Evaluation of the validation process of Q-fever PCR test on bulk tank milk samples from dairy goat farms

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Executive Summary

This report describes the evaluation of the validation process of the Real time Polymerase Chain Reaction (RT-PCR) for the detection of *Coxiella burnetii* in bulk tank milk samples of goat farms in the Netherlands. The evaluation has been performed by an international committee of experts on the basis of provided written documentation, published peer reviewed scientific papers and other information obtained through interviews or specific requests for information.

The committee has reached the conclusion that the TaqVet™ RT-PCR assay was already operational in late 2008. At that point in time, the TaqVet™ RT-PCR method was considered a valid assay by the research staff of the Gezondheidsdient-Animal Health Service (GD-AHS) for the monitoring of bulk tank milk from dairy goat farms to detect *C.burnetii* positive flocks. The research staff of the GD-AHS performed multiple studies to evaluate the performance of the TaqVet™ RT-PCR assay under field conditions and eventually summarized this in the RT-PCR test validation dossier.

The committee is of the opinion that the TaqVet™ PCR test validation dossier of the GD-AHS as presented in January 2010 provides only limited validation information.

The committee studied the timeline of events and has come to the conclusion that the choice and validation of the TaqVet™ RT-PCR assay for bulk tank milk of goat farms has not had an impact on the decision making process that eventually resulted in the culling of all pregnant goats on farms with a positive RT-PCR bulk tank test.

Therefore, the committee concludes that the validation process of the TaqVet™ RT-PCR assay at the GD-AHS was not time-critical for the decision making on *C. burnetii* control procedures and the utilization of other laboratories inside or outside of the Netherlands would not have resulted in a different decision making process with regard to *C. burnetii* control procedures.

Introduction

Coxiella burnetii, a causative agent of abortion in dairy goats, was demonstrated to be present in aborted goat fetuses for the first time in the Netherlands in 2005 (Roest et al. 2011). Retrospective seroprevalence data indicate that Q fever had been present in cows, sheep and goat for several decades and in fact was an endemic disease. Query (Q) fever in humans, which is caused by the same agent, was diagnosed prior to 2007 in approximately 20 human cases a year. This situation changed in 2007, when an increase in cases was observed in the town of Herpen, a small village in the south of the Netherlands. Herpen lies in an area which has a high density in dairy goat farms and some of these farms were known to have a high abortion rate at the time of increased incidence of Q fever among the residents of Herpen. It was suggested that the increase in human Q fever cases was related to abortions on dairy goat farms in the area.

At the time of the increase in human Q fever cases, C.*burnetii* infection in ruminants was not a notifiable disease in the Netherlands and there were no systematic data available on the prevalence of the infection at farm level. The suggestion to monitor all goat and sheep farms for prevalence of C. burnetii infection was first made in 2007 but it was not until October 2009 that obligatory screening of farms and obligatory reporting of positive farms would start. A complete history and timeline of the Q fever epidemic in the Netherlands can be found in the report by the commission van Dijk “Van verwerping tot verheffing”.

This evaluation focuses on the validation of the *C.burnetii* specific PCR that is used by the GD-Animal Health Service (GD-AHS) and that has been used since October 2009 for obligatory screening of goat herds in the Netherlands

Scope of this evaluation

The question presented to this committee by the Ministry of Economics, Agriculture and Innovation was as follows;

“Is de validatie van de test die nu wordt ingezet voor monitoren van tankmelk van geitenbedrijven op correcte wijze aangepakt en had, eventueel met gebruikmaking van internationale kennis, dit sneller gekund in de gegeven omstandigheden.”

Which translates in English as;

“Was validation of the test that is currently used in the Netherlands, starting October 2009, for monitoring of bulk tank milk of dairy goat farms performed correctly. Secondly, could this validation of the bulk tank PCR test have been performed quicker with the use of international experts”.

The evaluation process of the committee started on June 21th, 2012. A first draft report was presented by Dr. I. Claassen to two external experts (Dr. A. Rodolakis and Dr. Y.H..Schukken) for comments on June 24th, 2012. The final report was presented on June 26th, 2012.

The evaluation process of the committee focused on the period from early 2007 until October 10th, 2009 at which point in time obligatory bi-monthly bulk tank milk monitoring using a *C. burnetii* real time PCR test started. The evaluation is based on an interview held with Dr. P. Vellema, GD-AHS on June 24th, telephone interviews with Dr. C.van Maanen and Dr. G.Wellenberg both employed at GD-AHS, and on the basis of supporting documents provided by, GD-AHS, CVI-WUR, and the Ministery of Economics, Agriculture and Innovation either as hard copies or as electronic materials.

