Subclinical effects of Coxiella burnetii in dairy cows in the Netherlands

S. van Zalen

Department of Farm Animal Health, Utrecht University, PO Box 80.151, 3508 TD Utrecht, the Netherlands

Abstract

Coxiella *burnetii (Cb)* is an obligate intracellular bacterium which causes Q fever in humans and animals. The majority of Dutch dairy herds are considered infected based on bulk milk tank tests for antibody and Cb DNA. Commonly, infected cattle are without any clinical symptoms. However subclinical effects on fertility and udder health have been reported to be associated with Cb prevalence. The aim of this study is to investigate the presence of Cb in the genital tract and the udder in a random sample of 349 healthy slaughter cattle in the Netherlands and the possible relation with suboptimal fertility and mastitis. Therefore, different immunodiagnostic tests are used, such as Cb specific antibody detection, and Cb antigen specific interferon-gamma (IFN-gamma) release and related to the presence of bacterial DNA.

In Dutch dairy cattle a high animal level prevalence of Cb infection was observed based on serological diagnostic assays. Surprisingly, an even higher fraction of animals had antibodies against Cb in the milk. Using qPCR to detect Cb DNA, we were unable to detect Cb in genital tract tissue. However, Cb DNA could be readily detected in 15% of the milk samples. Whether Cb is indeed a causal agent in reproductive disorders of cattle is unclear. It is abundantly present in the udder, however no association was found between the presence of Cb and IMI. Also, we were unable to demonstrate a relation between (sub)clinical mastitis and the presence of the Cb in the udder or milk . Following from the results more research is needed to elucidate the clinical consequences of an infection with *Cb* in dairy cattle.

Introduction

Coxiella burnetii (Cb) is an obligate intracellular bacterium which causes Q fever in both humans as well as in animals. Ruminants are thought to be the most important reservoirs for human infections, although not all cases can be linked to direct contact with these animals (Tozer, Lambert et al. 2014). Cb is shed via milk, feces, vaginal mucus and birth products. Asymptomatic cows shed the bacteria in the milk, in contrast to ewes which shed the bacteria mainly in faeces and in vaginal mucus(Rodolakis, Berri et al. 2007). The transmission primarily occurs via inhalation of contaminated aerosols (Woldehiwet 2004). After the transmission, Cb targets alveolar macrophages and will bind to the α v β 3 integrin. After the binding, Cb enters the cell via RAC1-dependent phacocytosis. In the cell Cb exists in a vacuole that eventually will fuse with a phagolysosome(van Schaik, Chen et al. 2013). The immune defense against this intracellular pathogen depends on cellular immunity instead of humoral immunity (Ghigo, Pretat et al. 2009). This implies that to diagnose Q fever it might be more useful to determine the presence of T-cell immunity than the presence of antibodies.

The infection in humans is usually asymptomatic, but *Cb* can cause severe acute or chronic disease in sporadic cases. The symptoms of the acute form of Q fever are often similar to an influenza infection with potential complications like pneumonia and hepatitis. The chronic form of Q fever can occur at different time intervals after the acute illness but in some cases there is no history of acute illness at all. Chronic Q fever often presents itself as endocarditis (60-70%) in the absence of fever. Long term Q fever can cause chronic fatigue syndrome, cardiovascular disease and spontaneous abortion and prematurity (Parker, Barralet et al.).

In domestic animals, acute Q fever is generally considered subclinical or asymptomatic. The best known manifestation of Q fever in animals is the abortion storm on goat farms. In addition, in goats, the presence of Cb in a number of tissues from the genital tract has been proven in a study by Alsaleh et al(2011). In this study, flushing fluids from oviducts and uterine horns and genital tract tissues of twenty goats were tested on the presence of Cb through the use of a conventional PCR. In 40% of the flushing fluids as well as in 25% of the genital tract tissues Cb was present. Unfortunately,

this kind of data is not available for cattle, which is one of the reasons why the approach of the sampling of the genital tract in this research project is based on the study by Alsaleh et al.(Alsaleh, Pellerin et al. 2011).

