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Feasibility Study on Coxiella burnetii Phase-Specific Antibody Tests and Interferon-γ-Recall Assay in Dairy Cattle

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Abstract

The zoonotic pathogen *Coxiella burnetii* (C. *burnetii*) is characterized by two antigenic phases (PhI and II). PhI- and PhII-antibody tests and a PhII-IFN-γ-recall assay (IFN-γ-RA) with neutralization of IL-10 were developed for ruminants. Cows in an endemically infected farm were monitored for shedding of C. *burnetii* by quantitative polymerase chain reaction (qPCR) in milk (MS) and puerperal fluid (PS) samples over a period of two years. Subsequently, blood was collected. According to the shedding pattern cows were allocated to four groups: (1) MS+/PS+ (n=16), (2) MS+/PS- (n=36), (3) MS-/PS+ (n=17), and (4) Non-S (n=37, non-shedder). A fifth group consisted of primiparous cows that had been vaccinated as heifers prior tobreeding and thatwere tested after calving ((5) H/Vacc, n=11). As an additional parameter, the time since last detection of C. *burnetii* (< 6 months, ≥ 6 months) was also included in the analysis.

Group 1 was characterized by a higher level of PhI/PhII-antibodies and a lower level of IFN-γ compared to group 3, which was characterized by high level of IFN-γ, a moderate level of PhII-antibodies and absence or low level of PhI-antibodies. An inverse relationship of parameters of cellular and humoral immunity was observed for these two groups. An intermediate position was observed for group 2, i.e., cows with recent detection of C. *burnetii* tended to show a pattern similar to group 1, while those shedding C. *burnetii* more than 6 months ago were similar to group 3. Vaccinated heifers were characterized by strong IFN-γ-reactivity, moderate PhII-antibodies and absence of PhI-antibodies; low level shedding was observed in heifers despite vaccination.

ABBREVIATIONS

AG: Antigen; BSA: Bovine Serum Albumin; CAG: Control Antigen; *C. burnetii*: *Coxiella burnetii*; CFT: Complement Fixation Test; IFN-γ: Interferonγ; IFN-γ-RA: Interferon-γ-Recall Assay; ELISA: Enzyme-Linked Immune Sorbent Assay; H/Vac: Heifer Vaccinated; IgG: Immunoglobulin G; IL-10: Interleukin 10; LPS: Lipopolysaccharide; mab: monoclonalantibody; MS: Milk Sample; Non-S: Non-Shedder; OD: Optical Density; OTG: Octyl-ß-D-1-Thioglucopyranoside; PBMC: Peripheral Blood Mononuclear Cells; PBS: Phosphate-Buffered Saline; Ph: antigenic phase; PS: Puerperal fluid Sample; qPCR: quantitative Polymerase Chain Reaction; RT: Room Temperature; SC: Stimulation Control; TMB: 3,3‘,5,5‘-Tetramethylbenzidine.

INTRODUCTION

Coxiella (C.) burnetii, a small, gram-negative obligate intracellular bacterium, is the cause of Q fever in humans [1]. It exists in two antigenic phases, phase I (PhI) and phase II (PhII); this phase variation is comparable to rough/smooth variation in Enterobacteria. PhI- but not PhII-organisms are virulent [2,3]. PhI-antigen is a complete lipopolysaccharide (LPS), and it shields the outer membrane proteins from immune recognition. In contrast, a truncated LPS characterizes PhII-organisms from which PhII-antigen is extracted [4]. Both PhI- and PhII-antigens are used to distinguish acute and chronic infections in humans. PhII-antibodies are detected early after infection, whereas PhI-Immunoglobulin G (IgG) appears later [5]. Additionally, persistent and elevated PhI-titers are an indicator of chronic infection in humans and rodents [5,6]. Patients with chronic endocarditis are characterized immunologically by reduced *C. burnetii*-specific-IFN-γ-responses, high level PhI-titer and an increased level of IL-10, which is a result of the Th2-dominated immune response [4].

Cattle are frequently infected with *C. burnetii*- some of them persistently [7]. Recently, an inactivated PhI vaccine for vaccination of cattle and goats was registered in Germany (Coxevac™, Ceva, Düsseldorf, Germany). Vaccination reduced shedding of *C. burnetii* in milk and vaginal fluids in dairy cows - especially if non-pregnant and not-infected animals had beenvaccinated [8].

Compared to humans the knowledge about the immunobiology in ruminants is scarce. Repeated shedding of *C. burnetii* in milk was observed in individual cows and some were shedding >10^3 *C. burnetii*/ml as determined by qPCR. This repeated shedding has frequently been associated with increased antibody reactivity in ELISA [9] and an increased PhI-antibody titer was observed in milk samples of chronic shedders [10]. Acute infection seems to be characterized by aPhI-/PhII+ antibody pattern, as this pattern was frequently observed in primiparous cows after they

were introduced to the mature dairy herd [11,12]. However, this serological pattern was normally not associated with acute infection in multiparous cows [10,11]. Ph-specific complement fixation tests (CFT) were compared with qPCR and indirect ELISA; no correlation was observed between qPCR and CFT; however, indirect ELISA was correlated with PhI-CFT [13]. Roest et al. infected pregnant goats with *C. burnetii* and assessed the immune responses. PhII-IgG was detected 2 weeks *post infection*, while PhI-IgG appeared 5 weeks *post infection* [14]. No difference was observed for IgM. PhI- and PhII-ELISAs were also applied for monitoring purposes in an infected goat herdby Sting et al.; the early stage of infection was related to dominance of PhII-reactivity, while in the further course of infection PhI-reactivity dominated [15]. Cellular immune responses to *C. burnetii* infections have not been studied in cattle so far; however, they are crucial for control of Coxiellosis [4]. In the present study we established phase-specific antibody tests and an interferon-γ recall assay (IFN-γ-RA). Validation of immunodiagnostics is extremely difficult because serological reference tests currently being used have a low diagnostic sensitivity [12,16]. Therefore, a feasibility study was initiated to get an idea about the future use of these tests. Dairy cows had been monitored repeatedly for shedding of *C. burnetii* in milk or at calving for approximately 2 years. At the end of this period Ph-specific antibody titers and IFN-γ-reactivity were compared with the shedding history of *C. burnetii*-shedding.

