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The etiology remains unknown in many cases of bovine abortion in Switzerland. Bacteria of the Chlamydiales order are known abortive agents, therefore cases of bovine abortion from three representative regions of Switzerland were investigated in this study. Particularly Chlamydiaceae as well as the Chlamydia-like organisms Waddlia and Parachlamydia were of interest, especially because of their possible zoonotic potential. Placenta samples (n=343) were tested for these bacteria by different PCR-methods, immunohistochemistry and serology for Chlamydia abortus. Additionally an attempt for the isolation of Waddlia and Parachlamydia was made by co-cultivation in amoebae. In 67.3% of the 343 cases a necrotizing and/or purulent placentitis was found histologically. By real-time PCR, 0.9% (3/343) of the cases were positive for Waddlia, 13.4% (46/343) positive for Parachlamydia and 14.6% (50/343) positive or questionable positive for Chlamydiaceae. Of these samples, confirmation by immunohistochemistry was possible in 2/3 cases for Waddlia, 25/46 for Parachlamydia and 4/50 for Chlamydiaceae. Of the 50 cases positive or questionable positive for Chlamydiaceae, species-identification by ArrayTube Microarray or 16S rRNA PCR resulted in 41 cases positive for C. abortus whereas the presence of Chlamydia suis was confirmed in four and Chlamydia pecorum in one case. This study brought evidence for the importance of different members of Chlamydiales in different regions of Switzerland although Waddlia is not occurring in a high prevalence. On the other hand mixed infections with different Chlamydiales as well as with other abortigenic agents could be found.
Waddlia, Parachlamydia and Chlamydiaceae in bovine abortion

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Abstract

The etiology remains unknown in many cases of bovine abortion in Switzerland. Bacteria of the *Chlamydiales* order are known abortive agents, therefore cases of bovine abortion from three representative regions of Switzerland were investigated in this study. Particularly *Chlamydiaceae* as well as the *Chlamydia*-like organisms *Waddlia* and *Parachlamydia* were of interest, especially because of their possible zoonotic potential. Placenta samples (n=343) were tested for these bacteria by different PCR-methods, immunohistochemistry and serology for *Chlamydia (C.)* *abortus*. Additionally an attempt for the isolation of *Waddlia* and *Parachlamydia* was made by co-cultivation in amoebae.

In 67.3% of the 343 cases a necrotizing and/or purulent placentitis was found histologically. By real-time PCR, 0.9% (3/343) of the cases were positive for *Waddlia*, 13.4% (46/343) positive for *Parachlamydia* and 14.6% (50/343) positive or questionable positive for *Chlamydiaceae*. Of these samples, confirmation by immunohistochemistry was possible in 2/3 cases for *Waddlia*, 25/46 for *Parachlamydia* and 4/50 for *Chlamydiaceae*. Of the 50 cases positive or questionable positive for *Chlamydiaceae*, species-identification by ArrayTube Microarray or 16S rRNA PCR resulted in 41 cases positive for *C. abortus* whereas the presence of *Chlamydia (C.) suis* was confirmed in four and *Chlamydia (C.) pecorum* in one case.

This study brought evidence for the importance of different members of *Chlamydiales* in different regions of Switzerland although *Waddlia* is not occurring in a high prevalence. On the other hand mixed infections with different *Chlamydiales* as well as with other abortigenic agents could be found.

**Key words:** Bovine abortion, *Chlamydiaceae, Parachlamydia, Waddlia*
Introduction

The economic importance of bovine abortion is considerable. In Switzerland it results in a loss of 22-45 million Swiss Francs per year caused by 14’000-28’000 abortion cases calculated (Hässig et al., 2000). While the importance of some abortigenic agents such as *Neospora (N.) caninum* and bovine viral diarrhea virus (BVDV) has been shown, in many cases of bovine abortion (67.7%) the etiology remains unknown (Reitt et al., 2007). The infection of cattle with *Chlamydia (C.) abortus* is known to cause abortion during the 6th to 8th month of gestation with placentitis as the most consistent pathological feature reported among experimentally induced abortions (Idtse, 1984; Perez-Martinez and Storz, 1985). Zoonotic infection with *C. abortus* in pregnant women is known to cause spontaneous abortion (Pospischil et al., 2002a; Longbottom and Coulter, 2003). The *Chlamydia*-related organism *Waddlia (W.) chondrophila* was isolated from an aborted bovine fetus in the United States (Dilbeck et al., 1990) and in Germany (Henning et al., 2002). A possible zoonotic potential of *W. chondrophila* was suggested by an association of anti-*Waddlia* antibodies and contact with animals, where in women who had miscarriages the *Waddlia* seroprevalence was higher than in control women (p < 0.001) (Baud et al., 2007). *Parachlamydia (P.) acanthamoebae* is another member of the *Chlamydiales* that is involved in bovine abortion. *Parachlamydia* could be detected in bovine placenta samples originating from abortion cases from Grisons, Switzerland (Borel et al., 2007; Ruhl et al., 2009). The zoonotic potential of *P. acanthamoebae* is unclear but this agent might play an emerging role in human miscarriage (Baud et al., 2008).

