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Insights into the dynamics of endemic *Coxiella burnetii* infection in cattle by application of phase-specific ELISAs in an infected dairy herd

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Abstract

Serological diagnosis of acute and chronic Q fever in humans relies on detection of antibodies to phase I (PhI) and II (PhII) antigens of *Coxiella (C.) burnetii*. Although phase-specific antigens are available, they are not yet used in ruminants as they are in humans. This study focuses on phase-specific serology as a tool for analysis of the dynamics of infection in cattle. As a prerequisite, sero-prevalence in Bavarian cattle (1) and sero-prevalences for age-groups (2) were determined by ELISA (CHEKIT Q-Fever; mix of PhI/PhII-antigen). Subsequently, phase-specific antigens were coated onto ELISA plates individually and tests were simultaneously applied in an endemically infected herd with about 90 dairy cows and 250 calves/heifers in April 2005, March 2006 and retrospectively in May and October 2004. From April 2005 onward, placentas were analysed for *C. burnetii* by PCR (3).

(1) Sero- and herd prevalences based on 21,051 sera from 603 Bavarian dairy farms collected in 2003 were 14.8% +/-0.48% and 72.3% +/-3.6%, respectively. (2) Analysis of 3965 animals from 105 farms for which age was reported revealed a base level of sero-prevalence of less than 5% in 1-2 years old animals, it increased to 15% in 2-3 years old and reached a plateau (25-30%) in cows four years and older. (3) In May 2004 and April 2005 a peak of $PhI^{-}/PhII^{+}$-prevalence in

primiparous cows (2.0-3.5 years) was observed; but not in October 2004 and March 2006. The $PhI^-/PhII^+$-pattern in primiparous cows changed to negative (one-third), $PhI^+/PhII^+$ (1/3) or persisted (1/3). In contrast, sero-conversion was rare in multiparous cows (>3.5 years). If the $PhI^-/PhII^+$ pattern was detected, it was due to an infection in preceding years. This pattern persisted (2/3) or changed to negative (1/3); a change to $PhI^+/PhII^+$ did not occur. $PhI^-/PhII^+$ in heifers (1-2 yrs) always changed to negative. Detection of PhII-antibodies was significantly associated with PCR-positive placentas. Remarkably, 45% of sera with the $PhI^-/PhII^+$ pattern were negative for the CHEKIT Q-Fever ELISA, thus this test missed an important group of infected animals.

Key words: Q fever, serology, cattle, phase I, phase II

Introduction

C. burnetii is a small gram-negative obligate intracellular bacterium. Sporulation preserves its infectivity even under harsh conditions outside the host (McCaul, 1991). Acute (e.g., pneumonia) and severe chronic infections (e.g., endocarditis, granulomatous hepatitis) are of concern in humans (reviewed by Fournier et al., 1998). In contrast, subclinical infection is common in cattle. If infection occurs, it is generally related to reduced reproductive performance rather than to abortion (To et al., 1998; Sting et al., 2000; Rodolakis et al., 2007), although an increased rate of abortion in primiparous cows has been related to *C. burnetii* infection (Hässig and Lubsen, 1998). Sheep and goats are regarded as a major source of human infection with the organism (Dupuis et al., 1987; Hellenbrand, 2001; Berri et al., 2002; Henning et al., 2009; Delsing and Kullberg, 2008). In contrast, human infections are rarely attributed to cattle (Hellenbrand, 2001), although cattle, once infected and sero-positive, are regarded as persistently infected for life (Aitken, 1989; Lang, 1990).

Acute and chronic infections in humans have to be discriminated because of differences in prognosis and therapy. This is achieved serologically by utilising a phenomenon displayed by *C. burnetii* called phase variation: *C. burnetii* exists in two antigenic phases (PhI and PhII). Phase variation is similar to the rough-smooth variation in enterobacteria and is due to a partial loss of

lipopolysaccharide (LPS) (Hackstadt et al., 1985; Toman et al., 1996). PhII-antibodies are produced early after infection with virulent *C. burnetii*, whereas the increase of PhI-titre is delayed (Dupuis et al., 1985; Williams et al., 1986). A low level of PhI-antibodies and a decrease in levels of PhII-antibodies is a common finding months after acute infection. On the other hand, cases of chronic endocarditis are characterised by low or absent reactivity of IgM and a strong IgG- and IgA-response against both PhI and II antigens (Worswick and Marmion, 1985). The immunofluorescence antibody test (IFAT) is regarded as a gold standard for serological diagnosis of Q-fever in humans (Fournier et al., 1998). In contrast, the complement fixation test (CFT) is still frequently used for serological diagnosis in ruminants (Sting et al., 2004); furthermore, CFT is used as a standard for validation of ELISA, although its sensitivity is known to be low (Kittelberger et al., 2009).

Because of the endemic nature, we sought to analyse the dynamics of infection within dairy farms. Initially, sero-prevalence and age-related sero-prevalences were determined by a commercial ELISA (CHEKIT Q-Fever; Idexx, Ludwigsburg). Subsequently, one endemically infected dairy farm was analysed for phase-specific antibodies.

Materials and Methods

ELISA: Three types of ELISA were included in the present study: a commercially available indirect ELISA using a mixture of PhI and -II-antigens (CHEKIT Q-Fever, Idexx, Ludwigsburg) and two prototypes with PhI- and -II-antigens coated separately (PhI- and PhII-ELISA). PhI- and PhII-coated plates were provided by Idexx: antigens, their concentrations and coating conditions were the same as for CHEKIT Q-Fever. The same negative and positive controls were used. The test was performed according to CHEKIT Q-Fever instructions. OD% between 30-40% was scored as equivocal; OD% values less than 30% and higher than 40% were interpreted as negative and positive, respectively. The cut-off for the phase-specific ELISA was 40%. The monoclonal peroxidase conjugate recognises bovine IgG1.

PCR: DNA from placentas was purified using the QIAmp® Mini-Kit (Qiagen, Hilden). Polymerase Chain Reaction (PCR) was performed according to Willems et al. (1994).

Handling of blood samples: Blood samples included in this study were centrifuged (2000 rpm, 20 minutes); the serum was collected and frozen at -20°C until testing.

Assessment of seroprevalence: To determine sero-prevalence and herd prevalence of antibodies directed against *C. burnetii* in Bavaria, 21,051 sera from 603 herds were tested using the CHEKIT Q-Fever ELISA. Selection of herds was proportional to the number of cattle per district. Only herds with at least 10 animals were included; all animals per herd for which samples were available were tested. The age of the animals used was >18 months.

Assessment of sero-prevalence for age-groups: For analysis of age-related effects of sero-prevalence, 3,965 sera from 105 farms were analysed with the CHEKIT Q-Fever ELISA. Prevalence was plotted over age-groups.