MATERIALS AND METHODS

A farm with about 120 dairy cows (Simmental) was included in the study. The herd was known to be endemically infected with *C. burnetii* [10,11]. Cows were kept in two groups of almost equal size. The groups differed regarding milking process: an automated and a conventional system were used, respectively. Otherwise the management and feeding was the same. A direct contact between groups existed; moreover, dry cows of both groups were housed together and the same calving facility was used. We sought to characterize every cow by a shedding pattern of *C. burnetii*; therefore milk and puerperal fluids were tested by qPCR from March or August 2010, respectively [10]. The number of samples submitted for testing and the time of sample submission is indicated in Figure 1. Cows which had been tested at least once by a milk sample or a puerperal swab were included in this study (n=117). The study was performed irrespective of the state of pregnancy.

Starting in June 2012, groups of cows were vaccinated with Coxevac™ (Ceva, Düsseldorf, Germany). Blood samples were collected prior to vaccination and analyzed for Ph-specific antibodies and *C. burnetii*-specific IFN-γ-reactivity.

Vaccination of heifers already started in June 2011 at the age of 12-15 months while breeding started at about 18 months of age. The pregnant vaccinated heifers (n=11) were introduced into the dairy cow herd two weeks prior to the expected calving date starting in September 2012.

Blood sampling

Two blood samples (10ml) were collected from the jugular vein, one coagulated and one stabilized with Lithium-heparin (Li-heparin). Blood samples arrived at the laboratory within 3 hrs of collection. Immediately upon arrival, samples were processed. Li-heparin stabilized samples were promptly stimulated with antigens (see IFN-γ-assay) while serum was harvested by centrifugation (900g, 20 mins) from the coagulated blood. The collected serum was frozen at -20°C until testing.

Milk sampling and puerperal swabs

Metered composite milk samples of individual cows were collected during regular milking either by the automatic milking system or during the monthly milk quality sampling. Within 3 hrs the samples arrived at the laboratory, were stored between +2 - +8°C and analyzed with qPCR within 48 hrs upon arrival. Puerperal swabs (swab 60cm, Eydam KG, Kiel, Germany) were collected within 16 hrs after calving.

Quantitative Polymerase Chain Reaction (qPCR)

The qPCR was performed as previously described [10]. Briefly, the amount of the multi-copy target (*C. burnetii* transposase gene, IS1111a, Accession No. M80806) present in the samples was quantified using standard curves derived from the diluted DNA of the *C. burnetii* reference isolate Nine Mile RSA493 (generously provided by the Institute for Hygiene and Infectious Diseases of Animals, Justus-von-Liebig University, Giessen) via optical counting of stained cell culture material. The results of the qPCR were expressed as *C. burnetii* /ml (C.b./ml) milk and C.b./swab. Samples were considered positive when more than 1 C.b./swab or per ml milk was detected.

PhI- and PhII-ELISA

The previously used PhI- and PhII-ELISAs [10,11] manufactured by a commercial company were no longer available for this study. Therefore an in-house ELISA was developed. Test plates were coated separately with PhI-antigens (Dolfinin Q-fever ELISA2 bulk; B1EL 200; Dolfinin; Bratislava; Slovak Republic) and PhII-antigens (Dolfinin Q-fever ELISA1 bulk; B1EL 100; Dolfinin; Bratislava; Slovak Republic). Optimal dilutions of antigens were determined by checkerboard titrations. PhI- and PhII-antigen were used at a final dilution 1/5000 and 1/500, respectively.

Coating: Antigens were treated with octyl-thioglucopyranoside (OTG; Octyl-β-D-1-thioglucopyranoside; O6004; Sigma Aldrich; Sigma-Aldrich Chemie GmbH, Munich; Germany). First, a stock of OTG (40mg/ml in phosphate buffered saline (PBS Dulbecco w/o Ca^{2+} w/o Mg^{2+}; L1825; Biochrom GmbH; Berlin; Germany)) was prepared. Then PhI- and PhII-antigen were pre-diluted in PBS 1/500 and 1/50, respectively. Subsequently, OTG-stock was added to pre-diluted PhI- and PhII-antigen to obtain a final concentration of 0.5% and 0.16%, respectively. The mixture was incubated for 20 mins under agitation at room temperature (RT), followed by dilution of each antigen 1/10 in PBS (4°C) and 100µl/well were dispensed in testing plates (F96 PolySorpNunc-Immuno plate; 10449672;Thermo Fisher Scientific; Roskilde; Denmark) which had been cooled down to 4°C. Plates were sealed and kept overnight at 4°C. The next day plates were emptied and tapped dry. After plates had further dried for 3 hrs at RT the coated plates were vacuum sealed and kept at 4°C until use.

