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Pitfalls of molecular diagnostic testing for *Coxiella burnetii* DNA on throat swabs

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ABSTRACT

Introduction: *Coxiella burnetii*, the causative pathogen of Q fever, is regularly detected in throat swabs from patients without serological evidence of Q fever infection. *C. burnetii* is also frequently found in bulk tank milk from dairy cows. We evaluated the false positivity rate of polymerase chain reaction (PCR) for *C. burnetii* DNA on throat swabs and investigated whether recent consumption of *C. burnetii* DNA-positive cow milk could contribute to this phenomenon.

Methods: *C. burnetii* PCR was performed on throat swabs obtained from patients in whom a throat swab was ordered for other diagnostic purposes; patients with community-acquired pneumonia (CAP); and healthy volunteers after consumption of commercial *C. burnetii*-containing cow milk products.

Results: *C. burnetii* DNA was found in 5.0% of throat swabs ordered for other diagnostic purposes and in 15.3% of throat swabs from CAP patients without serological evidence of Q fever pneumonia. The positive and negative predictive value of *C. burnetii* PCR on throat swabs for Q fever pneumonia were 66.7% (95% CI, 38.0–88.2) and 48.9% (95% CI, 41.3–54.6), respectively. After consumption of commercial *C. burnetii*-containing cow milk products, *C. burnetii* DNA could be detected in throat swabs for as long as 30 min after ingestion.

Conclusion: *C. burnetii* PCR on throat swabs is of low diagnostic value for Q fever pneumonia and was false positive in 15.3% of CAP patients without Q fever pneumonia. Recent consumption of *C. burnetii*-containing products can influence the outcome of *C. burnetii* PCR on throat swabs. Therefore, diagnosis of *C. burnetii* infection should be made in combination with serology or PCR performed on blood.

1. Introduction

Q fever is a zoonosis caused by the intracellular bacterium *Coxiella burnetii*. Infection in humans occurs primarily through the inhalation of aerosols. Infection acquired through consumption of *C. burnetii* is considered rare (EFSA, 2010; Gale et al., 2015; Maurin and Raoult, 1999). The main reservoir of *C. burnetii* consists of cattle and small ruminants like goats and sheep. Primary infection with *C. burnetii* infection is in most cases asymptomatic, but can also present as acute Q fever in which case patients develop a flu-like illness, atypical pneumonia or hepatitis (Maurin and Raoult, 1999). Serological diagnosis of acute Q fever is based on detection of phase I and phase II *C. burnetii* antibody titers measured by indirect immunofluorescence assay (IFA). Seroconversion is usually detected from seven to 15 days after the onset of symptoms. Polymerase chain reaction (PCR) for detection of *C. burnetii* DNA in blood samples and various tissues is currently also commonly used for the diagnosis of Q fever. Whether *C. burnetii* PCR on throat swabs is a useful tool in diagnosing acute Q fever remains uncertain, since reports on its diagnostic value vary widely (Angelakis et al., 2014; Meijvis et al., 2011; Takahashi et al., 2004).

From 2007 to 2010, the Netherlands suffered a major Q fever outbreak with > 4000 reported symptomatic cases (van der Hoek et al., 2010). In order to control the outbreak, small ruminants were mandatorily vaccinated and pregnant dairy goats on *C. burnetii*-infected farms were culled. Large ruminants like cows were not vaccinated although *C. burnetii* DNA was found in 56.6% of bulk tank milk samples from dairy cow farms (Muskens et al., 2011). Multilocus variable-number tandem repeat analysis genotyping has shown that *C. burnetii* genotypes found

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in dairy cattle herds and dairy consumer products are distinct from the predominant genotype found in Dutch small ruminant dairy farms during the outbreak (Roest et al., 2011; Roest et al., 2013; Tilburg et al., 2012).