In contrast to goats, Cb infection in dairy cattle seems to be solely asymptomatic. Worldwide, the symptoms which are most frequently associated with *Cb* infection in dairy cattle are reproductive disorders like abortion, stillborn calves, infertility, metritis and mastitis. According to a monitoring study by the Dutch Animal Health Service (GD), 78.6% of the dairy farms in the Netherlands are ELISA-positive in tank bulk milk and 56.6% are PCR-positive in tank bulk milk. The individual animal prevalence is significantly lower with 16.0% ELISA-seropositive cows and 3.6% PCR-positive cows. In young stock a seroprevalence of 1.0% was found (Muskens, van Engelen et al. 2011).

Despite the high prevalence of Cb infection in cattle in the Netherlands, Cb infection is not considered an important cause of abortion based on routine diagnostic surveys of aborted fetuses and fetal membranes by the Dutch Animal Health Service (GD). In a study performed by the GD only 9 of the 100 abortions were Cb PCR positive, of which 4 fetuses also tested positive with the use of immunohistochemistry (Muskens, Wouda et al. 2012).

The aim of this study is to investigate the presence of Cb in the genital tract and the udder in a random sample of 349 healthy slaughter cattle in the Netherlands and the possible relation with suboptimal fertility and mastitis. Therefore, different immunodiagnostic tests are used, such as Cb specific antibody detection, and Cb antigen specific interferon-gamma (IFN-gamma) release and related to the presence of bacterial DNA.

Material and methods

Sample populations

Healthy cattle were randomly sampled in a slaughterhouse in the Netherlands in which dairy and beef cattle from all over the country are slaughtered. In this study, a total of 348 cows were sampled on 7 separate days between October 2012 and January 2014. On each day 46 to 50 cows were

consecutively sampled during 1 hour according to the scheme presented in table 1. The samples included simultaneously collected sera, whole blood (heparin), milk and the genital tract. The sera and milk samples were used to detect antibodies against *Cb*. The tissue samples and milk samples were used for the detection of the bacteria and the whole blood samples were used for a lymphocyte stimulation test.

In addition, bulk tank milk of twenty dairy farms with unexplained subfertility and good reproductive performance data recording were collected and tested for antibodies and detection of Cb. A subsequent cross-sectional study was performed on one of the Cb test positive farms to estimate within herd animal level prevalence. The cross-sectional study included all 219 animals that were present on the farm. Based on these results three dairy farms were selected for a longitudinal study of seven months. In this study heifers were tested with a serum ELISA ante partum before introduction in the dairy herd. After their first parturition and introduction in the dairy herd, the cows were tested every month until a second pregnancy was confirmed. During this sampling period serum and corresponding data of 76 cows was collected.

To further establish the moment of infection, serum samples and whole blood (heparin) samples of heifers (18 cows) and lactating cows (16 cows) were collected during the cross-sectional study. These samples were used for an IFN-gamma release assay to compare the different outcomes between the different age groups. The heifers included in this part of the research can be divided in one group of animals (five cows) that was not pregnant, one group of animals (five cows)that was eight months before parturition, one group of animals (three cows)that was five months before parturition and one group of animals (five cows) that was one month before parturition (table2).

Finally, to evaluate the potential association between Cb and (sub)clinical mastitis, 303 milk samples were tested for Cb antigen with the use of a PCR . The milk samples were obtained from cows with a known history and the results of bacteriological culture were available for analysis. Of this group, 180 cows were considered healthy and the other 123 were diagnosed with (sub)clinical mastitis.

Processing and Storage of the samples

The sampling method used to sample the genital tract is based on a study by Alsaleh et al. (Alsaleh, Pellerin et al. 2011), in which genital tracts of goat en sheep were examined for the presence of *Cb.* Of each genital tract, duplicate samples of the stromal tissue of the ovaries, oviducts and uterine horns were collected aseptically. In total, 498 ovaries, 498 oviducts and 498 samples of the uterine horns have been collected.

The samples of the ovaries, oviducts and uterine horns were stored at -20°C and in 4% buffered formalin. When the dam was pregnant, a cotyledon and caruncle were also sampled and stored similarly.

The serum and milk samples were collected following centrifugation at 1500 x g for 15 minutes. The sera and milk samples were stored in duplicate in deep well plates (2x 1mL) at -20°C until further analysis.

The whole blood samples (collected in heparin anticoagulant tubes) were processed for antigen stimulation on the same day of sampling to be able to perform a whole blood IFN-gamma release assay.

