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Occurrence of *Coxiella burnetii* DNA in bulk tank milk samples in Switzerland

Vorkommen von Coxiella burnetii DNA in Tankmilchproben schweizerischer Herkunft

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Summary

From May to November 2007, 872 bulk tank milk samples representing farms from almost the entire territory of Switzerland were collected and analysed by quantitative PCR for the presence of *Coxiella burnetii* DNA. Quantification of *C. burnetii* DNA revealed values that ranged from <10 to $<10^5$ counts per ml of bulk milk (Highest count: 24 256 cells per ml). The samples originated from three companies (A, B and C) that process milk from different regions of the country. In 255 of 872 samples (29.2 %) counts of $\geq 10^2$ per ml of milk were measured and in 89 of 872 (10.2 %) $\geq 10^3$ counts per ml. Furthermore, variations in contamination frequencies between the samples of the three companies indicated that geographic differences might exist with a certain trend to higher detection rates in the western part of Switzerland. Based on the obtained data, possible implications for food safety were discussed.

Keywords: Real time PCR, risk, epidemiology

Zusammenfassung

Von Mai bis November 2007 wurden 872 Tankmilchproben von Bauernhöfen aus nahezu der ganzen Schweiz mittels quantitativer PCR auf das Vorkommen von *Coxiella burnetii* DNA untersucht. Die Quantifizierung von *C. burnetii* DNA ergab Werte im Bereich von <10 to $<10^5$ Zellen pro ml Tankmilch (Höchster Wert: 24 256 cells per ml). Die Proben stammten von drei Unternehmen (A, B und C), welche Milch aus verschiedenen Regionen des Landes verarbeiten. In 255 von 872 Proben (29.2 %) wurden Keimzahlen von $\geq 10^2$ pro ml Milch und in 89 von 872 (10.2 %) solche von $\geq 10^3$ pro ml ermittelt. Im weiteren ergaben Unterschiede zwischen den Kontaminationsfrequenzen für Proben der drei in die Studie einbezogenen Firmen einen Hinweis auf mögliche geographische Unterschiede mit einem gewissen Trend zu höheren Kontaminationsraten im westlichen Landesteil. Abgestützt auf die erarbeiteten Daten wurden möglich Implikationen für die Lebensmittelsicherheit diskutiert.

Schlüsselwörter: Real time PCR, Risiko, Epidemiologie

Introduction

Coxiella burnetii, the agent of the zoonotic disease Q-fever, can cause infections in humans and in a wide range of animals including farm animals. Although, according to the reported cases, the clinical impact in both human and veterinary medicine is rather low, the presence of *C. burnetii* in some reservoirs seems to be significant. A previous study in Switzerland found 8 out of 27 (29.6 %) of milk cow farms positive for *C. burnetii* DNA (Fretz et al., 2007). Based on these findings, it was hypothesized that the presence of *Coxiella burnetii* might be fairly high in herds of Swiss dairy cows. Because our first study included only a small sample number from a specific geographic region and because conclusive data on the prevalence of *C. burnetii* in dairy cows are only scarcely available from other countries, we decided to launch an extensive screening of bulk tank milk samples covering almost the entire territory of Switzerland, using a method which allowed us to quantify *Coxiella*-contaminations. The obtained data will be useful in future risk assessments that are considered to be necessary as stipulated in a recent scientific opinion of the European Food Safety Authority (EFSA, 2010).

Materials and Methods

Bulk tank milk samples

A total of 872 bulk tank milk samples, each representing an individual farm, were made available for testing from May to November 2007 by three milk processing companies (A: 445, B: 200 and C: 227 samples) which covered almost the entire territory of Switzerland in the North of the Alps. The milk samples, approximately 50 ml per farm, were obtained either manually or by an approved inline sampler at farm pick-up following the standard procedures published by Swisslab, the official Swiss laboratory for milk quality control. After arrival at the laboratory, the samples were split and aliquots of about 10 ml were immediately deep frozen at –20 °C until further processing. For DNA extraction, tubes were transferred and held at room temperature until the content was thawed. Subsequently, the tubes were incubated for 20 min at 40 °C in a water bath and then mixed on a roller mixer (Stuart SRT9) at room temperature until further processing.