In the wake of the outbreak, we received multiple throat swabs for *C. burnetii* PCR at the Jeroen Bosch Hospital, which is located in the epicenter of the Dutch 2007–2010 Q fever epidemic. We noted cases in which throat swabs were positive for *C. burnetii* DNA in the absence of subsequent seroconversion. With the knowledge that cow milk often contains *C. burnetii* DNA, we hypothesized that throat swabs may become false positive after ingestion of cow milk containing products. To validate the significance of this problem the first objective of this study was to evaluate the incidence of *C. burnetii* DNA in throat swabs and to calculate the false positive rate. The second objective was to test our hypothesis by analyzing the presence of *C. burnetii* DNA in throat swabs of healthy volunteers after consumption of commercial cow milk products.

2. Materials and methods

2.1. Study design

In this study, we first evaluated the incidence of *C. burnetii* DNA presence in throat swabs and calculated the false positive rate. Therefore, PCR for *C. burnetii* DNA was performed on throat swabs sent to the diagnostic laboratory of the Jeroen Bosch Hospital for other diagnostic purposes. In addition, results of a previous prospective study cohort were used to calculate a false positive rate (van Gageldonk-Lafeber et al., 2013). In this study cohort, extensive laboratory analysis including paired *C. burnetii* serology and PCR for *C. burnetii* DNA on various clinical samples for patients with community-acquired pneumonia (CAP) had been performed and could therefore be used as gold standard for Q fever pneumonia. Further details, including the extensive microbiological testing performed on the different clinical samples obtained, are described elsewhere (van Gageldonk-Lafeber et al., 2013). Lastly, we tested our hypothesis that throat swabs can become positive for *C. burnetii* DNA after consumption of cow milk products by testing throat swabs from healthy volunteers before and after consumption.

2.2. Study population

Patients from whom throat swabs were obtained by their treating physician for other diagnostic purposes than testing for *C. burnetii* were included. Patients were included if they were 18 years or older and had not stated objection to the use of the remainder of their collected material for research purposes. We aimed to collect one hundred throat swabs from December 2017 to March 2018 and enrollment of patients ended after this number was reached.

The second study cohort originated from a prospective CAP study performed from November 2007 to January 2010 (van Gageldonk-Lafeber et al., 2013). Coincidentally, his study period spanned the years of the Dutch 2007–2010 Q fever outbreak. Patients aged 18 years or older who attended the emergency department of the Jeroen Bosch Hospital with CAP were included. Extensive laboratory analysis including detection of phase I and II *C. burnetii* antibodies and PCR for *C. burnetii* on various clinical samples was performed to identify causative pathogens. Further details of this study including ethical approval are described elsewhere (van Gageldonk-Lafeber et al., 2013).

2.3. Volunteers

To test the hypothesis whether ingestion of *C. burnetii* DNA-containing cow milk products can influence *C. burnetii* PCR on throat swabs, healthy human volunteers were recruited from the Department of Medical Microbiology and Infection Control at the Jeroen Bosch Hospital. Ten adult volunteers were approached by a student from the laboratory to rule out any use of pressure. After receiving information about this study and one week to consider participation written informed consent was obtained.

2.4. Cow milk products

Cow milk and goat milk products were tested for the presence of *C. burnetii* DNA by PCR. Random pasteurized milk and cheese products from different brands were purchased at various supermarkets throughout the Netherlands in areas with high and low Q fever incidence during the Dutch Q fever outbreak. Selection of cow milk products containing *C. burnetii* for our intervention was made based on the PCR cycle threshold (C_T) value with a lower C_T value representing a higher bacterial load (Schneeberger et al., 2010). The two products with the lowest C_T value were selected for intervention.

2.5. Intervention

Healthy volunteers ingested a glass of whole milk or a slice of bread with blue cheese. Throat swabs were obtained before consumption and 15 s, 15, 30 and 60 min after consumption of these *C. burnetii*-containing products. The intervention lasted 1 h during which volunteers were not allowed to eat or drink anything else after consuming the initial cow milk product.