Thus, the present study aimed to investigate (i) the role of *Chlamydiales* in different geographical regions of Switzerland, (ii) the role of *W. chondrophila* as an abortigenic agent, (iii) a possible co-infection of *Chlamydiales* and other abortigenic agents and
(iv) a correlation between placental infection and serological status on *C. abortus* in bovine.

**Materials and Methods**

**Abortion cases**

In total, 343 placental tissue samples of bovine abortions were collected for the present study. Of 102 cases (29.7%), fresh placenta samples were also collected and frozen at –80°C. In addition, maternal serum was available in 128 (37.3%) cases. All placenta samples (n=343) were fixed in 4% formalin and embedded in paraffin wax according to standard procedures. Histological sections of all placenta specimens (n=343) were stained with hematoxylin and eosin (HE) and examined for the type and degree of placentitis and/or vasculitis. In cases where a vasculitis was found, a Periodic-Acid-Schiff-staining (PAS) was performed for the detection of fungal structures. Smears of cotyledons or abomasal mucosa were stained by Koester (Bisping and Amtsberg, 1988) and Stamp (Stamp et al., 1950) and microscopically examined for the presence of *Coxiella (C.) burnetii* and *Brucella (B.) abortus* in all 343 cases. Immunohistochemistry for BVDV on mucosal, dermal or thyroideal tissue samples from the fetus was performed in 52 cases. Serum samples (n=298) from the dam were examined for the presence of antibodies to bovine herpesvirus infection (IBR-IPV) using an antibody-ELISA. Where indicated, selected cases (n=29) were investigated by a PCR for the detection of DNA of *Neospora (N.) caninum*.

Abortion cases (n=343) collected during the breeding seasons 2006 to 2010 originated from three different geographical regions in Switzerland:

Eastern part of Switzerland
183 placental samples came from the canton of Grison representing the mountainous regions of Switzerland (Eastern Alps) and were submitted from 2006 until early 2008 to the Cantonal Laboratory of Veterinary Bacteriology, Chur, Switzerland. All placental specimens were formalin-fixed and paraffin-embedded.

Western part of Switzerland

111 formalin-fixed and paraffin-embedded placental samples originated from the western part of Switzerland. Samples were collected by the Institute Galli-Valerio, Lausanne, Switzerland, in 2007 (n=35) and in 2009/2010 (n=76), respectively. The cases from 2009/2010 (n=76) included as well fresh placental tissue frozen at –80°C and serum samples.

Central part of Switzerland

49 abortion cases came from the central area of Switzerland. All samples (n=49) included formalin-fixed and paraffin-embedded placental specimens and serum from the dam. From 26 cases also fresh placental tissue was available.

DNA extraction and real-time PCR (rt PCR)

30μm sections of formalin-fixed and paraffin-embedded tissue samples were deparaffinized in xylene, centrifuged at 13’500 x g for 5 min and the xylene removed by a repeated extraction with ethanol followed by a second centrifugation and the removal of ethanol. The pellet was treated overnight with proteinase K on a thermomixer (55°C, 550rpm). The DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. All samples were examined for the presence of DNA of *Waddlia, Parachlamydia* and *Chlamydiaceae* by rt PCR.
Waddlia rt PCR

PCR reactions were performed as described previously (Goy et al., 2009). The ABI Prism 7000 (Applied Biosystems, Foster City, USA) was used to perform the amplification and detection of DNA with a cycling program of 3 min at 95°C and 45 cycles of 15 s at 95°C and 1 min at 60°C. Samples were tested at least in duplicates and were considered negative if no amplification was observed during all 45 cycles.