ELISA: Reagents were used at RT. Controls and samples

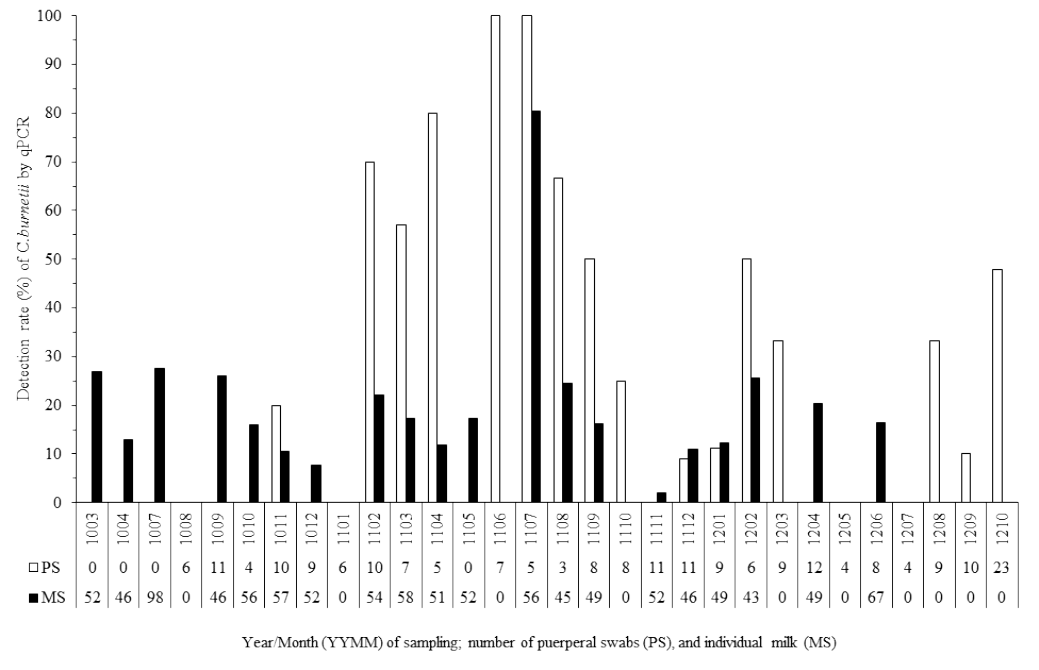

Figure 1 Puerperal swabs (PS) and milk samples (MS) were analyzed for *C. burnetii* (C.b.) using quantitative PCR (qPCR). The rates of positive results are shown over time. The x-axis indicates the year and month of sample collection (YYMM, top), and the number of PS and MS.

were diluted in sample diluent consisting of 1 M TRIS pH 9.0 (TRIS ultrapure; A1086; AppliChem GmbH; Darmstadt; Germany - Note: milk samples are diluted in 1 M TRIS pH 7.0), 1% fish gelatin (Gelatin from cold water fish skin; G7765-250ML, PCode: 1001551566; Sigma-Aldrich Chemie GmbH, Munich; Germany), 0.016% NaN_3 (Sodium azide; 1066870001; Merck KGaA; Darmstadt; Germany). Serum samples were pre-diluted 1/100, 1/1000, 1/10000 and 1/100000 in sample diluent; 100µl/well were transferred to the PhI- and PhII-testing plates, respectively. Samples were titrated in order to estimate which dilution of the sample might be used in a future test format based on a single dilution of the sample. The positive control was a strong positive milk serum at a dilution of 1/20; it indicated the plateau (i.e. maximum) of the ELISA-reactivity. The sample diluent served as a negative control. Plates were sealed and incubated for 90 mins at RT. Subsequently, plates were emptied and washed five times with PBS/0.05% Tween 20 (Tween 20 BioChemica; A1389; AppliChem GmbH; Darmstadt; Germany) after each washing step plates were tapped on a paper towel. Protein G peroxidase conjugate (Protein G, Peroxidase Conjugate; 539322; Merck KGaA; Darmstadt; Germany) was diluted 1/5000 in PBS/0.05% Tween 20 and was dispensed to the plates (100µl/well). Then the plates were sealed and incubated at 90 mins at RT. Plates were washed again as described. TMB-substrate and stopping solution were used from IDEXX test kits (TMB Substrate Nr. 12 / Stop Solution Nr. 3; IDEXX Switzerland AG; Liebefeld-Bern; Switzerland). Plates were washed, 100µl/well TMB-substrate was dispensed and after 20 mins100µl/well stopping solution was added. OD was read at 450nm.

Calculation of end-point titer: The end-point titer for each antigen was determined at 20% (OD%) of the positive control. Titers <100 scored negative and were set to 80. Titers exceeding 200.000 were set to this value.

Stimulation of blood samples for IFN-γ-recall assay (RA)

PhI-, PhII-antigen, negative-control-antigen and *Brucella abortus* LPS (Virion, Würzburg, Germany) were used at a final dilution of 1/900, 1/100, 1/500 and 1/5000, respectively. The dilution of PhII-antigen was determined in preliminary tests. For each antigen, endotoxin was determined with kinetic, chromogenic Limulus Amoebocyte Lysate test (LAL-Assay, Charles River Laboratories), finally PhI-antigen, negative control-antigen and *Brucella abortus*-LPS were diluted to the endotoxin concentration of PhII-antigen.

Pokeweed-mitogen (Sigma-Aldrich, Steinheim, Germany) at a final concentration of 0.55µg/ml served as stimulation control (SC); and PBS was included to control the unstimulated sample. An IL-10-neutralizing monoclonal antibody (mouse monoclonal anti-bovine IL-10 (CC320) Ab (IgG1), LifeSpanBioScience, Seattle, USA) and an isotype control (mouse monoclonal anti-T-2-mycotoxin (UNLB) Ab (IgG1), Southern Biotech, Birmingham, USA) were used at a final concentration of 0.1µg/ml. This concentration was determined in preliminary experiments.

Except for SC a pre-dilution of each antigen with each of the monoclonal antibodies in PBS was prepared in a 6-well plate. Subsequently, 20µl of antigen and 280µl Li-heparin blood were transferred per well to a 96-well sterile cell culture plate. Stimulation was performed in duplicate for each antigen. The stimulation plate was covered with a lid, thoroughly mixed and incubated in a humid chamber at 37°C and 5% CO_2. After 16-18 hrs of incubation the stimulation plate was centrifuged at 500xg

for 5 mins; 120-150µl plasma were harvested per well and transferred to a 96-polysterol plate.