Herd A, an endemically infected dairy farm: Herd A is a dairy farm with about 90 cows and 250 calves/heifers. Cows are kept in two groups: a freestall barn and a smaller tie-stall. Calving occurs in six boxes. Calves are removed from the cow after birth; they are kept single for the first days and are housed in groups in a barn separated from that with cows. Cattle older than four months are reared on a second location about 50 km away. They return the first time for breeding and again 6-8 weeks before calving. During these periods of return animals are kept away from cows in a separate barn; two weeks before calving pregnant heifers are introduced into the cow herd for acclimatisation.

C. burnetii had been detected sporadically over the past years in herd A. A case of abortion occurred (5.4.2005) and *C. burnetii* was detected by PCR in the aborted foetus. The farmer agreed to join the present study in April 2005.

Placental material (n = 67) was collected from a proportion of cows between April 2005 and March 2006 in order to determine the rate of cows shedding *C. burnetii* at birth.

Blood samples were collected in April 2005 and March 2006: 28.4.2005 (n=344) and 1.3.2006 (n=268). Sera from May (18.5.2004; n=92) and October 2004 (21.10.2004; n=88) had been stored at -20°C and were analysed retrospectively. These sera had been collected within a different project; therefore young animals were not included.

Sera were analysed by phase specific ELISAs. Samples were interpreted by phase patterns: $PhI^-/PhII^-$, $PhI^-/PhII^+$, $PhI^+/PhII^-$ and $PhI^+/PhII^+$.

Based on all animals for which age had been reported the prevalences (%) of the phase-patterns per age-group were calculated (respective phase pattern positive/analysed animals per group).

Those animals which had been tested on two subsequent points in time (i.e. May 2004 versus (vs) October 2004, October 2004 vs April 2005 and April 2005 vs March 2006) were selected and prevalence (%) of phase-pattern ($PhI^-/PhII^+$ and $PhI^+/PhII^+$) was determined and plotted over age-groups at the first respective point in time of sampling.

For individual animals the change of phase pattern was determined for the following groups: heifers (1-2 yrs), primiparous cows (2-3.5 yrs) and multiparous cows (>3.5 yrs). For 139 cows data of at least two subsequent samplings were available, whereby 62, 12 and 65 cows had been tested four, three and two times, respectively. 275 comparisons on 139 animals have been performed. The age at the first respective sampling was used for classification of age groups.

PCR- and phase pattern results were compared. For 32 cows of 67, which had been analysed by PCR, results on phase patterns in April 2005 and March 2006 were available.

Finally, phase patterns were compared to CHEKIT Q fever. Therefore results of 517 sera which had been tested with phase-specific ELISAs and CHEKIT Q fever were compared.

Statistical analysis of the data was performed with MedCalc® version 9.5.2.0. (MedCalc Software, Broekestraat 52, B-9030 Mariakerke, Belgium).

Results

Because data on antibody prevalence for Bavaria were incomplete and relied on CFT, sero- and herd prevalences were assessed as a prerequisite for any further investigations on the dynamics

129 of infection. Sero- and herd prevalences were 14.8% (CI95%: 14.3-15.3%) and 72.3% (CI95%:
130 68.7-75.9%), respectively. Fig. 1 shows the relative distribution of herds according to intra-herd-
131 prevalence (IHP%).
132 Analysis of 3,965 animals with the CHEKIT Q-Fever ELISA revealed age-related effects. In Fig.
133 2, rates of positive sera are plotted over age groups. A baseline level below 5% was observed in
134 animals 1 to 2 yrs old, followed by a sharp increase in the next age group, while further increases
135 were less pronounced.
136 In April 2005, material from an aborted foetus from herd A was submitted for analysis. Placental
137 material was positive for *C. burnetii* by PCR. The number of analysed placentas and the
138 cumulative number of positive samples, as determined by PCR, are summarised in Fig. 3. The
139 steepest increase was reported for July and August 2005, while no further increase was observed
140 between October 2005 and March 2006. Except for the initial case the detection of *C. burnetii* in
141 placental tissue was not associated with abortion.
142 Because an age-related sero-conversion was observed (Fig. 2), serological profiles of three
143 possible phase-patterns ($PhI^-/PhII^+$, $PhI^+/PhII^+$, $PhI^+/PhII^-$) were plotted over age groups (Fig. 4a-
144 d). A striking regularity of phase-patterns was visible: a peak in the $PhI^-/PhII^+$ -pattern was
145 observed in cows 2.6-3.0 yrs old in May 2004 and April 2005, but this peak was absent in
146 October 2004 and almost absent in March 2006. A second peak of this phase-pattern was
147 observed in cows 3.6 to 4.0 yrs old on both occasions in 2004. This second peak was present in
148 April 2005, as a shoulder of the first peak. A characteristic peak of the $PhI^+/PhII^+$-pattern in
149 animals 3.1-3.5 yrs old was regularly observed. Its dominance in March 2006 has to be stressed.
150 Finally, the $PhI^+/PhII^-$ -pattern was rarely detected.
151 Calves and young cattle were only analysed in April 2005 and March 2006, respectively. The
152 $PhI^-/PhII^+$-reactivity in cattle 1.0-2.0 yrs old was only observed in April 2005 and was
153 completely absent in the following year. In contrast to April 2005 (Fig. 4c), the $PhI^-/PhII^+$ -

154 pattern was absent in cattle between 1.0 and 2.0 years of age, and the $PhI^+/PhII^+$ pattern was
155 significantly reduced in animals 2.1-2.5 yrs old.
156 For better comparison of changes in phase-pattern between two samplings, further analysis
157 focused on those animals that were analysed at two subsequent points in time. The age at the
158 respective first sampling was used as the variable for presentation of prevalence (Fig. 5a-c). An
159 age-related regularity became obvious: in the 2.0-3.0 yrs age group, the prevalence of the PhI^-
160 $/PhII^+$ pattern decreased and that of the $PhI^+/PhII^+$ pattern increased (Fig. 5a and c). Notably, this
161 finding was not observed between October 2004 and April 2005 (Fig. 5b). The situation in cows
162 3.0-4.0 yrs old is best described as a nodal point, because prevalences were close together and
163 they did not change. In animals exceeding the age of 4 yrs, a clear divergence of both phase-
164 patterns was characteristic, with minimal change of prevalence between both points in time.
165 Surprisingly, a 10% decrease of $PhI^+/PhII^+$-prevalence was observed between October 2004 and
166 April 2005 (Fig. 5b).
167 Results of individual animals from two age-groups (2.0-3.5 years; >3.5 years) at two subsequent
168 points in time were compared (Table 1). Analysis of animals with the $PhI^-/PhII^+$ pattern at the
169 first point in time revealed three things: First, cattle 1.0-2.0 yrs old always lost their PhII-
170 reactivity and became negative (not included in Table 1). Second, for cattle 2.0-3.5 yrs old, three
171 outcomes with equal likelihood were possible: one-third switched to $PhI^+/PhII^+$, one-third
172 became negative and the last third remained as $PhI^-/PhII^+$. Finally, cows older than 3.5 yrs at the
173 time of the first test became negative (one-third) or remained $PhI^-/PhII^+$ (two-thirds), but a
174 transition to $PhI^+/PhII^+$ was never observed. A transition from negative to $PhI^-/PhII^+$ (n = 5) or
175 $PhI^+/PhII^+$ (n = 10) was observed in young cows (<3.5 yrs), whereas it was rare in old cows
176 (>3.5 yrs). Although the $PhI^+/PhII^-$ pattern was a rare finding, seven multiparous cows with the
177 $PhI^+/PhII^-$ pattern subsequently became negative. For 10 animals that were $PhI^+/PhII^-$ at the
178 second point in time, this pattern developed from $PhI^+/PhII^+$ (6) or was due to persistence (2).
179 Analysis of all animals with $PhI^+/PhII^-$ pattern revealed a median for PhI-OD% of 75%.