Timeline of relevant events

In this timeline a schematic overview is given of the relevant events in relation to bulk tank milk (BTM) monitoring and validation of the *C.burnetii* PCR assay for dairy goat herds.

2007

2007. The first commercial PCR kits to detect *C. Burnetii* were obtained by GD-Animal Health Services. A comparison of two commercially available RT-PCR assays was performed (internal document GD-AHS). The original aim was to test dairy cattle.

A number of research questions were formulated to fill gaps in knowledge that existed in the Netherlands regarding *C.burnetii* infection in small ruminants. Answering of these research questions would result in an increase of the knowledge on:

- introduction of the agent to farms,
- short and long term clinical signs
- diagnostic possibilities for several control scenarios
- amount and duration of C.burnetii shedding in animal excreta
- prevalence on sheep and goat-farms in the Netherlands
- transmission between herds and between different animals within herds (cattle, sheep, goat)
- effectiveness of intervention methods such as vaccinations, treatment with antibiotics and management procedures

2008

March The ministry of Agriculture, Nature and Food Quality (LNV), requested the GD-Animal Health Services to submit research proposals to answer some of these questions. April 25th, GD-AHS submits research proposals including a proposal to start BTM monitoring of dairy goats and dairy sheep using PCR. The submitted proposals by the GD-AHS included;

- *A study on the seroprevalence of C. burnetii in sheep and goats in the Netherlands utilising their existing sample flow of diagnostic and monitoring samples*. This research employed the existing sample flow for *Brucella melitensis* monitoring. The diagnostic method used for these samples was a commercial ELISA to measure *C.burnetii* antibodies. (research project 1; projectnr. 2080010)
- *A study to measure prevalence of C. burnetii in bulk tank milk (BTM).* This research used bulk milk samples provided by goat and sheep farmers on a voluntary basis. Testing of these samples was done using a, by commercial ELISA for antibodies and using a *C* commercial PCR kit to measure C*. burnetii* DNA in milk.(research project 2; projectnr. 208016)
- *A longitudinal study on confirmed Q fever positive farms.* The objective of this study was the collection of epidemiologically well-defined samples to improve diagnostic assays, and to study the course of infection of C. burnetii on known infected farms. The study involved the collection of vaginal swabs, blood samples, milk and bulk tank milk samples, environmental samples, wool and hair samples and in case of abortion expelled fetal material and placentae. Blood samples were tested for presence of *C.burnetii* specific antibodies by commercial ELISA. All other samples were tested for the presence of *C.burnetii* DNA using a commercial PCR kit. (researchproject 3; projectnr.208017)

All these proposals were eventually approved for funding on June 26th, 2008 (see below).

June 12th, Q fever becomes a notifiable disease for dairy farms with an abortion problem in at least 5% of pregnant animals in combination with a positive C. burnetii diagnosis using immunohistochemistry on aborted fetal material or placental samples. in farms with at least 50 animals. In small farms the critical abortion percentage is defined at 3% (Rapport van Dijk, pg 44).

June 26th, LNV approves funding of GD research proposals. No specific mention is made of the bulk tank PCR test (letters to A. Pijpers).

August 7th, GD-AHS invites farmers by means of a letter to participate voluntarily in BTM Q-fever prevalence study using TaqVet™ PCR (Available on web: http://www.capraovis.nl/?contentCode=navItem169).

August. Bulk tank PCR in known positive and known negative *C burnetii* flocks over time starts. This study is performed between August 2008 and June 2009 (Reported in PCR validation dossier, dated January 2010).

September 2008. BTM monitoring using TaqVet™ RT-PCR kit of dairy goat flocks on a voluntary basis starts.

October 2008. GD-AHS has performed a comparison of C. burnetii antibody detection in bulk tank milk with *C. burnetii* antigen detection in bulk tank milk using RT-PCR and reports a very good agreement with a kappa value of 0.80. (Reported in PCR validation dossier, dated January 2010).

December 2008. Results of bulk tank monitoring of goat dairy farms are presented in a meeting with LNV. At that point in time it was reported that over 30% of the farms that were tested using voluntarily submitted bulk tank samples were diagnosed to be contaminated with *C.burnetii*.

December 16th, GD-AHS sends PCR results of BTM monitoring to individual dairy goat farmers.

2009

May. BTM PCR results are discussed by experts (rapport van Dijk, page 51).

July. BTM PCR results on voluntarily submitted goat BTM samples are discussed again by experts and reported to the ministry of VWS (Letter from R.A. Coutinho to P.H.A.M. Huijts, dated 24 July 2009).

July. The discussion on obligatory BTM monitoring starts.