DNA extraction

DNA extraction was performed as described in the user manual of the LSI TaqVET *Coxiella burnetii* kit (Laboratorie Service International, 69380 Lissieu, France). According to these instructions, DNA was directly isolated from 200 µl of milk with the QIAmp DNA mini kit (Qiagen, Switzerland, cat. no. 51304) and kept frozen in 200 µl of kit elution buffer until used. An extraction series included 28 samples of bulk tank milk. The kit contained a standard (10^4 *C. burnetii* cells per ml of a reference strain from INRA, France) to set up the calibration curve needed for qPCR. From this standard, 200 µl were extracted in the same way as described for the milk samples.

In addition to the procedure given by the instructions for the PCR kit, an "extraction negative control" was integrated in each extraction series which consisted of a 200 µl aliquot of raw milk from an individual farm. Previously, ten extractions of DNA from this milk were shown to be free of *Coxiella burnetii* with the LSI Taq-Vet kit. The aliquots were kept at –20 °C and extracted in the same way as the test samples. Furthermore, an "extraction positive control" was processed. For that purpose, 20 ml of UHT-milk from retail was spiked with heat inactivated cells of the *Coxiella burnetii* strain Nine Mile RSA493 (Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig University, Giessen, Germany). UHT-milk was used for spiking because it is far more homogenous than raw milk. The concentration of *C. burnetii* in the spiked milk was calculated to be 5.58 x 10^3 cells per ml by analysis with the LSI Taq-Vet kit. Aliquots of 200 µl were kept at –20 °C until used. The DNA extraction procedure was the same as described above.

qPCR assay

Quantitative PCR was performed with the commercial LSI Taq-Vet *Coxiella burnetii* test kit which is a duplex PCR targeting the repetitive transposon-like region of *Coxiella burnetii* (Guatteo et al., 2007) and, as internal positive control, the endogenous gene GAPDH. The ready-to-use reagent mix contained for both targets forward primers, reverse primers and TaqMan® probes labelled in FAM-TAMRA for *Coxiella burnetii* and FAM-VIC for the internal positive control. The TaqMan PCR was assayed using the 7500 Real Time PCR system (Applied Biosystems, Switzerland). A 25 µl reaction volume consisted of 20 µl kit mix and 5 µl template DNA. The standard amplification protocol was 50 °C for 2 min followed by 95 °C for 10 min and subsequently 40 cycles of amplification (95 °C for 15 sec; 60 °C for 60 sec).

Each PCR run included DNA from three extraction series, one "PCR positive control" and two "PCR negative controls". For the "PCR positive control", DNA from an "extraction positive control" was aliquoted in portions of 7 µl kept at –20 °C until used. For the "PCR negative control", distilled and nuclease-free water (Sigma 53409145) was used.

The calibration curve needed to quantify the PCR products was generated from extracted DNA from a standard supplied with the kit (10^4 *Coxiella burnetii* cells per ml). Decadic dilutions in water afforded DNA concentrations representing 10^3, 10^2 and 10^1 cells per ml. The data was analysed by applying the default adjustments of the 7500 Taqman software (Version 2.0.3).

Results and Discussion

Performance of the test method

In total, 33 analyses of the "extraction positive controls" were carried out. The calculated mean value was 14 122 cells per ml with a standard deviation of 4070 cells per ml, revealing a relative repeatability standard deviation (RSDr) of 28.8 %. All 33 "extraction negative controls" showed negative results.

The "PCR positive control" was analysed twelve times revealing a mean value of 6323 cells per ml with a standard deviation of 1413 cells per ml. In this case, the RSDr was 22.3 %. Based on the results of the standards measured within the twelve experiments, a mean slope of –3.386 and a respective amplification efficiency of 98 % were calculated. The correlation coefficient of the standard curves (R^2 coefficient) was 0.997. According to a guideline of the European Network of GMO laboratories (ENGL), these data met the acceptance criteria for a valid quantitative PCR test (ENGL, 2009) and the performance of the app-

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lied test kit can therefore be considered to be appropriate. The "PCR negative control" revealed negative signals throughout the screening program. All samples, which were found to be negative for *Coxiella burnetii,* tested positive for the internal positive control of the test kit (GADPH gene). Based on these findings, false negative results due to co-extracted PCR inhibitors could be excluded. Since the kit is targeting a repetitive transposon-like region of the *C. burnetii* genome, a low sensitivity is possible which allows to get PCR signals with counts of <10 *C. burnetii* cells per ml. According to the instructions for the application of the test kit, exact quantification can be expected in the range from 100 to 10'000 counts per ml.