Persistence of the $PhI^-/PhII^+$-pattern was observed in March 2006 in 9 cows that were older than 3.5 yrs in April 2005. Samples from May and October 2004 were available and analysed for 7 and 6 cows, respectively (Fig. 6; N° 1-7): two cows (N° 1 and 5) had PhI-antibodies in May 2004 but OD% decreased to levels below the cut-off. Another three cows (N° 3, 6 and 7) had borderline PhI-antibodies: OD% decreased in cows N° 3 and 7, while for cow N° 6 an increase in OD% was observed for both phases between October 2004 and April 2005.

Throughout this paper, phase-patterns were used for classification of sera. Samples from 164 cattle collected in April 2005 were compared with respect to PhII and PhI-OD%. Reactivity between 30% and 40% was regarded as equivocal, whilst, in this study, sera with OD%≥40% were scored as positive (Fig. 7). It becomes evident that for a few animals, there is only a gradual difference between $PhI^-/PhII^+$ and $PhI^+/PhII^+$.

Sixty-seven placentas had been submitted for PCR. For 32 cows serological data from April 2005 and March 2006 were available. Phase-patterns in April 2005 and March 2006 were compared and PCR-positive animals are shown in Table 2. *C. burnetii* was detected in 11 cases: Four animals were 3.0-3.5, four 3.6-4.0 and three 4.9 to 5.3 years old in May 2005. *C. burnetii* could be detected by PCR in 9 of 17 placentas of $PhII^+$ cows in March 2006 but in only 6 of 13 PhI^+ cows. Analysis of these data with Fisher's exact test revealed that PhII-antibodies were significantly associated with detection of *C. burnetii* by PCR (p=0.027), whereas detection of PhI-antibodies was not (p=0.243). In two sero-negative cows, *C. burnetii* was detected by PCR. For these animals, data from 2004 were available as well. These data were negative at each testing (Fig. 6; N° 7 and 8).

CHEKIT Q-Fever ELISA was compared with phase-patterns (Table 3). Overall, 52 (45%) of 115 samples with the $PhI^-/PhII^+$ pattern were scored as negative with this test, when a cut-off of 40% was used. Seventeen samples showed reactivity between 30-40%. Median, first and third quartile for PhI (PhII) reactivity were 7.3 (79.5), 3.0 (58.0) and 14.0 (109.5), respectively. Samples with PhI-reactivity were always scored as positive. Finally, eleven (2.9%) of 381 animals with PhI^-

/PhII$^-$ pattern scored positive in CHEKIT Q-Fever ELISA. For eight samples CHEKIT Q-Fever OD% was between 40 and 50%, for the remaining three it was 69%, 97% and 111%, respectively.

Discussion

To our knowledge, this is the first report on the use of phase-specific ELISA for the detection of antibodies to *C. burnetii* in cattle. Because ELISA plates that came with the commercialised CHEKIT Q-Fever were coated with a mix of PhI- and PhII-antigens, the separate coating of these antigens was the easiest approach with which to assess the value of phase-specific serology. PhI- and -II-ELISAs were prototype tests and a proper validation could not be performed under field conditions. However, the same concentration of antigen as used for CHEKIT Q-Fever ELISA was coated to test plates; furthermore, the same test protocol, controls and cut-off values were used in this study.

Comparison of CHEKIT Q-Fever with prototype phase-specific ELISAs revealed that almost 50% of PhI$^-$/PhII$^+$ sera tested negative in the former (Table 3). Apparently, CHEKIT Q-Fever ELISA was inefficient in detecting PhII-antibodies. This is important since PhII-reactivity - not PhI - was significantly associated with detection of *C. burnetii* by PCR (Table 2). PhII-antigens are essential for serological diagnosis of Q fever. CHEKIT Q-Fever was criticised in the past for its low sensitivity because *C. burnetii* was detected by PCR in sero-negative ruminants; this was especially true for small ruminants but less obvious in cattle (Arricau-Bouvery and Rodolakis, 2005; Rodolakis et al., 2007; Kittelberger et al., 2009). A lack of sensitivity was attributed to the use of the tick-derived Nine Mile strain of *C. burnetii* and, consequently, the use of a ruminant strain in ELISA was recommended (Rodolakis et al., 2007). However, Guatteo et al. (2000) implemented an ELISA (Cox Ruminants®, LSI, Lisseux, France) based on a ruminant strain, and they reported that this ELISA failed to detect antibodies in cows that were positive for *C. burnetii* by PCR. Alternatively, the problem could be explained by a lack of PhII-antigens on ELISA plates. Inefficient binding of the truncated PhII-LPS as compared to the PhI-antigen to

ELISA plates and/or competition of both antigens for adsorption might be critical in the context of production (Hackstadt et al., 1985; Toman et al., 1996). Notably, an improved performance of CHEKIT Q-Fever had been demonstrated recently (Kittelberger et al., 2009). Moreover, it has to be kept in mind that ELISA was calibrated by CFT and on the other side the value of PCR-positive results must be analysed critically. Last but not least, the immune response towards an intracellular parasite - as discussed later - has to be considered, when differences between ELISA and PCR are discussed.

Following infection in humans and guinea pigs the PhII-IgG response is detected before the increase of PhI-IgG (Dupuis et al., 1985; Williams et al., 1986; Worswick and Marmion, 1985). The absolute dominance of the $PhI^-/PhII^+$-pattern over $PhI^+/PhII^+$, as observed in April 2005 (Fig. 4c) in cows 2-3 yrs old, as well as a change in phase-pattern prevalence in this age-group almost one year later (Fig. 4d, Fig. 5c), are in good agreement with early PhII- and late PhI-antibodies. The probability of detecting *C. burnetii* should be higher in acutely infected cattle. Indeed, a significant association between PhII-antibodies and detection of *C. burnetii* in placenta by PCR was observed. Most remarkably, our data support the finding of Hässig and Lubsen, (1998) that infection with *C. burnetii* was associated with abortion in primiparous but not in multiparous cows. The initially observed abortion and an associated shedding of Coxiellae might explain the dominance of $PhI^-/PhII^+$ in April 2005. But it doesn't explain the detection of $PhI^-/PhII^+$-pattern in heifers which are kept at another location.