2.6. Microbiological testing

Laboratory-developed real-time PCR, targeting positions 7635 to 7704 of the transposase gene, GenBank accession number AE016828, present in the multicopy IS1111 repetitive element of *C. burnetii* was performed on (the remainder of) throat swab samples by trained microbiological technicians at the Jeroen Bosch Hospital (NucliSENS easyMAG; bioMérieux, Marcy l'Etoile, France) and the National Institute for Public Health and the Environment, Bilthoven, The Netherlands (Light Cycler 480, Roche Molecular Biochemicals, Meylan, France) (Tilburg et al., 2010). Samples were analyzed in duplo and a C_T value of < 40 was considered positive (Schneeberger et al., 2010). Each PCR run was accompanied by one positive (*C. burnetii* DNA) and two negative controls (a mock isolation control and a water control). Serum samples for paired serology from patients included in the prospective study cohort were collected during the acute phase (day of hospital admission) and convalescent phase (> 28 days after admission). Phase I and phase II IgM and IgG *C. burnetii* antibody titers were measured using IFA (Focus Diagnostics, Cypress, CA, USA). Titers of $\geq 1{:}32$ were considered positive.

2.7. Data analysis

Descriptive data were generated with SPSS, version 21.0. True positive, false positive, true negative and false negative test results were evaluated. A 2×2 contingency table was constructed, by which the following was calculated: sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). The corresponding

95% confidence intervals (95% CIs) were calculated according to standard Gaussian distribution (1.96 * standard error).

2.8. Ethical approval

The local Medical Ethics Committee stated that this study needed no specific review. The Internal Review Board of the Jeroen Bosch Hospital approved the anonymous use of remnant clinical samples, in addition to data retrieved from the laboratory information system, and waived the need for informed consent from patients. However, informed consent was obtained from healthy volunteers.

3. Results

3.1. Incidence of C. burnetii in throat swabs

One hundred throat swabs from patients were collected at the diagnostic laboratory of the Jeroen Bosch Hospital. Median age was 68 years (interquartile range (IQR), 47–79 years) and 74 were male. Initial test results on these throat swabs were positive in 34 cases: influenza A (n = 3), influenza B (n = 17), rhinovirus (n = 3) or respiratory syncytial virus (n = 11). No pathogen was found in the 66 other throat swabs. PCR on these 100 throat swabs revealed the presence of *C. burnetii* DNA in five throat swabs (5.0%), with C_T values ranging from 32.1 to 37.5. Initial test results on these five throat swabs were respiratory syncytial virus (n = 2), rhinovirus (n = 1) and no pathogen found (n = 2).

Table 1
2 × 2 contingency table of throat swabs for the diagnosis of Q fever pneumonia.

	Q fever pneumonia	No Q fever infection	Total
Throat swab positive	8	4	12
Throat swab negative	23	22	45
	31	26	57

Positive predictive value 66.7% (95% CI, 38.0–88.2); negative predictive value 48.9% (95% CI, 41.3–54.6); sensitivity 25.8% (95% CI, 14.7–34.1); specificity 84.6% (95%CI 71.4–94.5).

Table 2
Presence of Coxiella burnetii DNA in various cow milk products.

Product	C_T value	Product	C_T value
Blue cheese	25.82	Whole milk	26.37
Gouda matured cheese	27.27	Semi-skimmed milk	28.23
Gouda young cheese	28.99	Drinking yogurt	28.57
Red smear cheese	29.24	Coffee milk	30.45
Old cheese	29.34	Low fat yogurt	30.75
Cumin cheese	29.41	Creamer stick	35.38
Goat cheese	Negative	Goat milk	Negative

C_T = cycle threshold value.