IFN-γ-RA

An ELISA-kit was used to determine IFN-γ in plasma (ELISA for Bovine IFN-γ; Mabtech, Uppsala, Sweden). ELISA-plates (Nunc-Immunoplates®, Maxisorp, Thermo Fisher Scientific) were coated with the catching monoclonal antibody MT17.1 in PBS pH 7.4 (100µl/well) overnight at 4-8°C. Plates were washed twice with 200µl/well PBS and subsequently blocked with 200µl/well PBS with 0.05% Tween 20 and 0.1% BSA (incubation buffer, 1 hr, RT). After each step plates were washed five times with 200µl/well PBS 0.05% Tween 20 (washing buffer). Per well 50µl washing buffer were distributed and 50µl/well plasma were added. A standard range covering 20-1,250 pg IFN-γ/ml was tested on each plate; samples and standard incubated 2 hrs at RT. Plates were washed and the monoclonal biotinylated detection antibody (MT307) was diluted to 0.25µg/ml in washing buffer; 100µl/well were dispensed followed by incubation for 1 hr at RT. Plates were washed as described above and 100µl/well of streptavidin-horse radish peroxidase diluted 1:1000 in washing buffer were dispensed (1 hr at RT). TMB-substrate and stopping solution were used from CHEKIT BHV1-bulk milk (Infectious Bovine Rhinotracheitis (BHV-1) Antibody Test Kit for bulk milk, Idexx). Plates were washed, 100µl/well of the substrate were dispensed and after appropriate color development 100 µl/well stopping solution were added. Optical density (OD) was read at 450nm.

The mean OD was calculated for duplicates. The net OD (ΔOD) was calculated for each antigen by subtracting the OD of the control antigen: ΔOD (AG_i-CAG_i), the suffix '*i*' indicates the variable monoclonal antibody (IL-10, isotype). Subsequently, ΔOD (AG_i-CAG_i) was expressed as per cent of ΔOD ($SC\text{-}PBS_i$). A preliminary cut-off of 15% was applied.

The following validation criteria were implemented: The ΔOD ($SC\text{-}PBS_{Isotype}$), ΔOD ($SC\text{-}PBS_{IL\text{-}10}$) had to exceed 0.5 while the OD of $PBS_{Isotype}$ and $PBS_{IL\text{-}10}$ had to remain below 0.5.

STATISTICAL ANALYSIS

Cows were grouped according to their shedding pattern: Group 1: Shedding of *C. burnetii* in milk (MS+) and at calving (PS+) (MS+/PS+), Groups 2 and 3: shedding only in milk or at calving (Group 2:MS+/PS-; Group 3: MS-/PS+), Group 4: non-shedding (Non-S), and group 5: primiparous cows that were vaccinated twice as heifers prior to breeding and were tested after first parturition (H/Vacc). Additionally, the time since the last detection of *C. burnetii,* independent of the shedding route, was included as a further variable (< 6 months/ ≥ 6 months). The number of cows per group and test is summarized in Table 1.

IFN-γ-reactivity against each antigen with/without IL-10-neutralization was compared by Wilcoxon-test for paired samples. The comparison of reactivity (PhII-IFN-γ, phase-specific titers) between groups of animals included the analysis for normal distribution of results. In case of normal distribution ANOVA was performed. If data were not normal distributed Fisher's exact test was used for positive and negative results. Due to the limited number of cases per group (shedding group x time since last detection) it was not possible to analyze the effect of shedding group and time since last detection. Moreover, if multiple tests were performed, e.g. comparison of phase-specific titers for shedding groups by Fisher's exact test, the Bonferroni-correction was not applied.

Statistical analysis was performed with MedCalc Statistical Software version 17.1 (MedCalc Software bvba, Ostend, Belgium; https://www.medcalc.org; 2017).

RESULTS AND DISCUSSION

Data on shedding are summarized in Figure 1. While a major peak of shedding at calving was observed in 2011, two minor peaks were detected in 2012. Only 3 cows shed *C. burnetii* at two successive parturitions. No distinct peak (except for June 2011) was observed for shedding of *C. burnetii* in milk. The rate of MS until June 2012 averaged 20.7% (CI95%: 18.1-23.6%).

A low level of *C. burnetii* ($10^{1.6}$-$10^{2.3}$C.b./swab) was detected in puerperal swabs from five of 11 vaccinated heifers (group 5 H/Vacc); an additional animal even shed *C. burnetii* at the second parturition in 2013 (data not shown).

IFN-γ-reactivity against Ph-specific antigens and control antigens with/without IL-10-neutralization is summarized in Figure 2 for 104 animals. The samples of additional 13 animals failed the validation criteria: Samples were excluded due to insufficient reactivity against SC (n=2) or because PBS-control exceeded the OD-value of 0.5 in case of isotype control (n=4) or IL-10-neutralization (n=7). Since the reactivity against the control antigen was subtracted, negative values for PBS, LPS, PhI- and PhII-antigen in Figure 2 are indicating reactivity against the control antigen. IFN-γ-reactivity against the LPS-antigen (*Brucella abortus*) was observed only once after IL-10-neutralization.

PhII-antigens resulted in the highest reactivity and IL-10-neutralization enhanced the PhII-specific IFN-γ-response significantly; in contrast, IL-10-neutralization had no effect on stimulation of peripheral blood mononuclear cells (PBMC) with PhI-antigen. Unspecific reactivity against the control antigen (increased negative values) was slightly enhanced by IL-10 neutralization.

To our knowledge this is the first study in which an IFN-γ-RA was used to assess the IFN-γ-response to *C. burnetii* in dairy cattle. Therefore, several key points have to be acknowledged. Firstly, conventionally IFN-γ is measured in pg/ml. However, this quantification does not reflect the general ability of individual animal's PBMC to produce IFN-γ. Indeed, blood samples/animals varied in their ability to respond to SC. Antigen-specific IFN-γ was hence expressed as percent of the SC per cow; a similar approach had been previously used for goats [14]. Secondly, antigens available for complement fixation test were used for the stimulation of PBMC. All antigens were adjusted to the endotoxin-concentration of PhII-antigen, which is regarded as a protein antigen. Additionally, purified LPS from *Brucella abortus* was included to assess any unspecific effect of LPS. A significant PhII-specific IFN-γ-response was detected compared to PhI-antigen (Figure 2). However, because of its higher endotoxin concentration PhI-antigen had to be used at a higher dilution than PhII-antigen (1/900 versus 1/100); this might explain the