Parachlamydia rt PCR

PCR reactions were performed as described previously (Casson et al., 2008). Briefly, the cycling program was the same as for the Waddlia rt PCR and the ABI Prism 7000 (Applied Biosystems, Foster City, USA) was also used. Samples were tested at least in duplicates and were considered negative if no amplification was observed during all 45 cycles.

Chlamydiaceae rt PCR

The extracted DNA of the samples was investigated on an ABI 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) using the 23S-based Chlamydiaceae family-specific real-time PCR described previously (Ehricht et al., 2006). This method includes primers Ch23S-F (5'-CTGAAACCAGTAGCTTATAAGCGGT-3'), Ch23S-R (5'-ACCTCGCCGTTAACTTACTCC-3'), and probe Ch23S-p (FAM-CTCATCA TGAAAGGCACGCCG-TAMRA) and an internal amplification control consisting of primers EGFP-1-F (5'-GACCACTACCAGCAGAACAC-3'), EGFP-10-R (3'-GACCCAGTGCCCTGAGCA-BHQ1). A 111-bp product specific for members of the family Chlamydiaceae is produced as well as a 177-bp product for the internal
amplification control. A final volume of 25 µl for each tested sample was achieved by adding 2.5 µl of extracted DNA to 12.5 µl of 2X TaqMan® Fast Universal PCR Master Mix (Applied Biosystems) and a final concentration of 5pmol/µl of each primer and the probe (Microsynth, Balgach, Switzerland). The cycling program is started by an initial denaturation (95°C, 20 sec) followed by 45 cycles of denaturation (95°C, 3 sec and 60°C, 30 sec), with an automatically calculated cycle threshold value. All samples were tested in duplicates. If the duplicates showed a Ct value of < 38, the sample was considered as positive and if the mean Ct value was > 38 it was considered questionable positive.

ArrayTube (AT) Microarray for species identification of Chlamydiaceae
Samples that were positive or questionable positive by rt PCR for Chlamydiaceae were further examined by the species-specific 23S ArrayTube (AT) Microarray (Borel et al., 2008).

16S rRNA PCR and sequencing
All samples positive or questionable positive by Chlamydiaceae rt PCR but negative by AT Microarray were investigated by another PCR method targeting the 16S rRNA gene (Everett et al. 1999, modified). DNA sequencing was performed as described previously (Zweifel et al., 2009).

Immunohistochemistry (IHC)
All placentas that were positive or questionable by PCR for Chlamydiaceae, Waddlia and Parachlamydia were further investigated by immunohistochemistry for the presence of the respective antigen in formalin-fixed and paraffin-embedded placental specimens.
**Waddlia IHC**

All cases (n=3) with at least two Ct-values for *Waddlia* by real-time PCR, were tested by IHC using a specific mouse monoclonal antibody directed against *Waddlia* at a dilution of 1:2’000. For this investigation the detection kit (Dako ChemMate, Dako, Glostrup, Denmark) was used according to the manufacturer’s instructions. Antigen retrieval was performed in citrate buffer (pH 6.0, 98°C) for 20 min.

After the primary antibody was incubated for 1h, the endogenous peroxidase activity was inhibited with peroxidase-blocking solution for 5 min at room temperature (RT).

The incubation with the link-antibody and the HRP-conjugated streptavidin was performed at RT for 10 min each, after which the slides were developed in 3-amino, 9-ethyl-carbazole (AEC) substrate solution for 10 min and then counterstained with hematoxylin. A negative control of each section was performed using only the antibody diluent instead of the primary antibody. For the positive control, cell pellets experimentally infected with *Waddlia* were used (Borel et al., 2009).

**Parachlamydia IHC**

On all cases (n=46) showing at least two Ct-values for *Parachlamydia* by real-time PCR, immunohistochemistry using a specific mouse monoclonal antibody directed against *P. acanthamoebae* was performed at a dilution of 1:1’000. The same detection kit (Dako) was used as for the *Waddlia* IHC and the procedure for the labeling is similar to the procedure described above. For the positive control cell pellets experimentally infected with *P. acanthamoebae* were used (Borel et al., 2009).