We assumed that we would observe a classical transition from sero-negative to $PhI^-/PhII^+$ (= acute infection) to $PhI^+/PhII^+$ (= chronic infection). Surprisingly however, serological transition of $PhI^-/PhII^+$ cattle was age-related: Animals that were one to two years old always became negative upon subsequent testing. PhII-reactivity in these heifers might be explained by maternally derived antibodies. We concluded that the $PhI^-/PhII^+$ pattern in heifers (10%) in 2005 reflected active infection with *C. burnetii* because PhII-reactivity was absent in the same age-group in March 2006. Two of these animals tested negative by PCR at calving; although

additional heifers with the $PhI^-/PhII^+$ pattern need to be tested for shedding of *C. burnetii* at parturition to confirm its absence, we assumed establishment of immunity in these animals. This view is supported by vaccination trials in which protection against persistent infection could be achieved by vaccination of non-pregnant animals before the first breeding (Woernle and Müller, 1986; Guatteo et al., 2008).

For primiparous cows (2.0-3.5 yrs), three possible outcomes were observed with equal likelihood: Transition to negative (1/3), transition to $PhI^+/PhII^+$ (1/3) or persistence of the $PhI^-/PhII^+$ pattern (1/3). Remarkably, detection of the $PhI^-/PhII^+$ pattern in multiparous cows (≥3.5 yrs) was due to persistence rather than to acute infection (Fig. 6). Only two possible outcomes were observed: One-third still became negative, while two thirds persisted in this pattern. A transition to $PhI^+/PhII^+$ was never observed.

A simple translation of $PhI^-/PhII^+$ and $PhI^+/PhII^+$ patterns of individual animals in terms like 'acute' and 'chronic', respectively, is inappropriate. However, the level of animals categorised as $PhI^-/PhII^+$ seems to reflect the extent of 'acute infection' at the herd level. The probability of detecting *C. burnetii* in PhII-postive animals at subsequent calving is 9/19 (47%; Tab. 2); it is independent of the $PhI^-/PhII^+$- or $PhI^+/PhII^+$ pattern.

The important role of primiparous cows in endemically infected herds does not generally exclude the possibility of infection and subsequent sero-conversion in older cows. Indeed, an increase of sero-prevalence with age is demonstrated in Fig. 2. However, despite intensive shedding in the summer 2005 and frequent seroconversion in primiparous cows about 60% of cows older than 4 yrs remained sero-negative (Fig. 5c and Table 1). Similar rates of sero-negative (ELISA) cows in endemically infected herds were reported by Guatteo et al. (2000). We assumed that the majority of these cows had already experienced infection and mounted protective immunity. Environmental contamination (as a result of shedding in faeces, urine, vaginal mucus or by placental material) with *C. burnetii* had already been demonstrated by Yanase et al. (1998). Thus it is assumed that all animals within a barn were exposed. The assumption that at least some of

these multiparous cows could have built up an efficient cellular immunity with low or undetectable levels of antibodies, as in mycobacterial infections, is supported by data obtained in humans: Ascher et al. (1983) reported that only 14 (61%) of 36 skin-test positive people were antibody positive by IFAT; a comparison with CFT revealed that only 52% of the people positive upon skin-testing were also antibody-positive (Hustson et al., 2000). A transient PhI^- /$PhII^+$-pattern, as observed for animals in this study, likely disappeared because *C. burnetii* was successfully controlled.

What is the event determining the outcome of infection with *C. burnetii*? Pregnancy might be important. During pregnancy, a shift of immunity towards T_H2-activity with elevated levels of immunosuppressive IL-10 occurs. It is probably further enhanced by *C. burnetii* itself (Capo et al., 1996). Such a polarisation of the immune response was described as the T_H1/T_H2-paradigm in mice and humans (Wegmann et al., 1993; Romagnani, 1997). Not only does T_H2-activity direct the immune response in an unfavourable direction to control an intracellular parasite, but IL-10-mediated immunosuppression could also activate *C. burnetii* in persistently infected cows during pregnancy. On the positive side, the benefit could be prevention of immune-mediated placentitis and subsequent abortion. The hypothesis that pregnancy is an important trigger relies on non-immune heifers at the time of first pregnancy.

About 50% of the foetuses in the endemically infected herd – PCR-positive results in $PhI^-/PhII^+$ and $PhI^+/PhII^+$ cows at calving (Table 2) - were considered to have been exposed to *C. burnetii* during pregnancy. The amount of bacteria present in such cases exceeds that necessary for *post-natal* infection by a multiple. What is the biological consequence of an *in-utero* infection? Sero-prevalence decreased almost to zero in calves between 6-12 months (Fig. 2 and Fig. 4c), but a transient antibody response due to *in-utero* infection would have been masked by maternally derived antibodies. Moreover, such transient antibody responses were observed in older animals (e.g., heifers). Additionally, a decrease of antibody titre in humans has to be regarded as a common event; it is associated with a favourable prognosis. What is the impact of immunity after

310 foetal or *post- partum*-infection? From an evolutionary point of view, such immune animals
311 characterised by cellular immune response in the absence of detectable antibodies could be
312 crucial for maintenance of endemic infection in dairy farms with *C. burnetii*: These immune
313 animals would give birth to non-exposed, non-immune calves that could be infected as pregnant
314 primiparous cows, with subsequent persistence of infection. The steady-state between infected
315 sero-positive cows and immune sero-positive/–negative cows would sustain endemic infection at
316 the herd level. This would explain why within-herd prevalence rarely exceeded 50%, and why
317 prevalence that exceeded this value rather indicates active infection at the herd level (Fig. 1).
318 Any discussion of pregnancy as a possible trigger has to include the possibility that *in-utero*
319 infection and a shift towards T_H2-immunity might favour the establishment of oral tolerance.
320 Finally, systemic immunotolerance as described for BVDV (Peterhans et al., 2003) has to be
321 considered when sero-negative and PCR-positive animals are discussed (Table 2; Fig. 6: N° 8
322 and 9).
323 The situation in one endemically infected dairy farm was described. Whether or not these
324 findings can be extrapolated remains to be determined. As a prerequisite of our work on phase-
325 specific serology, we analysed sero-prevalence in dairy cattle. Sero-prevalence was assessed in
326 earlier studies, but they were restricted to certain regions and relied on complement-fixing
327 antibodies: sero- and herd prevalences in the northern parts of Bavaria in 1986 were 7.6% and
328 30%, respectively (Roth and Bauer, 1986). Analysis of cattle sera south of the Bavarian Forest
329 revealed a prevalence of 12% (Rehacek et al., 1993). In the present study, an ELISA was
330 implemented: *C. burnetii*-specific antibodies were found in 14.8% of animals older than 18
331 months and in 72% of the tested herds. The higher prevalences observed in this study are most
332 likely explained by higher sensitivity of the ELISA (Kittelberger et al., 2009). Although infection
333 is widespread, IHP% was less than 10% in 64.1% of herds and exceeded 50% in only 6.1% of
334 the herds. The prevalence of sero-negative but immune cows, as hypothesised above, probably
335 limits that of persistently infected sero-positive animals, which might be important for steady-

state infection within herds. As a consequence, IHP% exceeding 70% is rarely observed (Fig. 1) and might indicate recent herd infection.