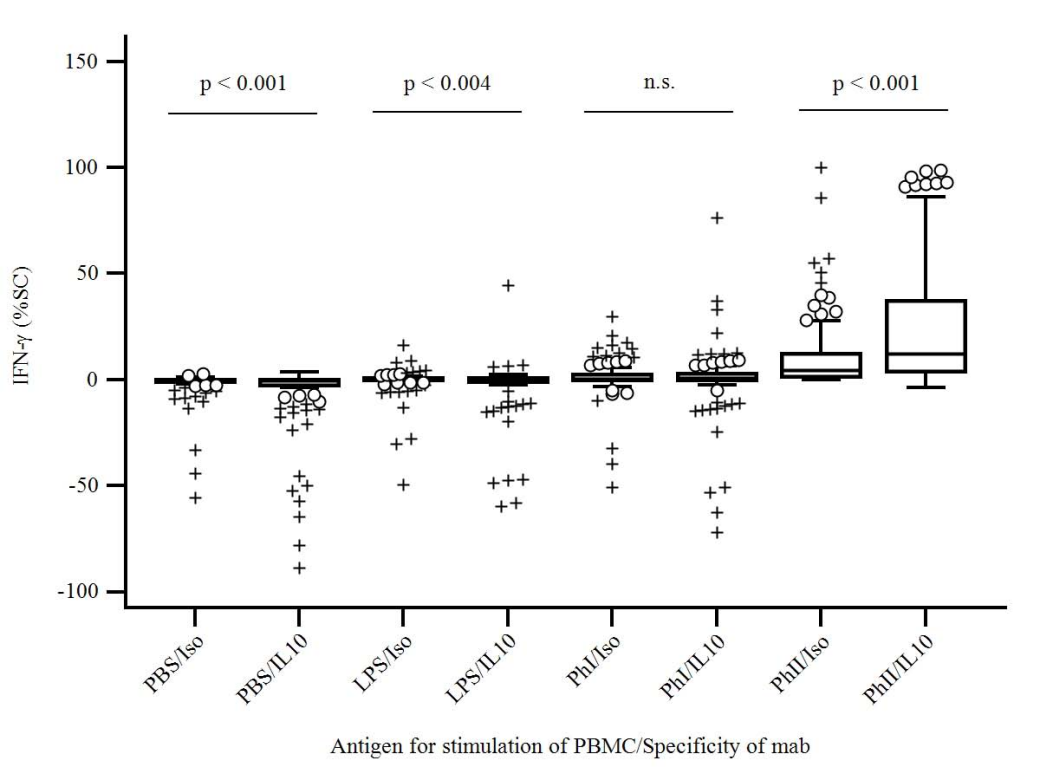

Figure 2 The Box-Whiskers-Plots show the Interferon-γ- (IFN-γ) response after stimulation of PBMC with different antigens/controls in the presence of an IL-10-neutralizing monoclonal antibody (mab) or an isotype control (Iso). PBMC were stimulated with PBS, LPS-control, phase I (PhI) and phase II-antigen (PhII). Interferon-γ- (IFN-γ) response was determined by an ELISA and expressed as per cent of the stimulation control (%SC). The optical density of the respective control antigen (CAG) was subtracted from that of PBS, LPS, PhI and PhII, it resulted sometimes in negative values. Outliers from each distribution are characterized by "+". The effect of IL-10-neutralization was compared with the isotype control for each antigen/control by Wilcoxon-test, significant differences are indicated.

lower reactivity observed for PhI-antigen. Indeed, ELI Spots were performed in humans and goats with the same antigens and no difference for PhI- and PhII-antigens was observed; however, any effect of endotoxin-concentration was neglected in these studies [14,17]. Thirdly, based on experience from paratuberculosis in ruminants neutralization of IL-10 by a monoclonal antibody enhances the antigen-specific IFN-γ-response [18,19]. Unfortunately, occasionally IL-10 neutralization increased the reactivity in unstimulated PBMC of goats. In order to improve any control of unspecific reactivity, the panel of controls was extended (PBS, LPS and an isotype control for the IL-10-neutralising monoclonal antibody) and validation criteria were established. The latter excluded samples with low SC-reactivity and elevated background activity (PBS). As expected, IL-10-neutralization did enhance IFN-γ-reactivity against the negative control antigen in some cases (negative values in Figure 2); but after subtraction of the respective OD_{CAG} from the OD of other antigens (PBS, LPS, PhI and PhII), negative values were only observed for PBS, LPS or PhI, but not for PhII-antigens. On the other hand, PhII-specific IFN-γ-responses were significantly enhanced by IL-10-neutralization, and most remarkably, differences between shedding groups were only observed if IL-10 was neutralized (Figure 3). This observation might support the crucial role of IL-10 in the pathogenesis of Q-fever [21-23]. In some rare instances the net PhII-specific IFN-γ-response was lower after IL-10-neutralization than that of the respective isotype control; this effect was even enhanced if a higher concentration of the IL-10-neutralizing antibody (1µg/ml) was used (data not shown).

The following analysis of IFN-γ-reactivity refers to PhII-IFN-γ-RA with IL-10-neutralization. We compared groups of cows to evaluate the data from PhII-IFN-γ-RA and phase-specific serology. The distribution of PhII-IFN-γ-reactivity, PhI- and PhII-titers is shown in Figure 3, Figure 4a,b respectively. Percentages of positive samples are summarized in Table 1.

PhII-IFN-γ-reactivity increased in the order of the groups 1 (MS+/PS+), 2(MS+/PS-), and 3 (MS-/PS+). Additionally, when the time since last shedding was taken into account, this increase in reactivity was particularly driven by cows that had stopped shedding (Figure 3). There were no significant differences of PhII-IFN-γ-reactivity among shedding groups (ANOVA, p=0.07) or the reactivity of animals with or without cessation of shedding (ANOVA, p=0.29). The comparison of the proportions of each group testing positive for IFN-γ ($p<0.05$) are shown in Table 1. Significantly weaker IFN-γ-reactivity was observed in group 1 (MS+/PS+) compared to group 5 (H/Vacc) and in group 2 (MS+/PS-) compared to group 3 (MS-/PS+), respectively.