Overall positive rate for bulk tank milk and distribution of *Coxiella* counts

From 872 bulk tank milk samples, 431 (49.4 %) were shown to be positive for *Coxiella burnetii.* The measured *Coxiella*-counts in the 431 positive samples were distributed over a large range of five $\log_{10}$ (Fig. 1). In five samples (1.2 %), counts of $>10^4$ cells per ml were demonstrated. Two samples were from company A (12 115 and 13 606 counts), one from company B (13 161 counts) and two from company C (12 952 and 24 256 counts). The occurrence of high counts of around 10^4 cells per ml, which were found in a part of the examined samples, are in accordance with the observations of Guatteo et al. (2006). These authors tested bulk milk from 14 dairy herds with the same test system and demonstrated estimated *Coxiella* counts of up to 3.5 to 4.0 Log_{10}.

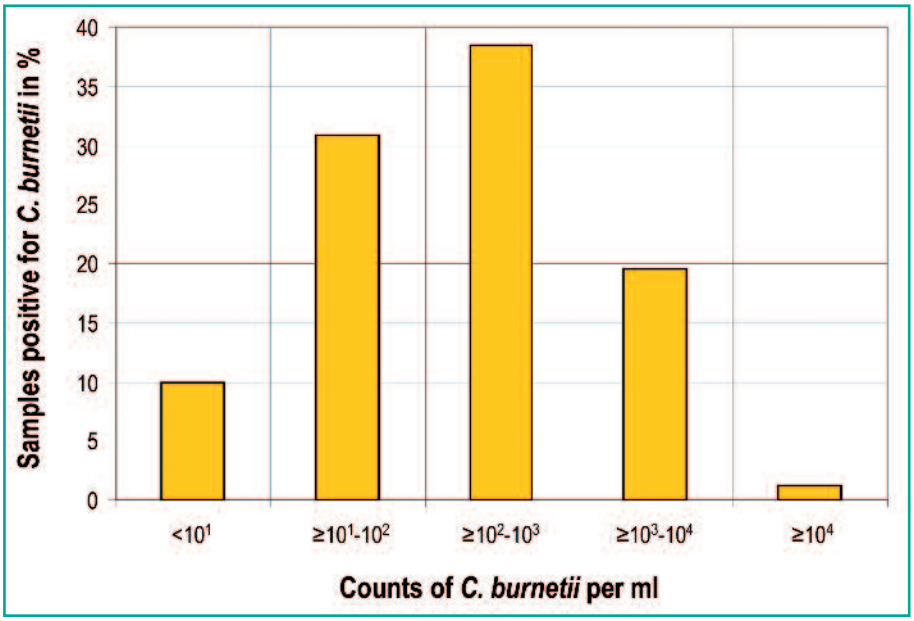

FIGURE 1: *Coxiella burnetii-counts in 431 bulk milk samples measured with quantitative PCR.*

In another study, the same researchers sampled individual cows seven times over three months and observed maximum titers of >5 Log_{10} in milk (Guatteo et al., 2007).

Percentages of positive samples might be overestimated to a certain degree in the categories with low counts of $<10^1$ and $\geq 10^1$–$<10^2$. This is due to the fact that possible cross contamination by the inline sampling device cannot completely be excluded. The possible carry-over varies in dependence of the sampling system and the amount of milk loaded at a single farm. The maximum amount of carry-over, which is tolerated for approved sampling devices, are 1.2 l at 40 l. Generally, well functioning sampling devices have a carry-over of less than 1 %.

The bias due to this possible cross-contamination between consecutive samples has an influence on the estimated overall positive rate. In a former study, a positive rate of 29.6 % was calculated based on data generated with a less sensitive PCR-method and a smaller sample size (Fretz et al., 2007). Since the percentage estimated in the present study is overestimated to a certain degree for the mentioned reason, the true overall positive rate for *C. burnetii* in Swiss tank milk has to be expected between 30 and 50 %. Compared to the results of Kim et al. (2005), it can be concluded that in Switzerland the frequency of *C. burnetii* in bulk tank milk is high but visibly lower than in the U.S. A higher percentage of contaminated bulk tank milk samples were also reported in France by Guatteo et al. (2006) who found 31 of 36 (86.1%) samples positive for *C. burnetii* with real-time PCR.