This is the first report on phase-specific serology in cattle. The increase of sero-prevalence in animals between two and three years (Fig. 2) was associated with an increased prevalence of PhI^- /$PhII^+$-pattern. However, it has to be kept in mind that categorical classification as used in this study simplified the data. In some cases only gradual differences were observed between certain phase-patterns (Fig. 7). The authors are aware about critics that a validation of phase specific ELISAs is still outstanding. However, validation relies on defined negative and positive sera; but how to define these categories? Facing this uncertainty analysis of phase patterns for age-groups was a rather practical approach. Currently, a representative number of dairy farms are under investigation and a quantification of phase-specific antibodies, as in human medicine, will be performed.

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438

Table 1: Transition of phase pattern: Animals in herd A were tested in May 2004, October 2004, April 2005 and March 2006. For 139 cows data of at least two subsequent samplings were available, whereby 62, 12 and 65 cows had been tested four, three and two times, respectively. 275 comparisons on 139 animals have been performed. As an additional variable the age at the first respective sampling was included: Analysis was performed for primiparous cows (2.0-3.5 yrs) and multiparous cows (>3.5 yrs; in brackets). The total of three comparisons (May04 vs Oct04, Oct04 vs Apr05 and Apr05 vs Mar06) is given whereby the first respective point in time of sampling is "1st Test" and the subsequent date is "2nd Test".

		2nd Test				
		$PhI^-/PhII^-$	$PhI^+/PhII^-$	$PhI^-/PhII^+$	$PhI^+/PhII^+$	∑
1st Test	$PhI^-/PhII^-$	33 (60)	1 (0)	5 (1)	10 (1)	49 (62)
	$PhI^+/PhII^-$	0 (5)	0 (2)	0 (0)	0 (0)	0 (7)
	$PhI^-/PhII^+$	10 (7)	1 (0)	11 (14)	1 (0)	32 (21)
	$PhI^+/PhII^+$	2 (3)	3 (3)	4 (2)	31 (56)	40 (64)
	∑	45 (75)	5 (5)	20 (17)	51 (57)	121 (154)

Table 2: Comparison of phase-pattern and PCR-results: Between April 2005 and March 2006 67 placentas were submitted for analysis of *C. burnetii*-specific subgenomic sequences by PCR. For 32 cows phase patterns in April 2005 and March 2006 were available. The number of PCR-positive animals and the number of analysed placentas (in brackets) are recorded. Only those possibilities for which results were available are shown.

		2nd Test (Mar 2006)				
		$PhI^-/PhII^-$	$PhI^+/PhII^-$	$PhI^-/PhII^+$	$PhI^+/PhII^+$	∑
1st Test (Apr 05)	$PhI^-/PhII^-$	2(12)				2(12)
	$PhI^+/PhII^-$	0(1)				0(1)
	$PhI^-/PhII^+$	0(2)		2(3)	2(4)	4(9)
	$PhI^+/PhII^+$			1(1)	4(9)	5(10)
	∑	2(15)		3(4)	6(13)	11(32)

Table 3: Comparison of CHEKIT Q-Fever (CHEKIT QF) and phase-specific ELISAs: CHEKIT QF test plates are coated with a mix of PhI and PhII antigens. For phase-specific testing antigens were coated individually onto ELISA plates. Results in CHEKIT QF were compared with phase patterns.

CHEKIT QF	$PhI^-/PhII^+$	$PhI^+/PhII^+$	$PhI^+/PhII^-$	$PhI^-/PhII^-$
neg	52	0	0	381
pos	63	57	6	11

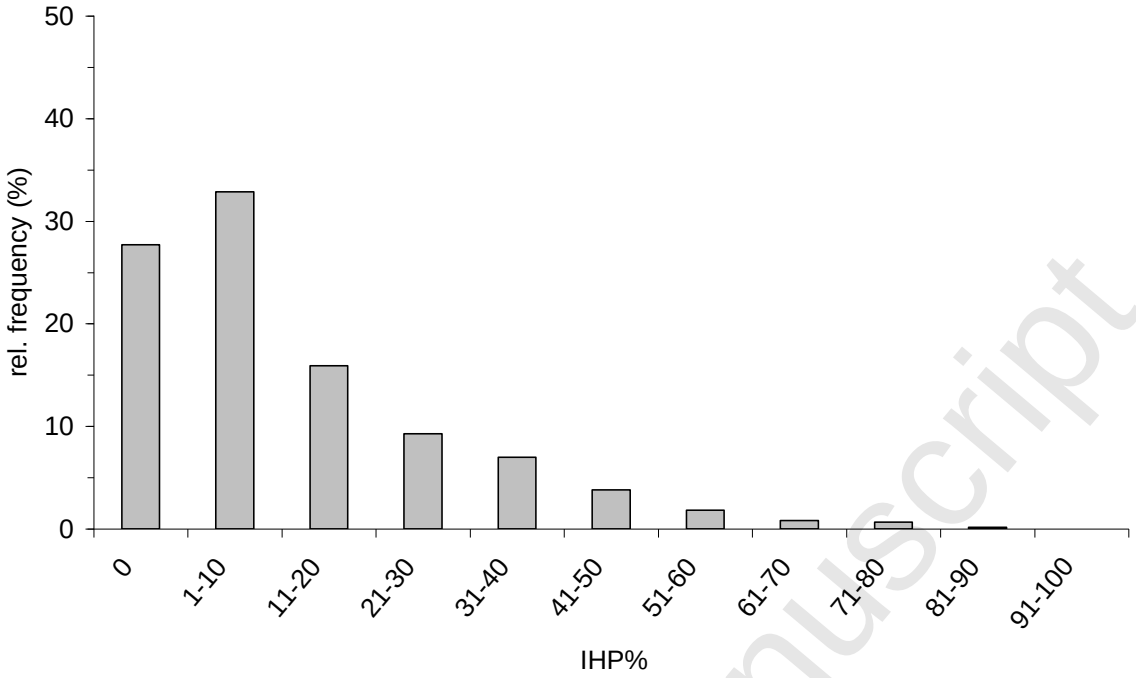


Figure 1: A total of 21,051 sera from 603 Bavarian dairy herds were analysed for antibodies to *C. burnetii* (CHEKIT-Q-Fever ELISA; Idexx, Ludwigsburg). The minimum sample number per herd was 10. For each herd, the relative intra-herd-prevalence (IHP%) of positive samples was calculated. The distribution of herds over classes of IHP% is shown.