Titers of PhII-antibodies are illustrated in Figure 4b. Detection of *C. burnetii* did not always result in PhII-antibodies. For example, in group 3 (MS-/PS+) only 58.8% of cows sero

Table 1: Percentages of animals positive in serology and in PhII-IFN-γ-RA with IL-10-neutralization.

Shedding group	last shedding	Phase-specific serology					PhII-IFN-γ-RA			∑ PhII+ and IFN+)[3]
		n	neg	PhI-/PhII+	PhI+/PhII+)[1]	∑ PhII+)[1,2]	n	neg	IFN+)[1]	(p)
(1) MS+/PS+	<6 mon	7	0.0	28.6	71.4	100.0	6	83.3	16.7	0.0122
	≥6 mon	9	11.1	22.2	66.7	88.9	6	50.0	50.0	0.2832
	∑	**16**	**6.2**	**25.0**	**68.8**[a,b,c,d]	**93.8**[a,b]	**12**	**66.7**	**33.3**[a]	**0.0028**
(2) MS+/PS-	<6 mon	13	15.4	30.8	53.8	84.6	13	76.9	23.1	0.0059
	≥6 mon	23	34.8	43.5	21.7	65.2	18	55.6	44.4	0.3102
	∑	**36**	**27.8**	**38.9**	**33.3**[a,e]	**72.2**	**31**	**64.5**	**35.5**[b]	**0.0057**
(3) MS-/PS+	<6 mon	9	44.4	22.2	33.3	55.6	8	37.5	62.5	n.a.
	≥6 mon	8	37.5	37.5	25.0	62.5	8	25.0	75.0	n.a.
	∑	**17**	**41.2**	**29.4**	**29.4**[b]	**58.8**[a,c]	**16**	**31.3**	**68.8**[b]	**0.8144**
(4) Non-S	n.a.)[4]	37	43.2	37.8	18.9[c]	56.8[b,d]	35	60.0	40.0	0.2342
(5) H/Vacc	n.a.	11	0.0	100.0	0.0[d,e]	100.0[c,d]	10	20.0	80.0[a]	0.4150
total	**∑**	**117**	**29,1**	**41.0**	**29.9**	**70.9**	**104**	**53.8**	**46.2**	**n.a.**

[1]shedding groups irrespective of last shedding were compared by Fisher's exact test ($p<0.05$), groups with the same index are significantly different.
[2]Sum of PhI-/PhII+ and PhI+/PhII+; PhI+/PhII- was not observed.
[3]Proportions observed for ΣPhII+ and IFN+ within groups were compared.
[4]n.a. not applicable

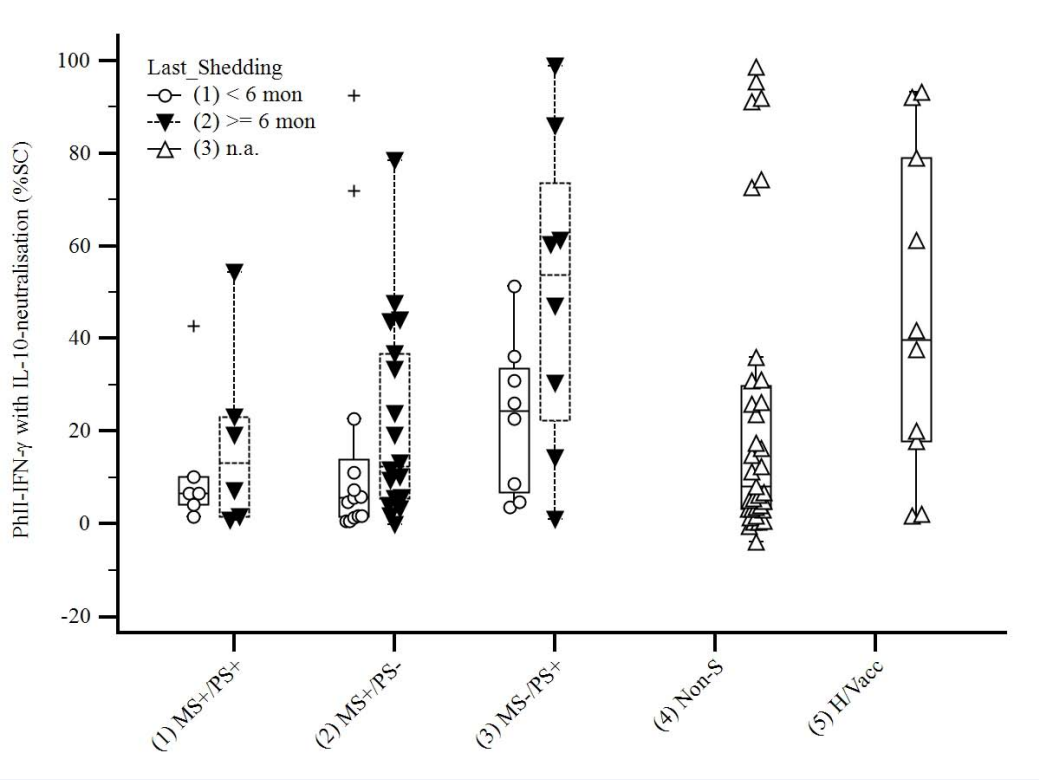

Figure 3 The Box-Whiskers-Plots show the phase II-specific Interferon-γ-responses after IL-10-neutralization as percent of the stimulation control (PhII-IFN-γ (%SC)) for five groups of cows. The time between blood sampling and the last shedding of *C. burnetii* irrespective of the route (detection of *C. burnetii* within the last six months or for more than six months before testing, n.a. not applicable) serves as an additional parameter. Groups were defined by detection of *C. burnetii* in milk samples (MS) or puerperal fluid swabs (PS), non-shedders (4 Non-S) and primiparous cows which had been vaccinated before first breeding (5 H/Vacc).