Detection of antibodies against Coxiella burnetii in serum and milk (ELISA)

Antibodies specific to Cb were measured using a commercially available ELISA (IDEXX Q fever Ab Test) according to instructions provided by the manufacturer. In short, the sera were diluted to 1:400 and the milk samples to 1:5 in ELISA wash buffer. The resulting optical density values were used to calculate the sample to positive ratios (S:P), using the formula (ODsample-ODnegative control)/(ODpositive control-ODnegative control) x 100%). The S:P values were expressed as percentages and divided into three categories: negative (<30%), suspect (between 30%-40%) and positive (>40%) as per manufacturer's instructions.

Detection of Coxiella burnetii antigen in tissue and milk (qPCR)

The QIAamp® DNA Mini Kit (Qiagen S.A., France) was used for all the DNA isolations performed in this study following instructions provided by the manufacturer. Minor adjustments were made in the lysis procedure of the different types of samples. The frozen tissue samples were cut in pieces of 20-30 mg and the tissue was lysed for 1,5 hour in a heat block heated to 70 °C. Milk samples were lysed for 30 minutes in a heat block heated to 70 °C.

After the random selection of 25 seropositive and 25 seronegative animals in group #1 (see table 1) the qPCR was performed on the genital tract. A pooling strategy on the samples of the genital tract was used to perform the PCR. The samples were distributed over 76 pools, of which 50 pools contained three tissues (one pool contained the ovary, oviduct and uterus horn of the left, the other contained those of the right) from an ELISA positive animal. Additionally, 25 pools contained six samples, namely the two ovaria, oviduct and uterus horns, from an ELISA negative cow. Last, 1 pool contained the placentome tissue. The control samples consisted of pools with previously determined Cb DNA positive ovine samples, which were diluted to 1:3 and 1:6 with a negative sample. The milk samples were tested individually.

The qPCR test was performed with an internal control (GAPDH, a housekeeping gene of ruminant cells) to ensure proper DNA isolation per sample. The DNA isolation was considered successful when a characteristic amplification curve was observed for GAPDH. Samples that met this requirement and were showing a typical amplification curve as well as a Ct value below 40 were regarded positive for *C. burnetii*.

Interferon – gamma release assay

To measure the cellular adaptive immune response against *Cb* a whole blood interferon-gamma release assay was performed. The heparinized whole blood was incubated at 37^0C for 20-24 hours with culture medium (RPMI supplemented with 10% fetal calf serum (FCS) and penicillin plus streptomycin) as negative control to determine spontaneous release of INF-gamma. Poke Wheat

Mitogen (PWM) was used as a positive control at 2.5 ug/ml and different concentrations of killed Cb varying from 2.25 units/ml till 18 units/ml. These units were isolated from the COXEVAC vaccine (CEVA, France) using a 10kD spin filter. To replace the thiomersal of the vaccine, PBS without calcium and magnesium was added three times before centrifugation. After the incubation period, the supernatant was collected and used in an IFN-gamma ELISA as supplied with the Bovigam assay (Prionics, Switzerland) according to instructions provided.

The OD450 blank value was subtracted from the test values. Subsequently, the blank corrected OD450 values were corrected for spontaneous release of IFN-gamma by subtracting the medium control. The sample to positive (S/P) ratio was calculated as OD450(plasma stimulated with antigen)/OD450(plasma stimulated with PWM.) The cut off value used in the manufacturer's manual to decide the sample was positive was 0.1; this value is also used in this study.

Results

First screening of twenty selected dairy farms

The results of the first screening of the twenty dairy farms are shown in table 3. The herd prevalence based on ELISA in bulk tank milk (BTM) in twenty dairy farms is 55%. The prevalence based on PCR is 35%. All of the milk samples that were positive in the qPCR were also positive or suspect in the ELISA. One sample was only positive in the ELISA.

Cross-sectional study at a selected test positive dairy farm

One of the dairy farms which was positive in both the BTM ELISA and the BTM qPCR had fertility problems at the moment of sampling. To establish the individual seroprevalence and to research the possibility of a link between Cb infection and fertility problems a cross-sectional study was performed at this specific dairy farm.