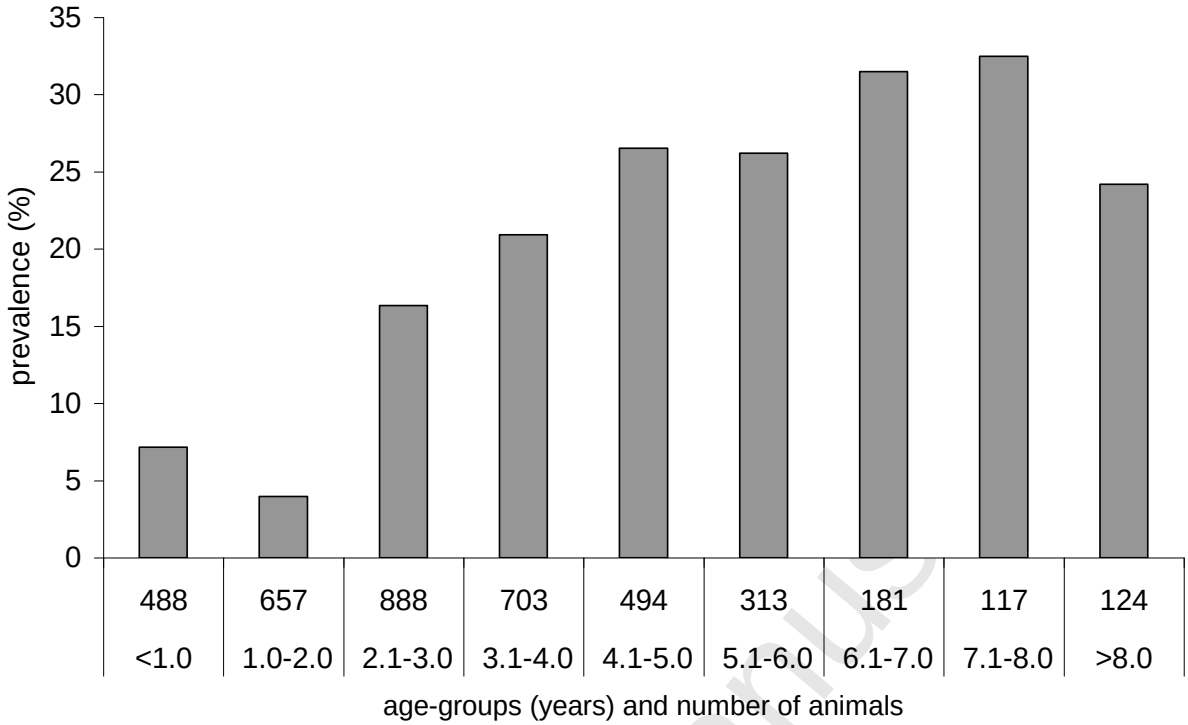


Figure 2: Relative frequency of sero-positive animals by age group. For analysis of age-related effects, 3,965 sera from animals were analysed for antibodies against *C. burnetii* in CHEKIT-Q-Fever ELISA.

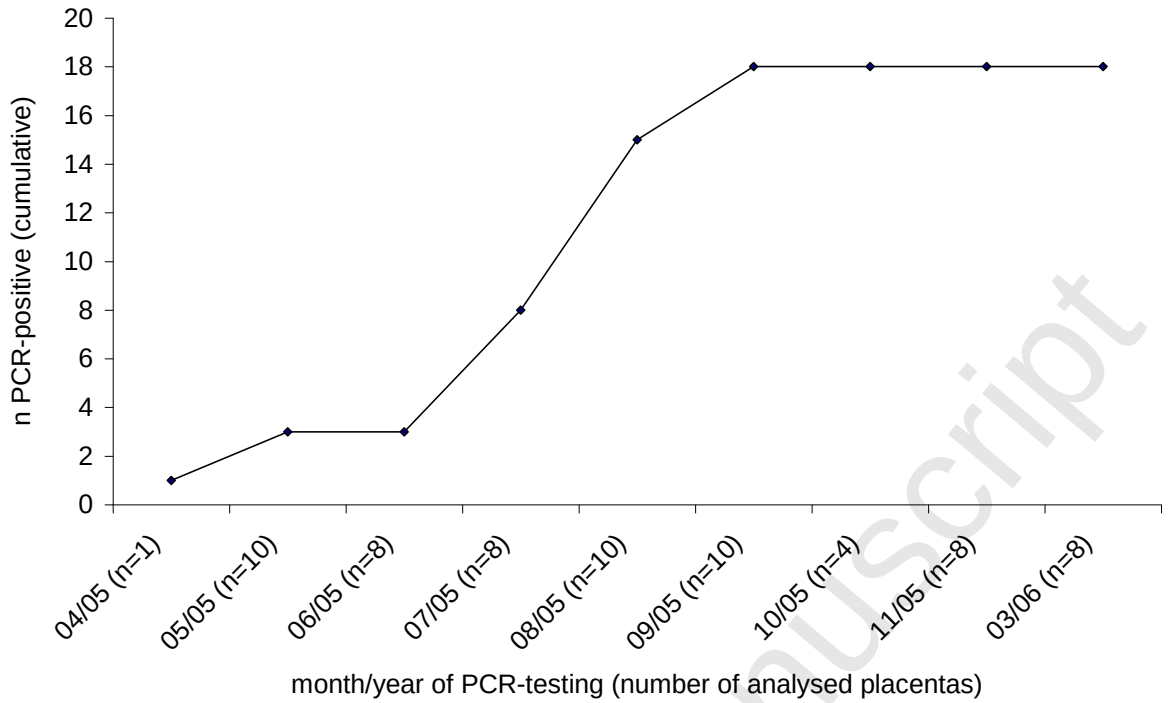


Figure 3: The cumulative number of PCR-positive placentas over time (month/year) and the number of analysed placentas per month in a dairy herd (herd A) in which *C. burnetii* was detected by PCR in April 2005.