Our results are in line with a large seroepidemiological study carried out by the biological service of the Swiss army in the years 1981 and 1982. In this study, bulk tank milk samples were tested for antibodies against *C. burnetii* with a capillary agglutination test and 602 of 1634 samples (36.8 %) were found to be positive (Metzler et al., 1983). More data from other countries would be needed to interpret our results in a broader context and to better understand those factors that allow the spread of *C. burnetii* in dairy cows.

Occurrence of *Coxiella burnetii* in samples of different companies

Table 1 displays the quantitative data according to the three companies which supplied milk samples. For company A, *C. burnetii* DNA was demonstrated in 202 of 445 (45.4 %), for company B in 100 of 200 (50.0 %) and for company C in 129 of 227 (56.8 %) samples. The most pronounced difference was manifest between companies A and C, indicating at a first glance geographic variations of contamination frequencies. Samples from company A were collected in the eastern and central cantons of the country (Aargau, Bern, Luzern, Solothurn, Thurgau, Zürich, Zug). Samples from company C were collected mainly in the western and some central cantons (Bern, Fribourg, Jura, Neuchâtel, Schaffhausen, Solothurn, Vaud, Zürich). The existence of geographic variations was observed in a former study, where we found a clear difference in the overall positive rate for *C. burnetii* in milk from farms supplying two cheese factories which were about 20 km air apart the crow flies, a difference which remained constant over an extended period of time (Fretz et al., 2007). By exclusion of the data for low level contaminated samples ($<10^1$ and $\geq 10^1$–$<10^2$ counts per ml), where false positives due to carry-over effects have to be expected, the hypothe-

TABLE 1: *Numbers and percentages of Coxiella burnetii DNA positive bulk tank milk samples from milk producers delivering to three different milk processing companies.*

Company	Number of samples						
	Total	nd	<10[a]	≥10–<10²	≥10²–<10³	≥10³–<10⁴	≥10⁴–<10⁵
A	445	243 (54.6 %)	24 (5.4 %)	65 (14.6 %)	86 (19.3 %)	25 (5.6 %)	2 (0.5 %)
B	200	100 (50.0 %)	10 (5.0 %)	28 (14.0 %)	40 (20.0 %)	21 (10.5 %)	1 (0.5 %)
C	227	98 (43.2 %)	9 (4.0 %)	40 (17.6 %)	40 (17.6 %)	38 (16.7 %)	2 (0.9 %)
A+B+C	872	441 (50.6 %)	43 (4.9 %)	133 (15.3 %)	166 (19.0 %)	84 (9.6 %)	5 (0.6 %)

[a]: Range of *C. burnetii* counts per ml; nd: not detectable.

sis of a certain geographic variation could be maintained. Under this restriction, the positive rate for company A is 25.4 % and for for company C 35.2 %. However, further and extended field test are needed to confirm this observation taking factors into consideration as for example cow races, density of animals or direct and indirect contacts between farms.

Epidemiological impact

Our data show that *C. burnetii* is highly prevalent in Swiss dairy herds over the whole territory and that significant amounts of the agent may enter milk processing facilities such as cheese factories. Nevertheless, the impact in veterinary medicine seems to be rather low regarding the small numbers of reported cases of coxiellosis detected after abortions. From 1991 to 2010, an average of 49 cases per year of bovine coxiellosis was reported to the Swiss Federal Veterinary Office (Swiss Federal Veterinary Office, 2010). On the other hand, 28 014 milk-producing farms were registered in Switzerland in 2008. According to our findings, DNA from *C. burnetii* can be found in bulk tank milk samples in at least a third of them. However, the design of our study did not allow a distinction between viable possibly infective agents and the debris of DNA from non-viable cells. In addition, it was not possible to trace back the origin of DNA in the bulk tank milk samples. But, according to Rodolakis et al. (2007), cows most often release *C. burnetii* into milk. Therefore, it can be assumed that on most farms that tested positive, at least one or several cows harboring *C. burnetii* were present.