3.2. Diagnostic value of throat swabs for Q fever pneumonia

Throat swabs were obtained from 63 of 339 patients (18.5%) who presented with CAP at the emergency department and were analyzed in the previously mentioned prospective study (van Gageldonk-Lafeber et al., 2013). Six patients (9.5%) were excluded from analysis because a second serum sample was not obtained making serological testing for an acute Q fever infection inadequate. Thus, 57 patients were available for analysis. Of these patients, median age was 67 years (IQR, 54–78 years) and 36 (63%) were male. In 31 patients (54.4%), evidence of Q fever pneumonia was found consisting of *C. burnetii* DNA positivity in seronegative acute phase sera and/or appearance of phase I and/or phase II IgM antibodies with titers of at least ≥1:256 in convalescent phase sera. The other 26 patients (45.6%) showed no evidence of Q fever pneumonia. Eight of 31 (25.8%) throat swabs from patients with evidence of Q fever pneumonia were *C. burnetii* DNA positive with C_T values ranging from 34.1–37.9. In patients without Q fever pneumonia, four of 26 (15.3%) throat swabs were *C. burnetii* DNA positive with C_T values ranging from 36.8–38.6. Table 1 displays the 2 × 2 contingency table of throat swabs for diagnosing Q fever pneumonia with the calculated PPV, NPV, sensitivity and specificity and their corresponding 95% CI.

3.3. Cow milk products

Table 2 displays the results of *C. burnetii* PCR on various milk and cheese products and their corresponding C_T values. Based on these results, whole milk and blue cheese were selected for the intervention.

3.4. Throat swabs after consumption of C. burnetii DNA-containing cow milk products

Written informed consent was obtained from ten volunteers. None of the ten throat swabs obtained before consumption contained *C. burnetii* DNA (see Table 3). Five volunteers ingested a glass of *C. burnetii* DNA-containing whole milk. In two throat swabs, *C. burnetii* DNA was found 15 s after consumption with C_T values of 33.3 and 35.0. All other throat swabs were negative. The five other volunteers consumed a slice of bread with blue cheese. In all five throat swabs obtained 15 s after consumption, *C. burnetii* DNA was found with C_T values ranging from 31.3 to 33.5. *C. burnetii* DNA was also found in a throat swab obtained 15 min after consumption and in another throat swab obtained 30 min after consumption with C_T values of 33.0 and 33.5, respectively. After one hour, *C. burnetii* DNA was absent from all throat swabs.

Table 3
Results of throat swabs before and after ingestion of Coxiella burnetii DNA-containing products.

Results	Before ingestion	t = 15 s	t = 15 min	t = 30 min	t = 60 min
After ingestion of *C. burnetii* DNA-containing whole milk					
C. burnetii DNA negative	5	3	5	5	5
C. burnetii DNA positive	0	2	0	0	0
After ingestion of *C. burnetii* DNA-containing blue cheese					
C. burnetii DNA negative	5	0	4	4	5
C. burnetii DNA positive	0	5	1	1	0

4. Discussion

The overall diagnostic value of throat swabs for the diagnosis of Q fever pneumonia is low. Only 25.8% (8/31) of Q fever pneumonias were diagnosed correctly by *C. burnetii* PCR on throat swabs and 15.3% (4/26) were false positive. Recent consumption of blue cheese resulted in *C. burnetii* DNA-positive throat swabs for a maximum of 30 min. One hour after consumption, *C. burnetii* DNA could not be detected in throat swabs. Diagnosis of Q fever pneumonia cannot rely only on PCR of throat swabs, but must always be made in combination with serological analysis.

For this study, we used two different patient populations and a group of healthy volunteers. The first patient group consisted of patients for whom the treating physician ordered a throat swab in most cases for detection of respiratory viruses. In three patients with a positive throat swab for *C. burnetii* DNA a viral pathogen had initially been detected, while no other pathogen was detected in the other two *C. burnetii* DNA-positive throat swabs. While respiratory co-infections can occur, we thought this was unlikely in these cases considering the extremely low incidence (0.1 per 100.000 inhabitants) of Q fever in the Netherlands during the study period (2017–2018) (https://www.atlasinfectieziekten.nl/q-koorts). In this population, no serum samples were available to exclude *C. burnetii* infection. In the second patient group, serum was available for confirmation of *C. burnetii* infection with serology or PCR. Although titers for phase I and phase II IgM were considered positive at ≥1:32, patients with a serologically confirmed *C. burnetii* infection had titers of at least ≥1:256. A *C. burnetii* DNA-positive throat swab in the absence of subsequent seroconversion or PCR-positive blood samples was observed in 15.3% (4/26) of patients, suggesting false positivity of the throat swab result.