In this cross-sectional study 29 of 219 animals were found to be seropositive (shown in table 4), indicating an individual seroprevalence of 13%. When an age distribution (figure 1) was made a peak

appeared to exist in seroconversion following the introduction of heifers in the dairy herd. No association was observed between recorded fertility problems and any of the seropositive animals.

Longitudinal study on three test positive dairy farms

In this part of the study the main question was to determine the moment of seroconversion based on the hypothesis that the first contact with Cb follows the first introduction of heifers in the dairy herd. For a period of 7 months, 76 pregnant heifers were sampled for seroconversion against Cb. None of the animals were seropositive at the time of enrolment. However, during this longitudinal study only one animal seroconverted (71,6 %). These results give no distinct answer as to the moment of exposure to Cb and to the occurrence of associated seroconversion. This observation leaves open the questions regarding the tissue location of Cb, the timing of exposure and the immune response of the animal to exposure.

Cb DNA is absent in genital tract tissue

Using the cross-sectional samples from slaughterhouse cattle of the ELISA performed in sample group #1 (see table 1) an individual seroprevalence of 19% (table 5) was established. The age distribution (figure 2) seems to differ between the negative and positive group. The negative group resembles the age distribution of all animals. However, the positive group is different with an additional peak of seropositive animals around an age of 40 months.

In the first qPCR analysis of the genital tract, all but 3 out of 76 samples tested had amplification of GAPDH with an average cycle threshold of 22,53. The Cb positive control series gave a typical amplification curve with a threshold for Cb depending on which dilution of the positive control sample was used. Six samples with a positive GAPDH amplification were suspect for Cb. A second qPCR analysis was performed to analyse the six doubtful samples and the three GAPDH negative samples for a second time. In the second run, two pools stayed negative for GAPDH and were

206 therefore excluded from further analysis. All of the samples tested in the second run were negative
207 for Cb.
208
209 *Cb is present in milk*
210 In the sample population #2 (see table 1) a seroprevalence of 24,4% has been found. The antibody
211 prevalence of positive milk samples was 55,5%. The qPCR of the milk samples were performed
212 individually. All of the samples had a typical amplification curve of GAPDH. In the sample population
213 #2 18% of the animals were tested positive. These results confirm the presence of Cb in milk, as
214 already has been shown in the first screening of BTM samples of the twenty dairy farms. Table 6
215 shows the combined test results of the ELISA in milk, serum and the qPCR of the milk.
216 The results show a substantial number of animals (three out of nine) that are positive in the qPCR,
217 but seronegative in the ELISA.
218
219 *Milk samples of healthy cows versus cows with a (sub)clinical mastitis*
220 Based on the high prevalence of Cb in milk samples, we tested the samples collected in a mastitis
221 pathogens prevalence study and tested these samples for the presence of Cb DNA. In the Cb qPCR
222 analysis of 303 milk samples, all of the samples were positive for GAPDH with an average Ct of 27,54.
223 The Cb positive control, using the standard dilution 1:1000, gave a typical amplification curve with a
224 threshold for Cb resembling the Ct of the other experiments. In this sampling group only two samples
225 tested positive for Cb. One of the samples tested positive for coagulase-negative staphylococci, the
226 other one was cultured negative.
227
228 *Relation between presence of Cb DNA, Cb antibody and Cb specific IFNg release*
229 To test the hypothesis that the immune response initially starts with a cell mediated type of response
230 a third set of samples was collected which included heparanized blood. In the sample population #3
231 (see table 1) a seroprevalence of 19% has been found. The antibody prevalence of positive milk

samples was 44%. The qPCR of the milk samples were performed individually. All of the samples had amplification of GAPDH with an average cycle threshold of 23,87. In the sample population #3 14% of the animals were tested positive.

Using a modified version of the Bovigam interferon release assay to detect antigen specific interferon gamma T cell responses in cattle substituting tuberculin antigens with Cb antigen we determined Cb specific IFN-gamma release. The S/P ratios of the negative samples are similar to the S/P ratio reached with medium. The S/P ratios of the positive samples stimulated with Cb antigen are higher when compared with the control samples and reach a minimal level of 0.1. Following the interpretation of the IFN-gamma release assay five animals tested positive. Of the five positive animals which were considered positive, one also was positive with PCR, another one in both ELISAs and the last three were only positive in the lymphocyte stimulation test.