**Chlamydiaceae IHC**
The presence of chlamydial antigen in paraffin sections was investigated on cases positive or questionable positive for *Chlamydiaceae* by rt PCR (n=50) using a *Chlamydiaceae* family-specific mouse monoclonal antibody directed against the chlamydial lipopolysaccharide (LPS, Clone ACI-P, Progen, Heidelberg, Germany). A detection kit (Dako) was used according to the manufacturer's instructions for detection. After deparaffinization in xylene and rehydration through graded ethanol to water, the antigen retrieval was performed by a 10 min enzyme digestion (Proteinase K, Dako). The endogenous peroxidase activity was inhibited as described above, then slides were incubated for 60 min with the primary antibody diluted 1:200 in antibody diluent (Dako). The incubation with the link-antibody and the substrate solution as well as the counterstaining with hematoxylin was performed as described above. Negative control of each section was performed as described above. For the positive control intestinal tissue from gnotobiotic piglets experimentally infected with porcine *C. suis* strain S45 was used (Guscetti et al., 2000).

Serology for *Chlamydia abortus*

All available maternal serum samples (n=128) from the central and the western part of Switzerland were tested with a commercial antibody-detecting ELISA assay (Institute Pourquier, Montpellier, France) specific for *C. abortus*. This test is validated for cattle, sheep and goats and was performed according to the manufacturer's instructions. The ratio between the corrected optical density (OD) of the sample (S) and the mean corrected density of the positive control (P) determines the final value and is expressed as S/P%. For cattle, sera with an S/P% higher or equal to 100% were interpreted as positive, sera with an S/P% between 90% and 100% were classified as doubtful and sera with an S/P% lower or equal to 90% were considered negative for antibodies against *C. abortus*. 
Co-cultivation of *Waddlia* and *Parachlamydia* in amoebae

Fresh placenta material for samples positive for these agents by real-time PCR and the majority also positive by immunohistochemistry was available in 12 cases: one *Waddlia* positive case (number 3) and eleven *Parachlamydia* positive cases. To try to isolate *Waddlia* and *Parachlamydia*, we used the amoebae strain *Acanthamoeba castellanii* ATCC 30010 in a co-culture system (Corsaro et al., 2009; Lienard and Greub, 2011). Amoebae were grown in peptone-yeast extract glucose at 28°C in humidified atmosphere and washed in Page’s amoeba saline (Lienard and Greub, 2011). Then, 24-well amoebal microplates were prepared by plating 5x 10^5 amoebae/ml in Page’s amoeba saline and Peptone Yeast-extract glucose, respectively. Homogenized, sterile-crushed placenta samples were inoculated by spinoculation at 1790 xg for 10 minutes. Then, co-cultures were examined daily for amoebal lysis and both *Waddlia* and *Parachlamydia* PCR was performed after 7 days of co-culture, to exclude or confirm growth of any of these two *Chlamydia*-related bacteria (Casson et al., 2008; Goy et al., 2009).

**Results**

**Abortion cases**

The histological investigation of placental tissue from all cases (n=343) resulted in acute necrotizing and/or purulent lesions in 231 cases (67.3%). In 45 cases (13.1%) a vasculitis could be found additionally. 90 cases (26.2%) were not interpretable due to autolysis and in 22 cases (6.4%) no pathological lesions could be found. PAS-staining of the 45 cases with vasculitis revealed fungal structures in one case (2.2%).
The investigation of placental smears on *B. abortus* and *C. burnetii* resulted in two cases (0.6%) positive for *C. burnetii*. 2/52 cases (3.8%) positive for BVDV in fetal organs were found using immunohistochemistry. Investigation for *N. caninum* by PCR resulted positive in 7/29 cases (24.1%). All the sera from the dam investigated for IBR-IPV were negative.

**Waddlia** rt PCR

By the *Waddlia* real-time PCR, three cases out of 343 cases (0.9%) had at least two Ct values. These cases originated from the western part of Switzerland. In the remaining 340 samples no cycle threshold value could be achieved. Details of cases positive for *Waddlia* are given in Table 1.

**Parachlamydia** rt PCR

By the *Parachlamydia* real-time PCR, 46 out of all the 343 cases (13.4%) were positive. Only nine of them (2.6%) showed a Ct below 40 and thus most cases exhibited less than 5 DNA copies (data not shown). Most cases originated from the western part (n=30), nine cases were collected in the central region and another seven cases in the eastern part of Switzerland. Thus, among 46 positive cases, 30 out of 111 were from the western part of Switzerland, whereas only 16 out of 232 were from other regions (p-value less than 0.0001). In the remaining 297 cases no cycle threshold value could be achieved in the duplicate or the result could not be reproduced by repetition. Details of cases positive for *Parachlamydia* are given in Table 2.