We confirmed our hypothesis that recent consumption of *C. burnetii* DNA-containing products can influence the result of a PCR test for *C. burnetii* on throat swabs. *C. burnetii* DNA was present in throat swabs longer after consumption of blue cheese in comparison with whole milk, the first having an initial lower C_T value. *C. burnetii* DNA disappeared from all throat swabs within an hour after consumption of the two types of cow milk products. We have demonstrated that the presence of *C. burnetii* DNA in throat swabs can be a non-specific finding and does not in all cases indicate Q fever infection. However, we have not shown that false positive throat swabs occurring in clinical practice are the result of recent consumption of cow milk products. *C. burnetii* DNA detected in false positive throat swabs may originate from other sources. It has been demonstrated, for instance, that *C. burnetii* is fairly common in the environment (post offices, stores, schools, etc.) in the United States (Kersh et al., 2010). It would be interesting to know whether the relative high percentage (15.3%) of false positive throat swabs during the Dutch Q fever epidemic in 2007–2010 versus the lower percentage (5%) in 2017–2018 corresponded with environmental loads of *C. burnetii* during these periods.

C. burnetii DNA was found in all cow milk-containing products available at various supermarkets in the Netherlands. Milk products in other countries have also been shown to contain *C. burnetii* DNA (Tilburg et al., 2012). As mentioned before, genotyping showed that *C. burnetii* genotypes in dairy consumer products are distinct from the predominant genotype found in Dutch small ruminant dairy farms during the outbreak (Roest et al., 2011; Roest et al., 2013; Tilburg et al., 2012). Consumption of pasteurized *C. burnetii*-containing products and occupational exposure can lead to seroconversion, but no conclusive evidence exists that consumption has resulted in clinical Q fever in humans (EFSA, 2010; Schimmer et al., 2014; van Engelen et al., 2014). No *C. burnetii* DNA was found in products containing goat milk, probably due to the Dutch vaccination program of goats and sheep.

C. burnetii DNA was found in throat swabs obtained for other diagnostic purposes, from patients without serological evidence of Q fever pneumonia and from healthy volunteers after consumption of cow milk products. C_T values of these positive throat swabs varied between 31.3 and 37.9, which is well below the cutoff of 40. The laboratory-developed real-time PCR assay targets the multicopy IS1111 repetitive element ensuring sensitive detection of *C. burnetii* (Klee et al., 2006). Previously, the assay was demonstrated to have a high specificity and sensitivity for detection of *C. burnetii* DNA in human serum (Schneeberger et al., 2010). Recently, it was shown that IS1111-like elements also occur in *Coxiella*-like endosymbionts in ticks (Duron, 2015). Whether such elements would be detected in the IS1111 *C. burnetii* assay used in our study remains to be established, just as the clinical impact of infection with *Coxiella*-like bacteria in humans. In our experiments, each PCR run was accompanied by one positive and two negative controls. The mean C_T value of positive controls (n = 22) was 29.2 and all negative controls (n = 44) were negative. Therefore, no additional *C. burnetii* targets were assessed with PCR.

A limitation of this study is the small number of patients with Q fever pneumonia resulting in sensitivity, specificity and predictive values with broad confidence intervals. However, the timing and location of the prospective study coincided with the epicenter of the largest Q fever outbreak reported, making it the best available cohort for our study objective.

Testing ten volunteers proved that *C. burnetii* can be detected in throat swabs after ingestion of *C. burnetii* DNA-containing cow milk products. This test population is too small to establish a maximum detection time.

5. Conclusions

The diagnostic value of throat swabs for Q fever pneumonia is low and false positive results occur in 15.3%. Recent consumption of *C. burnetii*-containing dairy products can result in *C. burnetii* DNA-positive throat swabs for 30 min after consumption. Diagnosis of *C. burnetii* pneumonia should be based on serological and molecular diagnostic results.

Declaration of interest

The authors declare that there are no conflicts of interest.

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