Dairy heifers versus adult dairy cows in an IFN-gamma release assay

The IFN-gamma data suggested that a T-cell driven response precedes the antibody response to Cb similar to what is observed with other persistent intra-cellular bacterial pathogens such as Mycobacterias, before seroconversion takes place.

The results of the IFN-gamma release assay are shown in table 2. Although the sampling groups are small, at least one individual in every age group showed a response in the IFN-gamma release assay.

Discussion

The aim of this study was to investigate the presence of Cb in the genital tract and the udder in a random sample of 349 healthy slaughter cattle in the Netherlands and the possible relation with suboptimal fertility and mastitis. Therefore, different immunodiagnostic tests are used, such as Cb specific antibody detection, and Cb antigen specific interferon-gamma (IFN-gamma) release and related to the presence of bacterial DNA.

In this study has been tried to describe the presence and location of *Coxiella burnetii* in the genital tract of slaughter cows. This kind of study was performed before with goats, but to our knowledge this design has not been carried out in dairy cattle before. The number of animals used in this study was based at the individual seroprevalence in the Netherlands, determined by the Dutch Animal Health Service Deventer. The number of animals which were chosen to use for the antigen detection was based on the study performed with goats(Alsaleh, Pellerin et al. 2011).

The antigen detection of the tissues from the genital tracts with the PCR can be considered valid because of the positive internal control GAPDH and the typical amplification curves of the positive controls. We found none of the samples positive, so it seems unlikely that the genital tract is the place where Coxiella burnetii hides.

On the other hand, we did find *Cb* antigen in 12% and 18% of the individual milk samples and also in bulk tankmilk of 35% of the dairy farms. In comparison, the Animal Health Service Deventer tested 8,7% of the cows qPCR-positive in the milk(Muskens, van Engelen et al. 2011). In this study and in other studies Coxiella burnetii has also been found in the milk and mammary gland tissue (Ho, Htwe et al. 1995). Although we did find high prevalences of Cb in milk, no relationship was found between the presence of Cb and (sub)clinical mastitis.

It seems more likely that the residency of C. burnetii is placed in the mammal gland than in the genital tract. However, according to other studies it can be related to fertility problems like abortion and placentitis in dairy cattle, which raises the question, what Coxiella burnetii triggers to move in the direction of the genital tract when the animal is pregnant. More research is needed to answer those questions and to find a possible relationship between unexplained subfertility and Q fever in dairy cattle.

Thirdly, the current seroprevalence of Q fever in dairy cattle in the Netherlands has been established. The serology showed an individual seroprevalence of 19%. This resembles the prevalence of 16%, found by the Animal Health Service Deventer(Muskens, van Engelen et al. 2011). Interestingly enough, the ELISA results of the sera and milk samples do not match. The milk ELISA showed a high

prevalence of 44%, which differs a lot from the seroprevalence. All animals, but one, are positive in serology as well as in the milk. These results seem to indicate a local production and/or distribution of antibodies.

When the age distribution of the seropositive animals is compared with the negative animals or all animals, there is quite a difference around the age of 40 months. These results seem to suggest that the seroconversion of most animals takes place around this specific age. It raises the question why there is a peak around 40 months old. A reason for the seroconversion could have been the introduction to the dairy herd, but in the longitudinal study none of the included dairy cows did seroconverse during this period. Another possibility is a different kind of immune response, which is quite likely because of the intracellular life of Cb. In the last part of this study (table 2) we did try to prove the existence of this type of immune response in different age groups from one year old until the introduction in the dairy herd and to compare this with the results in lactating cows. These results demonstrated at least one individual in every age group that showed a response in the IFN-gamma release assay. These preliminary data open the possibility that young stock acquire the Cb infection early in life and before introduction into the dairy herd. Further research is recommended to confirm this data. Also, more studies need to be carried out to establish the route of infection, the exact timing of the infection and the influences of Cb at the health status of young stock.

Acknowledgements

Many thanks go to Ad Koets and Peter Vos, who led me through the process and tried to keep me on track. I also want to thank Mirjam Nielen, Tine van Werven and Ineke Daemen for their support during this research project.