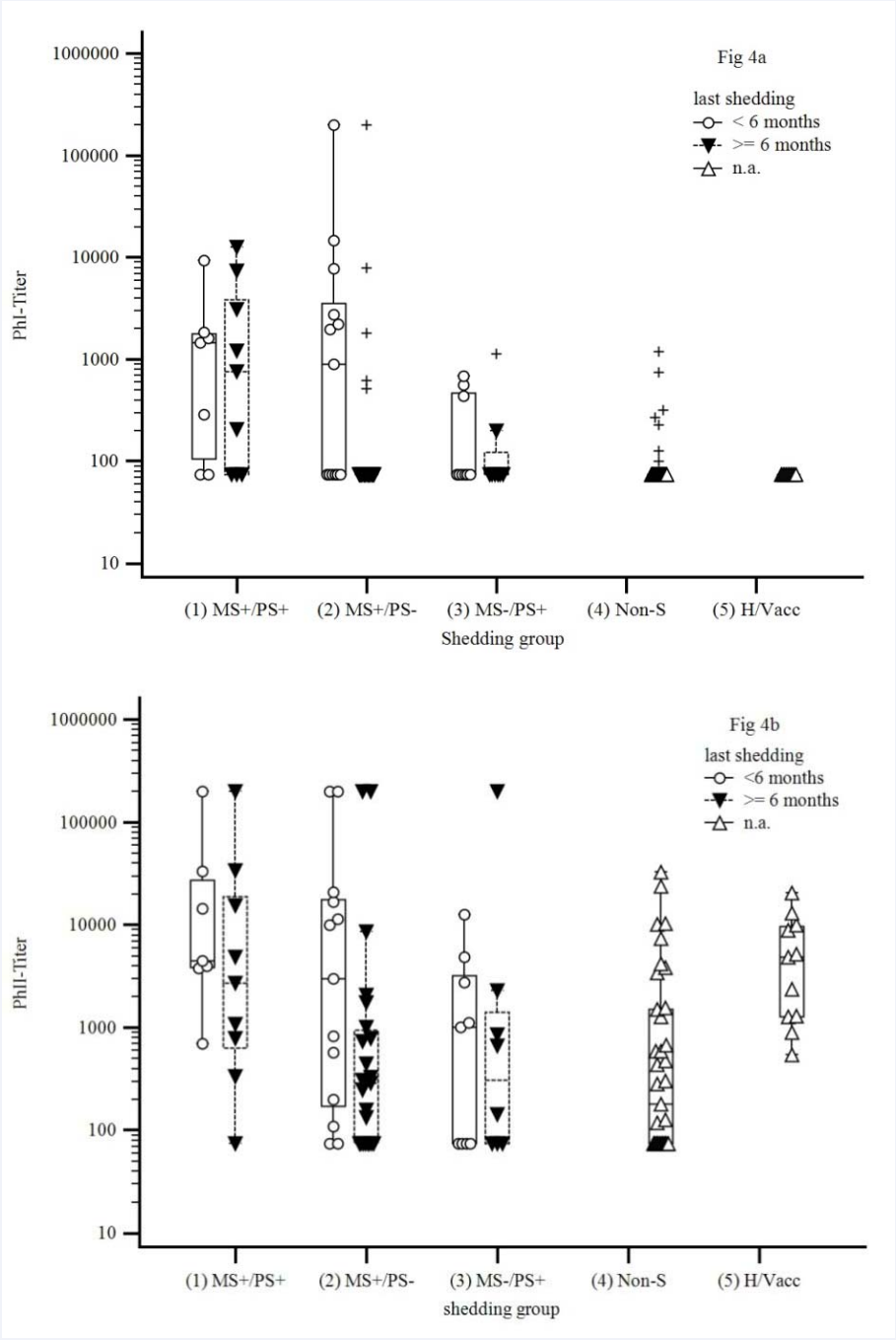

Figure 4 PhI- **(a)** and PhII-antibody titers **(b)** are shown for groups of cows and the time since the last detection of *C. burnetii* irrespective of the route of shedding (n.a. not applicable). Groups are described in Figure 3.

converted. The proportion of IFN-γ-positive animals was slightly higher (68.8%). Differences in PhII-antibodies (Σ PhII+) between groups can also be seen in Table 1. The proportions of PhII+-samples in groups 1 (MS+/PS-) and 5 (H/Vacc) were significantly higher than in groups 3 (MS-/PS+) and 4 (Non-S).Compared to PhII-antibodies PhI-antibodies were less frequently observed (Figure 4a); PhI-antibodies were primarily detected in cows that shed *C. burnetii* in milk. Here group 1 (MS+/PS+)differed from all other groups (Table 1, PhI+/PhII+); remarkably, higher percentages of PhI+/PhII+ animals were observed in the group 2 (MS+/PS-)when shedding was recently detected (<6 months). It indicated that PhI-reactivity - at least in some animals - is related to concurrent detection of *C. burnetii* in milk. Consequently, animals in groups 1 (MS+/PS+) and 2 (MS+/PS-) should not necessarily be considered as chronically infected. The occurrence of transient or intermittent shedding of *C. burnetii* in milk had been previously reported. In addition, studies showed that PhI-titer can be used as a suitable indicator to distinguish chronically infected cows from transient shedders [10].

Lastly, the ratio of percentages of PhII-titers (Σ PhII+) and

IFN-γ-reactivity were compared within groups (Table 1). These ratios differed within groups 1 (MS+/PS+) and 2 (MS+/PS-). In contrast, they were comparable within group 3 (MS-/PS+). Additionally, an inverse relationship of the percentages of PhI+/PhII+ and IFN-γ-results for groups 1 (MS+/PS+) and 3 (MS-/PS+) was apparent: 68.8% PhI+/PhII+ and 33.3% IFN-γ^+ in group 1 (MS+/PS+), and 29.4% PhI+/PhII+ and 68.8% IFN-γ^+ in group 3 (MS-/PS+), respectively. An intermediate position was observed for group 2 (MS+/PS-). However, animals with detection of *C. burnetii* shedding (< 6 months) and (> 6 months) tended to belong to group 1 (MS+/PS+) or group 3 (MS-/PS+), respectively.