August 20th. GD-AHS and CVI are asked to draft a protocol for BTM monitoring

September, A change in policy is announced regarding positive diagnosis of Q fever at farm level. Farms will be regarded as positive after repeated PCR positive BTM samples, even in the absence of abortion problems (Rapport van Dijk pg 55).

October 10th, obligatory two monthly BTM monitoring of C. burnetii using PCR for all sheep and goat dairy farms. Monitoring of BTM samples starts. Flocks are considered officially positive for C. burnetii based on either BTM PCR results or abortion in 5% of pregnant animals with a positive C. burnetii diagnosis. (Rapport van Dijk pg 55).

December. Control procedures on C. burnetii infection in dairy goat farms are discussed. Multiple scenarios are suggested to the ministiry of LNV.(Letters on expert meetings by R.A. Coutinho to A. burger, dated 4 and 10 december 2009).

December 14th. Obligatory BTM monitoring frequency increased from every two months to every two weeks. (Rapport van Dijk pg 58).

December 16th Mandatory killing of all pregnant does on bulk tank PCR positive farms is decided upon. (Rapport van Dijk pg 58). This precautionary measure leads to the culling of approximately 85,000 pregnant goats.

2010

January, Final version of GD-AHS validation dossier on C burnetii TaqVet™ PCR assay is completed.

January 8th, official transfer of *C.burnetii* PCR from GD-AHS to Dr.Van Haeringen Laboratorium bv. (VHL) (Validation dossier PCR assay, C. van Maanen – GD)

May 26th, Dutch Accreditation Council (Raad voor Acreditatie, RvA) visits VHL to extend the scope of accredited tests with the C burnetii PCR test (information provided by GD-AHS)

2011

March 4th, official accreditation by RvA of C. burnetii PCR at VHL (letter of RvA to VHL)

PCR kit LSI TaqVet™ *Coxiella burnetii*

The LSI TaqVet™ *Coxiella burnetii* PCR kit is the only assay used by GD-AHS to detect C.burnetii DNA in BTM samples in the period 2007-2009. According to the manufacturers information (product leaflet, 2009) the kit is suitable for the detection of *C.burnetii* DNA in placentae, foetal liquid and tissue, milk, vaginal mucus, vaginal and cervical swabs, and faeces.

The assay is a real time PCR. Internal control is based on the detection of a household gene GAPDH to control for inhibition of the PCR reaction in different testing matrices.

Detection level of the assay is 1-10 bacteria per gram of sample. The assay can be used in a quantitative way in a range from 100 bacteria per ml to 10.000 bacteria per ml.

History and evaluation of the PCR TaqVet™ validation process by GD-AHS

In 2007 GD-AHS selected a real time PCR assay for the detection of C.burnetii DNA in samples. Two commercial kits (LSI Taqvet *Coxiella burnetii* and Adiavet Cox realtime) were compared (rapportage GD-AHS) and the LSI TaqVet™ *Coxiella burnetii* kit which targets the repetitive transposon-like region of Coxiella burnetii was selected. According to the manufacturers product information this kit had been validated for individual milk samples. The kit has a sensitivity of 1-10 bacteria per ml sample and can be used in a quantitative mode reliably in the range of 100-10.000 bacteria per ml. Sensitivity generally is lower in testing matrices such as milk, faeces, blood, or placental material compared to simple testing matrices such as water. High levels of C.burnetii, up to 1000.000.000 bacteria per gram, can be found in abortion material (placenta) from infected goats.

A 2007 publication by Guatteo et al. reported on the use of the TaqVet™ kit on bulk milk of dairy cows (Guatteo et al. 2007). In 2006 the same group reported on the use of the kit on individual cow samples (Guatteo et al. 2006). In 2008 the TaqVet™ kit was also used by the GD to monitor BTM samples from dairy cows.

In 2008, GD-AHS, at the request of LNV, started several research projects to fill knowledge gaps on Q fever in small ruminants in the Netherlands. No routine screening of large quantities of BTM samples was foreseen and specific validation for this application of the TaqVet™ kit was not considered an issue. In October 2008, GD reported on a comparison of C. burnetii antibody detection in bulk tank milk with C. burnetii antigen detection using TaqVet™ Rt PCR in bulk tank milk and reports a very good agreement with a kappa value of 0.80. For the proposed research goal, to measure prevalence of *C.burnetii* DNA in BTM, the assays performance was therefore considered adequate. The BTM surveillance study also served to collect a set of positive and negative BTM samples that are necessary to validate the assay for routine application. These samples were used when the assay was transferred from GD-AHS to VHL. This assay transfer took place in January 2010.