The loose relation between clinical cases and the number of animals, where *C. burnetii* has to be expected, clearly illustrates that bovine coxiellosis is a disease of minor importance in Switzerland. Recently, the Swiss Federal Veterinary Authorities downgraded bovine coxiellosis from a disease to be combated to a disease to be monitored (Schweizerischer Bundesrat, 2010). The reasons were that effective therapeutic options for herds with dairy cows that were positive for *C. burnetii* do not really exist and that a full control of the pathogen would only be possible with the application of disproportional measures such as culling of infected animals. This decision to put into perspective the importance of *C. burnetii* in the veterinary legislation is also justified in the light of our findings.

The importance of *C. burnetii* in human medicine seems to be even lower than in the context of dairy cows. Since the annual number of cases of human Q-fever was very low in Switzerland, official reporting of sporadic infections was discontinued in 1999. As can be seen from Table 2, the bovine cases (abortions with subsequent detection of *C. burnetii* in clinical materials) decreased from 1991 to 1998 and so did the human cases. Statistical comparison of the human and bovine coxiellosis case data of the years 1991 to 1998 revealed a significant but weak correlation (p=0.031, Bonferroni probabilities). The question remains open of why the widespread pathogenic agent *C. burnetii* has such a low clinical impact in both the bovine and human population. Airborne transmission by dust contaminated with *C. burnetii* seems to be by far the most important route of infection in humans. In this context, sheep holders are particularly exposed as it was shown in an epidemiological investigation in North Dakota (Guo et al., 1998). In Switzerland, the only verified outbreak of human Q-fever in the past thirty years resulted from sheep that migrated from the Alps to locations in the valley (Dupuis et al., 1987). Another reason could be differences in virulence among the *C. burnetii* strains that circulate in the bovine population. Recent studies indicated that strains of *C. burnetii* with different levels of virulence for humans might occur (Enserink, 2010). A subject of further research will be to test the *C. burnetii* strains in samples found to be positive in the present study with modern typing methods (Arricau-Bouvery et al., 2006). We hope that these analyses will help us to come to a more conclusive epidemiological interpretation of our results. However, already at this stage, it is legitimate to question the epidemiological importance of *C. burnetii* and to consider it more as an agent that rarely causes disease and then, only under particular conditions. This conclusion is also supported by a recent publication of Kersh et al. (2010) who showed for the US that *C. burnetii* is fairly prevalent in the environment and that human exposure may be more common than is suggested by the reported cases of coxiellosis.

There is still an on-going debate concerning contaminated foods as a possible vehicle of transmission of *C. burnetii,* but generally it is believed, as shown by infection trials with humans (Benson et al., 1963) that this way of transmission is not relevant. Additionally, raw milk is not considered as a ready-to-eat food by the current legislation and raw milk drinking might be limited to local consumers at farms. There are no studies available about the survival of *Coxiella burnetii* in raw milk cheese. However, there are a

TABLE 2: *Annually reported cases of bovine and human coxiellosis in Switzerland, 1991–2010.*

Year	1991	92	93	94	95	96	97	98	99	2000	01	02	03	04	05	06	07	08	09	10
Human cases	34	19	10	16	14	13	16	11	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Bovine cases	105	92	58	64	66	45	28	34	37	39	26	26	38	24	30	45	54	57	66	42

NR: Not reported

few data indicating that *C. burnetii* survives not more than 48 h in a sour environment as occurs in kefir or whey (Mitscherlich and Marth, 1984). During the production process of extrahard and hard cheeses from raw milk, a low pH of around 5 is combined with high heating temperatures of the curd (Emmental cheese at least 52 °C and Gruyère cheese 57 °C). It was shown that pathogens such as *Salmonella* Enterica or unwanted toxigenic bacteria such as coagulase positive staphylococci quickly die off under these harsh production conditions (Bachmann and Spahr, 1995). Based on these observations, it is not unrealistic to assume that *C. burnetii* cannot keep its infectivity in such types of cheese. Therefore, we believe that the rare cases of human coxiellosis in Switzerland must rather be linked to aerosols than to contaminated milk or products thereof and that *C. burnetii* in connection with cow milk is not of relevant public health concern.

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