Repeated and high-level milk-shedding of *C. burnetii* seemed to indicate a chronic infection in cows. It has been associated with strong antibody responses [9,10]. In the present study cows shedding *C. burnetii* in milk showed a tendency towards reduced IFN-γ-responses and increased titers of PhI-specific antibodies compared to cows shedding *C. burnetii* exclusively at calving (group 3, MS-/PS+). Taken together the detection of *C. burnetii* in milk, an increased PhI-specific antibody titer and a weak IFN-γ-response suggests a chronic infection comparable to chronic Q fever endocarditis in humans [4]. In contrast, cows that shed *C. burnetii* at calving were characterized by a favorable immune response, i.e., a stronger IFN-γ-reactivity, the presence of PhII-antibodies as well as weak or mostly absent PhI-antibody titers. The difference between these two groups is further substantiated by their immune response after vaccination with Coxevac™: Cows shedding *C. burnetii* only at calving showed a strong IFN-γ-response already after the first vaccination, whereas that of cows shedding *C. burnetii* in milk was weaker and retarded (Schumacher et al., manuscript in preparation). Based on these data we hypothesized that shedding at calving resulted in an increased level of herd immunity.

The low IFN-γ-reactivity in group 4 (Non-S) might be explained by uninfected, susceptible cows. However, only 12 of 37 animals in this group were sero negative. An alternative explanation might be that this reflected the contraction of lymphocytes to few memory cells after the infection was successful controlled. This assumption is further substantiated by the observation that antibody-positive non-shedders vigorously responded in the PhII-IFN-γ-RA already after primary vaccination (Schumacher et al, manuscript in preparation). However, this hypothesis of a down-regulated immune response below detection level is in disagreement with the observation that PhII-IFN-γ-responses increased when the pathogen-shedding ceased more than 6 months ago. Moreover, this increase of PhII-IFN-γ was at least in group 2 (MS+/PS-) associated with a decrease of antibodies. Instead, one might argue that *C. burnetii* induces the immunosuppressive cytokine IL-10 [22] which then explains low IFN-γ-reactivity in cows in which the pathogen was recently detected (Figure 3). IL-10 is probably down-regulated in the further course of successful control of infection and this might result in an increased IFN-γ-reactivity. Once the pathogen/antigen is completely eliminated, the IFN-γ-reactivity decreases again - resulting in the immune status as exemplified by the group 4 (Non-S).

In group 5, cows vaccinated as heifers (H/Vacc), strong IFN-γ-responses and only PhII-specific antibodies were detected, but puerperal swabs tested positive. Thus vaccination did not prevent them from infection. Consequently, at this time it cannot be determined whether the strong IFN-γ-response is due to vaccination or infection. However, it is clear that vaccination reduces the level of puerperal shedding, as reported previously [8,24]. Obviously, subsequent infection did not result in PhI-antibodies; and these cows did not develop chronic milk-shedding of *C. burnetii* (data not shown).

A common assumption is that an infection during pregnancy can trigger the development of chronic infections [25,26]. In contrast, we observed a remarkably strong IFN-γ-response after puerperal shedding of *C. burnetii* as long as it was not detected in milk; but puerperal shedding does not necessarily result from infection of the pregnant uterus. Currently, the infection of the udder and associated lymph nodes is regarded as a consequence of systemic infection. However, inspired by Marrie et al. [27], who emphasized that the route of infection determines the clinical outcome of an acute infection, we hypothesize that the udder is an additional route of primary infection for *C. burnetii*. The pathogen may enter the animal by galactogenic infection of the udder - a common route for mastitis pathogens. Additionally, control of intracellular pathogens in the udder as a mucosal immune-compartment might be regarded as insufficient [28]. A low IFN-γ-response in cows with concurrent detection of *C. burnetii* in milk supported this idea of an impaired immune response; it might be explained by pathogen-induced IL-10/TGF-ß during acute infection [22]. As described for the pathogenesis of chronic endocarditis in humans [20] in cows an initial IL-10-polarised immune response might be aggravated by a subsequent inappropriately timed infection of the pregnant uterus, so that an initially misdirected immune response is subsequently boosted during pregnancy. Earlier we reported that chronic milk-shedding was established in the course of first lactation; and that it was associated with shedding of *C. burnetii* at the second calving [10].

As a practical consequence, chronically infected cows need to be removed from the herd while susceptible heifers should be vaccinated prior to first breeding in order to reduce the susceptibility for infection. Although vaccination does not necessarily prevent infection, it reduced the amount of pathogen excreted and diminished the likelihood of a chronic infection of the udder [10,29].

CONCLUSION

Any improvement of our understanding about endemic circulation of *C. burnetii* within dairies depends on reliable diagnostics. Therefore, we analyzed the value of a quantitative Ph-specific serology and a *C. burnetii*-specific IFN-γ-RA in an endemically infected dairy cow farm [10,11].

Validation of immune diagnostics for Coxiellosis is a challenging task because reliable gold standards are not available [13,16]. However, Ph-specific serology and IFN-γ-testing revealed remarkable and unexpected differences between groups of infected cows. Thus, a misdirected immune response (low IFN-γ and PhI-antibodies) was observed in cows supposed to be chronically infected, while a more favorable response was detected in cows shedding *C. burnetii* at calving (group

3, PS+/MS-). Due to the limited number of animals per group significant differences could not always be assessed. However, it was the primary objective of this pilot study to advance our understanding of this infection in cattle and to generate new hypotheses. For instance, we included a possible hypothesis regarding the epidemiological role of chronic milk-shedders in maintaining long-term persistence of *C. burnetii* within herds and the possibility of a galactogenic infection. Our data are indicating that PhII-antibody testing is a promising tool to identify infected herds e.g.by surveillance of primiparous cows [11]. In contrast, PhI-antibody titers are of advantage to identify chronically infected animals: by a PhI-screening of individual milk samples animals at risk to be chronically infected might be preselected; these preselected samples are subsequently tested for *C. burnetii* by qPCR. By this procedure the costs for searching chronically infected cows are reduced [10]. A remarkably similar situation exists for bovine para tuberculosis, in which chronically infected "high shedders" are also characterized by strong antibody responses [30]. In both cases those "high shedders" need to be discriminated from cows intermittently excreting the respective pathogen.

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