In 2008 the GD-AHS staff evaluated the performance and suitability of the TaqVet™ PCR assay and identified the following test limitations:

- Shedding of *C.burnetii* bacteria in milk is intermittent, meaning that animals do not shed bacteria in the milk continuously when they are infected. Samples taken at different time points from the same animal may therefore show different results (positive versus negative)
- Repeat samples (the same sample tested two times independently), sometimes gave contradictory results. Cell clustering that resulted samples that were not fully homogeneous was considered a possible explanation for this phenomenon (G. Wellenberg, P.Vellema, interview information).
- Shedding of bacteria in individual animals and in herds is a seasonal phenomenon, likely related to the kidding season of goats. Therefore seasonality should be taken into account when designing a longitudinal BTM monitoring program.
- No reliable data are available on the relationship between PCR positive BTM samples and *C. burnetii* infection prevalence in a herd. The relationship between herd infection prevalence (the percentage of infected animals in a herd) and BTM PCR result is unknown. On one hand this means that false negative BTM PCR results may occur when *C. burnetii* is present in the herd but the BTM PCR test is negative. On the other hand positive BTM PCR results may be present in farms that have a true prevalence that varies between one single animal and all or most animals present in the herd.

Research of the GD-AHS in a limited number of farms also showed that vaccination of herds against Q fever affects shedding of the bacteria in milk. Vaccination as intervention was first used in 2008 using a provisionally licensed vaccine and was more widely practised in 2009, when it became obligatory for dairy goat farms in a large region in the Netherlands. Vaccination provides a degree of protection against clinical signs of Q fever and generally suppresses replication of the pathogen that causes disease. Research performed by GD-AHS showed that after vaccination of positive farms, the number of bacteria/ml decreases. On two vaccinated, known *C. burnetii* infected, herds that were longitudinally followed using BTM, the RT-PCR actually showed temporarily negative BTM PCR results. However, during the kidding period these two farms became positive again in BTM (PCR validation report, January 2010).

The TaqVet™ kit was used by GD-AHS in the course of 2008-2009 to test a wide variety of samples, including BTM samples of dairy goat farms. Data from these studies were used in the test validation dossier that was finalized in January 2010 (see below).

The original use that was envisaged for the Q fever PCR was to declare flocks free of Q fever (P.Vellema personal communication). Studies to support this application were executed during 2008 and 2009. Two kidding periods were included in this study because shedding, in milk and into the environment, is highest in the period around kidding. Hence, a lengthy validation program, to demonstrate fitness for purpose, was considered necessary. Based on the known seasonality of *C. burnetii* shedding in goats, declaration of freedom of a farm from *C. burnetii* infection can only be

provided after a full years monitoring results have been collected, and all tested BTM samples are negative in the PCR test.

Identifying *C. burnetii* infected flocks is a much easier process as any single BTN positive test would be sufficient to define a flock as infected. Validation of a positive PCR result would require a formal validation of the test itself and not a long-term longitudinal follow up of known infected flocks. Such a validation would require the quantification of test sensitivity and specificity against a known gold-standard. Although the GD validation dossier describes several studies, no data is provided from a formal estimation of sensitivity and specificity of the TaqVet™ PCR kit as used for dairy goat BTM.

In fact, when it was discussed and decided in July-September 2009, that obligatory monitoring to detect *C.burnetii* infected farms, the assay that was present could be used immediately. In a meeting at the ministry of LNV, held at July 28th, 2009, GD-AHS representatives mentioned that based on their experience it was possible to detect positive farms on the basis of one or a few animals shedding *C. burnetii* bacteria. Positive farms were confirmed by testing of independent samples from the same farm at the Central Veterinary Institute (CVI-WUR). CVI-WUR had previously developed an RT-PCR test specifically for this purpose. Test performance of the GD-AHS and the CVI-WUR assays were compared using negative milk samples spiked with a range of inactivated *C.burnetii* bacteria. However, no data on test sensitivity, specificity or test comparison of the TaqVet™ kit and the CVI PCR test were presented in the GD test validation dossier of January 2010. In the same validation dossier, a comparison of PCR results from monitoring dairy goat BTM using the commercial TaqVet™ kit with the CVI in-house PCR is reported with little detail, dates and precision (RT-PCr validation dossier January 2010).

At some point in time, a comparison between the non-automated DNA extraction as prescribed by the kit manufacturer with a high throughput automated DNA extraction method was performed and the use of the high throughput automated DNA extraction method was validated (RT-PCR validation dossier, 2010).