**Chlamydiaceae** rt PCR
Of the 343 cases investigated for *Chlamydiaceae* by rt PCR, 39 cases (11.4%) were considered positive. In 11 more cases (3.2%) the mean Ct values were slightly higher than 38 and considered as questionable positive.

The positive samples originated from the eastern (n=26), the central (n=11) and the western part of Switzerland (n=2). The questionable cases originated from the eastern (n=8), the central (n=2) and the western part of Switzerland (n=1). In the remaining 293 cases no cycle threshold value could be achieved in the duplicate or the result could not be reproduced by repetition. Details of cases positive for *Chlamydiaceae* are given in Table 3.

ArrayTube (AT) Microarray for species identification of *Chlamydiaceae*

Samples that were positive or questionable positive by rt PCR for *Chlamydiaceae* (n=50) were investigated by the species-specific 23S ArrayTube (AT) microarray. In 34/50 cases (68.0%) the presence of *C. abortus* was confirmed whereas in 3/50 (6.0%) cases a double infection with *C. abortus* and *C. suis* could be found. One case each (2.0%) revealed *C. suis* and *C. pecorum*, respectively. In 11 cases (22.0%) the results were negative.

16S rRNA PCR and sequencing

Cases negative in the AT (n=11) but positive or questionable positive by the rt PCR were further investigated by 16S rRNA PCR and sequencing. In four cases (36.4%) *C. abortus* was detected whereas for the other seven cases (63.6%) no specific sequence result could be obtained.

Waddlia IHC
For the three cases that were at least twice in the *Waddlia* rt PCR, IHC for the *Waddlia* antigen was performed. Both cases clearly positive for *Waddlia* by rt PCR were also positive in the immunohistochemistry. For the one case with a mean Ct value of 39.6 the immunohistochemistry was negative.

*Parachlamydia* IHC

Immunohistochemistry was performed on all cases with at least two cupules positive by rt PCR for *Parachlamydia* (n=46). Of these cases 25/46 were positive (54.3%). An example of a case (no. 12) presenting prominent histological lesions in the placenta and positive IHC is shown in Figure 1a & b.

*Chlamydiaceae* IHC

On all cases positive or questionable positive by rt PCR for *Chlamydiaceae* (n=50) immunohistochemistry for *Chlamydiaceae* was performed. Of these cases 4/50 were considered positive (8.0%). An example of a case (no. 74) presenting the typical histological lesions in the placenta and positive IHC is shown in Figure 2a & b.

Serology for *Chlamydia abortus*

The maternal serum samples (n=128) investigated with the commercial ELISA assay (Institute Pourquier) all had a ratio S/P% lower to 90% and were therefore considered as negative.

Co-cultivation of *Waddlia* and *Parachlamydia* in amoebae

All amoebal co-cultures (n=12) remained negative.

**Discussion**
Most cases of bovine abortion remain of unknown etiology although pathologic changes such as purulent and/or necrotizing placentitis are often observed and indicate the presence of infectious agents (Borel et al., 2006). Therefore this study brought into focus *Chlamydiaceae* as well as *Chlamydia*-related emerging abortigenic agents such as *W. chondrophila* and *P. acanthamoebae*.

*Waddlia*

This is the first description of *Waddlia* in bovine abortion in Switzerland. However, this agent seems unlikely to be of much importance in this country since only three samples out of 343 (0.9%) were positive by real-time PCR for *Waddlia*. False positive PCR results were excluded since two cases could be confirmed by *Waddlia*-immunohistochemistry. Histologically one case showed a necrotizing placentitis without vasculitis whereas in the other two cases a purulent to necrotizing placentitis with vasculitis could be seen. One of the cases was negative for all other investigated abortigenic agents. The other two cases were also positive for *Parachlamydia* both by real-time PCR and immunohistochemistry. However, the infectious agents could not be isolated from the samples. This might be due to (i) sampling errors as the agent is not necessarily present throughout the whole placental tissue, (ii) to long duration either of transport or storage or (iii) to autolysis because of delay between abortion and sample processing. A mixed infection with *Waddlia* and *Neospora* has been reported in Germany (Henning et al., 2002) but the present cases are the first reports of mixed infection with *Waddlia* and *Parachlamydia*. It remains unclear if both or only one agent caused bovine abortion.