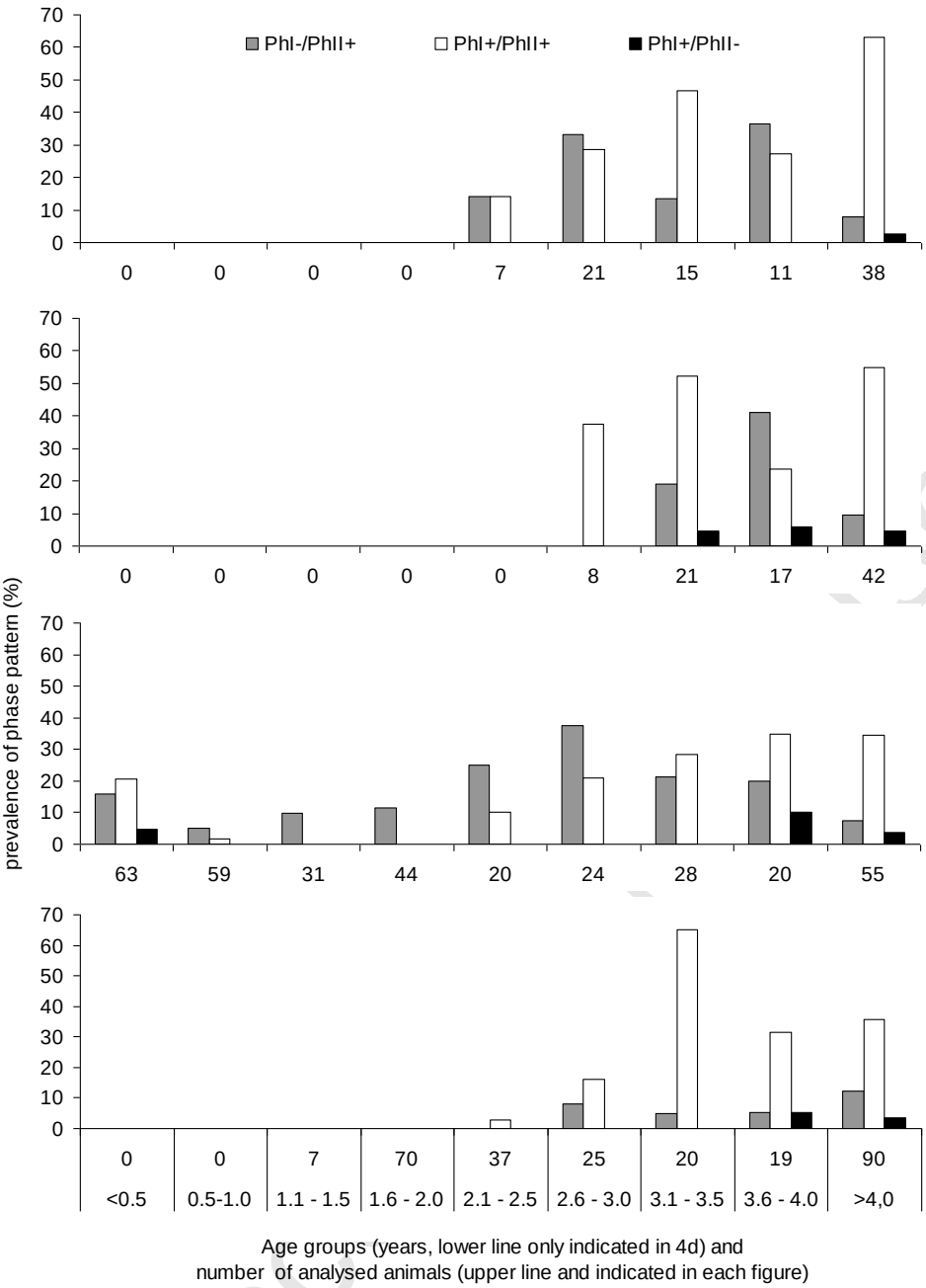


Figure 4a-d: In an endemically infected dairy farm, a cross-sectional analysis of sera for antibodies against *C. burnetii* in phase-specific ELISAs was performed on four occasions: May (a) and October 2004 (b), April 2005 (c) and March 2006 (d). Prevalences for three possible phase patterns ($PhI^+/PhII^+$; $PhI^-/PhII^+$; $PhI^+/PhII^-$) were calculated for each age group. Age groups and the number of analysed animals are indicated on the x-axis of 4d and each figure, respectively. Note: Not necessarily the same animals are represented in figures 4a-d,; for this it is referred to Fig. 5a-c.

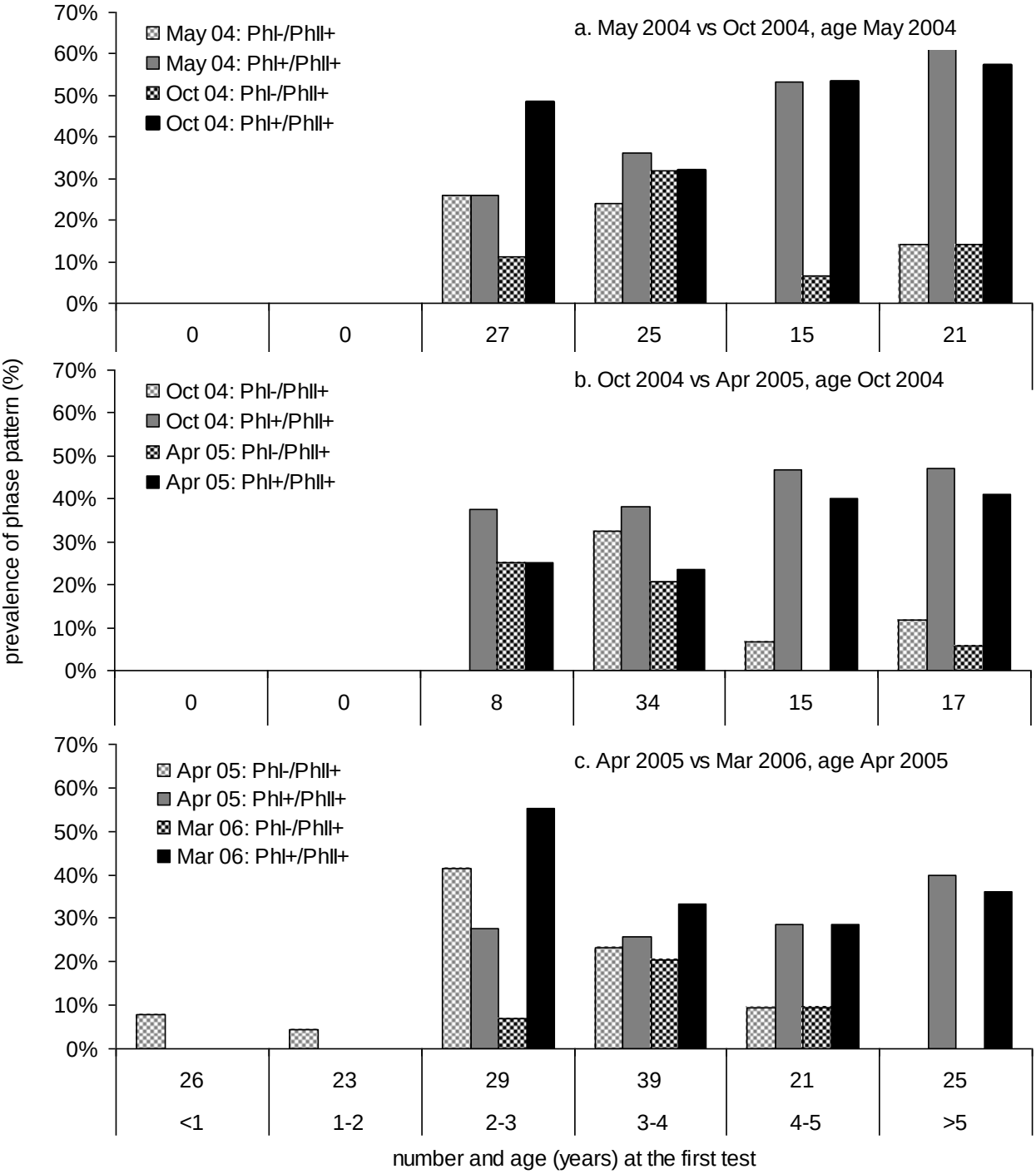
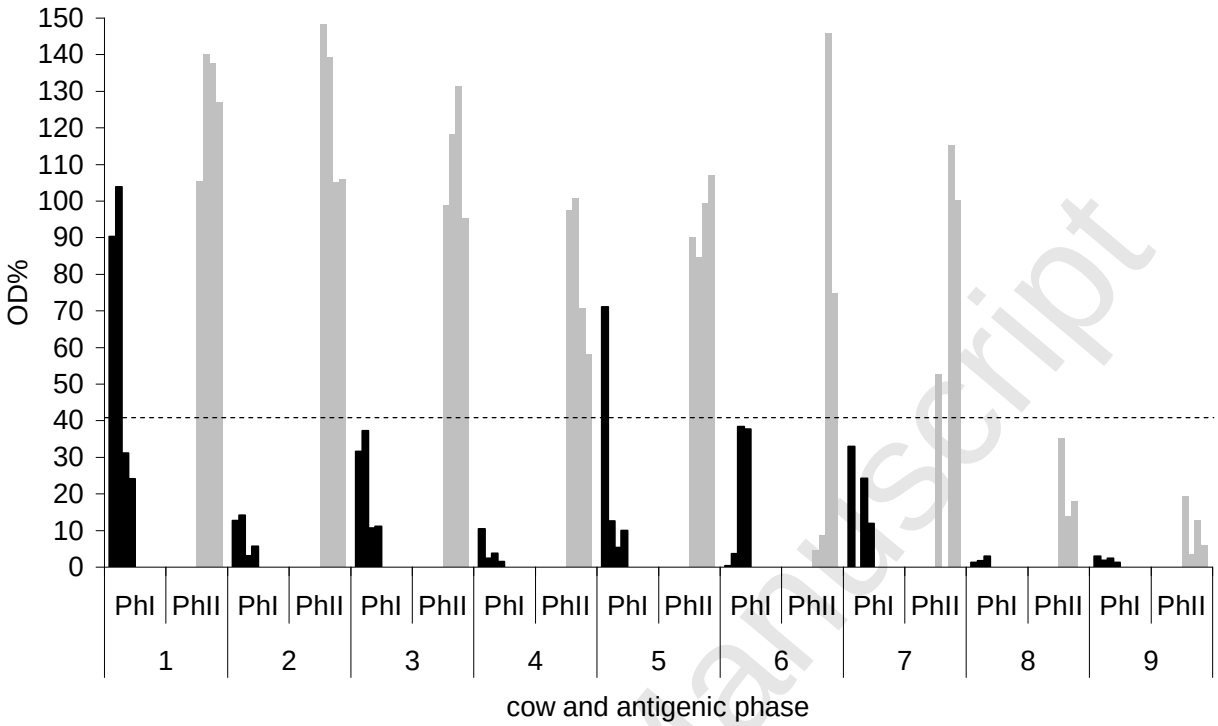