Soon after the transfer to VHL the accreditation process for ISO-17025, the international ISO standard for laboratory testing, was started on the basis of the validation and performance in the laboratory. During the first visit by RvA, in March 2010 additional information was requested regarding reproducibility of the assay and second and third line controls. After providing the additional information, accreditation was formally granted in 2011.

Confirmation testing of positive farms is performed at CVI-WUR, the reference laboratory for *C. burnetii* diagnostics in the Netherlands.

Committee opinion

The committee is of the opinion that the dossier on test validation as reported in January 2010 appears to be somewhat haphazard. The dossier on validation (dated January 2010) is not very strong as it lacks precise data and dates. However, from the dossier, and other sources, it is clear to the committee that the PCR test is able to identify *C.burnetii* in BTM of goat flocks and that the execution of the test can be done both at GD-AHS and the related VHL laboratory. The test is accredited according to ISO-17025 on the basis of an evaluation visit in March 2010.

An in-house validation of commercial kits, to demonstrate performance, repeatability and fit for purpose characteristics, like robustness, diagnostic sensitivity and diagnostic specificity, is normal practice for diagnostic laboratories. However, when the assay is used for research purposes this validation is not necessarily done in a formal manner.

From the provided information it becomes clear that the TaqVet™ PCR test has been used since at least 2008. In October 2008, a comparison between ELISA and PCR in bulk tank milk was done on 202 samples and a good test association between the two tests was observed (Kappa = 0.8). Even though this result provides some assurance that the PCR kit provided relevant results, a comparison against an antibody ELISA cannot be considered sufficient validation for a real-time PCR test for *C.burnetii* antigens.

As the commercial kit was to be used for a National screening program, the committee is of the opinion that the use of the TaqVet™ kit for goat BTM screening had not been described in literature or in product information and therefore a more formal validation would be considered necessary.

The results of the bulk milktank PCR screening in 2008-2009 on voluntarily submitted bulk tank samples were known in early 2009 to both experts and policy makers and were used in the development of policies.

The first application that was considered for the bulk milktank screening was to declare goat herds free of *C.burnetti*. The committee is of the opinion that a lengthy validation program, including one kidding period, is needed to account for seasonality variations in bacteria shedding.

Not until December 2009 there was a developing consensus among the experts that identification of *C.burnetii*-positive herds using the bulk tank PCR and the subsequent killing of all pregnant animals on these farms was the preferred control method.

The development of *C. burnetii* infection control policies was based on ongoing discussion among all involved parties that were fed by continuously updated information on the *C.burnetii* epidemic in people, *C.burnetii* data in animals, the possible relationship between *C.burnetii* in animals and people, logistical limitation with regard to testing and vaccination and a continuously changing pressure because of valid public health concerns on decision makers.

Even though the validation of the real time PCR test for *C.burnetii* antigens could have been performed and reported in a more formal and precise manner, the committee is of the opinion that the process of validation was not time-critical for the decision making with regard to *C. burnetii* control measures on dairy goat farms. It is the opinion of the committee that a faster and more formal validation process of the TaqVet™ PCR would not automatically have resulted in different infection control policies or in a different timeline in the implementation of the *C. burnetii* control policies.

Closing remarks

This evaluation report has been written on the basis of the documents and other information presented to the committee. Within the short time frame available to the evaluation committee it was impossible to execute an in depth analysis of the events and available information. Moreover, all relevant information was available in Dutch language only, and therefore could not be evaluated by the French member of the committee Dr. A Rodolakis. The committee feels however that this has had no influence on the outcome of this evaluation.

The committee appreciates the open atmosphere of the discussions and the swiftness with which available information was provided.

Consulted documents

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19. Eerste analyse testvergelijking drie ELISA’s en twee commerciele PCR kits voor detectie vaan *Coxiella burnetii* in tankmelk monsters van Nederlandse melkveeebedrijven (2007 internal GD documen, not dated)
20. Offerte voor onderzoek GD dd 25 april 2008, ref 20080425-00002566
21. Opdrachtverstrekking LNV ten behoeve GD onderzoek dd.26 juni 2008
22. GD-AHS letter to sheep and goat farmers containing invitation for BTM investigation, dd August 7th, 2008 ref.20080807-00000236
23. GD-AHS letter to sheep and goat farmers with result of BTM testing dd.december 18th, 2008.
24. GD-AHS Eindrapport Seroprevalentie meting Q-fever bij schapen en geiten in Nederland, gebruik makend van bestaande monsterstroom. March 2009, project 2080010, P.Vellema and L.Moll
25. GD-AHS Eindrapport Q-fever tankmelkonderzoek op melkschapen en melkgeitenbedrijven. November 2010. Project 2080016.P.Vellema, L.Moll, R.van der Brom, S. Luttikholt,.
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