This project was financially supported by CEVA, France

References

ALSALEH, A., PELLERIN, J., RODOLAKIS, A., LARRAT, M., COCHONNEAU, D., BRUYAS, J. and FIENI, F., 2011. Detection of Coxiella burnetii, the agent of Q fever, in oviducts and uterine flushing media and

in genital tract tissues of the non pregnant goat. *Comparative immunology, microbiology and infectious diseases,* **34**(4), pp. 355-360.

GHIGO, E., PRETAT, L., DESNUES, B., CAPO, C., RAOULT, D. and MEGE, J.L., 2009. Intracellular life of Coxiella burnetii in macrophages. *Annals of the New York Academy of Sciences,* **1166**, pp. 55-66.

HO, T., HTWE, K.K., YAMASAKI, N., ZHANG, G.Q., OGAWA, M., YAMAGUCHI, T., FUKUSHI, H. and HIRAI, K., 1995. Isolation of Coxiella burnetii from dairy cattle and ticks, and some characteristics of the isolates in Japan. *Microbiology and immunology,* **39**(9), pp. 663-671.

MUSKENS, J., VAN ENGELEN, E., VAN MAANEN, C., BARTELS, C. and LAM, T.J., 2011. Prevalence of Coxiella burnetii infection in Dutch dairy herds based on testing bulk tank milk and individual samples by PCR and ELISA. *The Veterinary record,* **168**(3), pp. 79.

MUSKENS, J., WOUDA, W., VON BANNISSEHT-WIJSMULLER, T. and VAN MAANEN, C., 2012. Prevalence of Coxiella burnetii infections in aborted fetuses and stillborn calves. *The Veterinary record,* **170**(10), pp. 260.

PARKER, N.R., BARRALET, J.H. and BELL, A.M., Q fever. *The Lancet,* **367**(9511), pp. 679-688.

RODOLAKIS, A., BERRI, M., HECHARD, C., CAUDRON, C., SOURIAU, A., BODIER, C.C., BLANCHARD, B., CAMUSET, P., DEVILLECHAISE, P., NATORP, J.C., VADET, J.P. and ARRICAU-BOUVERY, N., 2007. Comparison of Coxiella burnetii shedding in milk of dairy bovine, caprine, and ovine herds. *Journal of dairy science,* **90**(12), pp. 5352-5360.

TOZER, S.J., LAMBERT, S.B., STRONG, C.L., FIELD, H.E., SLOOTS, T.P. and NISSEN, M.D., 2014. Potential Animal and Environmental Sources of Q Fever Infection for Humans in Queensland. *Zoonoses and Public Health,* **61**(2), pp. 105-112.

VAN SCHAIK, E.J., CHEN, C., MERTENS, K., WEBER, M.M. and SAMUEL, J.E., 2013. Molecular pathogenesis of the obligate intracellular bacterium Coxiella burnetii. *Nature reviews.Microbiology,* **11**(8), pp. 561-573.

WOLDEHIWET, Z., 2004. Q fever (coxiellosis): epidemiology and pathogenesis. *Research in veterinary science,* **77**(2), pp. 93-100.

Table 1: Overview of collection dates of samples at the slaughterhouse. In 2012 the first sampling group[1] (#1) was collected, this included 249 animals of which serum and genital tracts were taken. In December 2013 the samples of the second group[2] (#2) were collected. Finally, in 2014 the samples of the third group[3] (#3) were collected.

Date	10-30-2012[1]	11-2-2012[1]	11-7-2012[1]	11-14-2012[1]	11-21-2012[1]	12-4-2013[2]	01-22-2014[3]
Total animals	50	50	50	50	49	49	50
Serum	50	50	50	50	49	49	50
Heparanized blood	-	-	-	-	-	-	46
Milk	-	-	-	-	-	49	49
Genital tract tissue	50	50	50	50	49	-	-
- **Pregnant**	2	0	2	2	0		

Table 2: A overview of the sampling group and the test results of the interferon-gamma release assay. In this part of the study the cellular immune response against Cb of heifers was compared with the cellular immune response of lactating cows.(a.p.: ante partum)

Group	#cows	Reaction INF-gamma test (number of animals)
Not pregnant	4	1
8 months a.p.	5	2
5 months a.p.	4	2
1 month a.p.	5	4
Dairy cows	16	5

Table 3: ELISA and PCR results of the first screening of the twenty dairy farms of the University Farm Animal Health practice.