Figure 5a-c: Comparison of the prevalence of phase-patterns at two subsequent samplings: Cows in herd A were sampled in May and October 2004, April 2005 and March 2006. For animals sampled at two subsequent points in time prevalence of phase pattern was calculated and plotted over the age at the first respective date of sampling. The number of compared animals per age-group is indicated beneath each x-axis, whereas age-groups only once are shown at the bottom x-axis.

1



2

3 Figure 6: Multiparous cows with the $PhI^{-}/PhII^{+}$ pattern in Apr 2005 and Mar 2006 (N°1-7) and
4 two negative cows with PCR-positive results (N°8+9) were analysed for their phase-specific
5 reactivity over time. Each bar represents a sampling time point: May 2004, October 2004, April
6 2005, March 2006. No sample was available for cow N° 7 in Oct 2004. A preliminary cut-off of
7 40% (dashed line) is indicated.

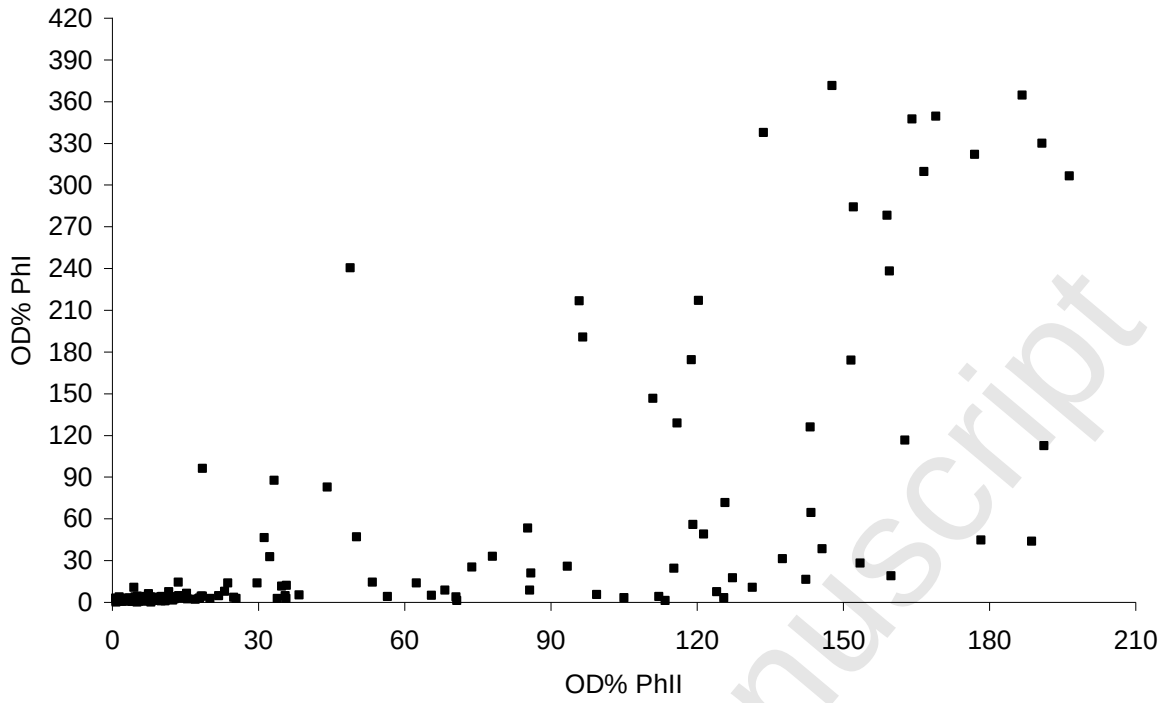


Figure 7: Comparison of optical densities (OD%) of 164 animals (herd A) for PhI and PhII antibodies in April 2005.