance from Williams (1991)

C. burnetii survived in milk (dried 37°C) for 30 – 60 days and in cheese made from infected milk for 17 – 46 days (Babudieri and Moscovici 1950). *C. burnetii* survived in sterile milk at room temperature for 125 days (Zubkova 1957). Babudieri (1953) note that they have found that *Coxiella* in cheese is no longer a source of infection after 46 days presumably referring back to the Babudieri and Moscovici (1950) study.

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