		ELISA			
		Positive	**Suspect**	**Negative**	**Total**
PCR	**Positive**	**7**	**0**	**0**	**7**
	Suspect	**3**	**2**	**0**	**5**
	negative	**1**	**0**	**7**	**8**
	Total	**11**	**2**	**7**	**20**

Table 4: The ELISA results of the cross-sectional study at the first dairy farm selected from the twenty dairy farms.

	#cows	*Prevalence(%)*
Seropositive	*29*	*13%*
Seronegative	*190*	*87%*
Suspect	*0*	*0%*
Total	*219*	*100%*

Figure 1: Parity distribution of the cross-sectional study at one dairy farm, divided in three groups; (1) all animals, (2) all seronegative animals and (3) all seropositive animals

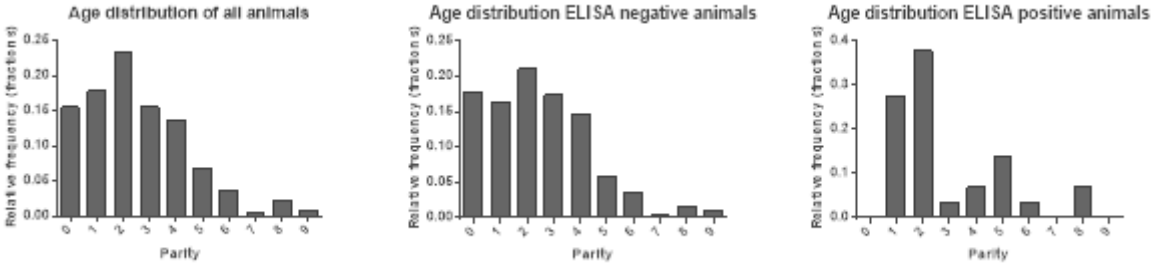

Table 5: ELISA results of group #1 of the slaughterhouse sample population. Also shown is the average age of the different result groups.

	#cows	%	Age (months)
Seropositive	48	19,27	79,0
Seronegative	198	79,52	79,8
Suspect	3	1,20	87,6
Total	249	100	79,7

Figure 2: Age distribution of group #1 of the slaughterhouse sample population, divided in three groups; (1) all animals, (2) all seropositive animals and (3) all seronegative animals

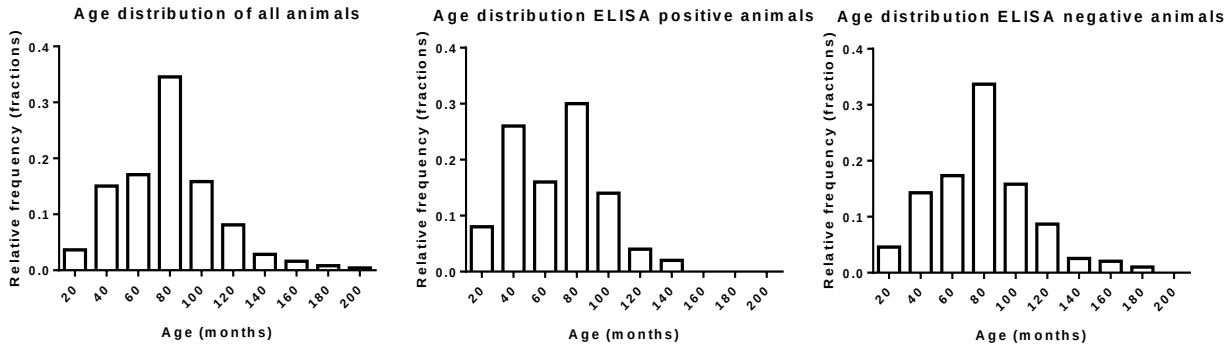

Table 6: the results in the second sampling group of the slaughterhouse. In this table the ELISA results (sera and milk) are combined with the qPCR (milk) results

ELISA results	qPCR results		
	Positive	Negative	Total
serum+/milk+	5	6	11
serum+/milk-	1	0	1
serum-/milk+	2	14	16
serum-/milk-	1	20	21
Total	9	40	49