Interestingly, all cases positive for *Waddlia* originated from the the western part of Switzerland. Although this agent does not seem to be of much importance and does
not occur in a high prevalence in bovine abortion in Switzerland it still could be of some future interest because of its possible zoonotic potential (Baud et al., 2007).

**Parachlamydia**

Of the 46 out of 343 cases (13.4%) positive by the *Parachlamydia* real-time PCR, 36 cases showed histologically a purulent and/or necrotizing placentitis but only in four of these cases an additional vasculitis could be found. One case showed no pathological changes in the placenta whereas 9 more could not be interpreted due to autolysis. In contrast to the purulent to necrotizing placentitis including vasculitis that can histologically be found in abortions caused by *C. abortus*, the prominent feature in placentas infected with *Parachlamydia* is a necrotizing placentitis without vasculitis. These findings are consistent with the findings in the study of Ruhl et al. (2009), where 18.3% of the investigated samples were positive for *Parachlamydia*. Confirmation by *Parachlamydia* immunohistochemistry was possible in 25/46 cases (54.3%) so the positive results achieved by real-time PCR are unlikely to be false positive, even when positivity was observed only after > 40 cycles in 37 out of 46 cases. When considering cases with low copies number (Ct > 40) as questionable, then this study would anyway demonstrate the occurrence of *Parachlamydia* in 2.6% of samples, of which 44.4% were confirmed by immunohistochemistry.

Besides the cases of mixed infection with *Waddlia* and *Parachlamydia* already described above, three cases positive for *Parachlamydia* and *C. abortus* could be found. A mixed infection of *Parachlamydia* and *C. abortus* has already been described in ovine abortion (Ruhl et al. 2008) but this are the first cases described of mixed infection of these agents in bovine. One of these and three more cases were also positive on *N. caninum* by PCR. Henning et al. (2002) described a mixed infection of *N. caninum* and *Waddlia* in a bovine abortion but our study presents the
first cases describing a mixed infection of *N. caninum* and *Parachlamydia* in bovine abortion. All four cases originated from the central area of Switzerland, the most urban and densely populated of the investigated regions. In the cases of mixed infection it remains unclear if both or only one agent caused bovine abortion.

Strikingly, the majority of cases positive for *Parachlamydia* originated from the western region of Switzerland (p-value less than 0.0001). Interestingly first evidence of maternal-fetal transmission of *Parachlamydia* was found in a case of prematurely ended pregnancy in a woman from the western region of Switzerland where also a zoonotic infection was suspected (Baud et al., 2009a). Additionally the only three positive cases in a study on the seroprevalence of *Parachlamydia* in young men from Switzerland originated from the same rural area and in two of those cases an exposure to farm animals took place (Baud et al., 2009b). It is remarkable that all cases positive for *Waddlia* and most of the cases positive for *Parachlamydia* originated from the western part whereas most cases positive for *Chlamydiaceae* were collected in the eastern and central region of Switzerland; this regional difference is a new finding of yet unknown significance.

The importance of *Parachlamydia* considering bovine abortion is not completely confirmed. The cultivation in amoebae was negative, the possible reasons being the same as discussed above for *Waddlia*. In conclusion, to determine the possible zoonotic potential of this agent that can cause pneumonia in humans (Baud et al., 2009a), further investigations should be considered.

*Chlamydiaceae*

The combination of real-time PCR and ArrayTube Microarray or of real-time PCR and 16S rRNA and sequencing resulted in 41/343 cases (12.0%) positive for *C. abortus*. 


From the cases positive for *C. abortus* (n=41), 27 originated from the eastern, 11 from the central and only three from the western part of Switzerland.

Immunohistochemistry for *Chlamydiaceae* performed on all positive and questionable positive cases by real-time PCR resulted in only four positive samples, this may be due to low antigen concentration in bovine placentas unlike in the tissues from sheep and goat. Furthermore focal accumulation of the agent in the organ itself could give false negative results due to sampling error.

The definition of an abortion caused by *C. abortus* includes the histological features of the placenta, which are a purulent and/or necrotizing placentitis ideally including vasculitis, as well as confirmation of the agent either by PCR or immunohistochemistry (Borel et al., 2006; Sachse et al., 2009). In this study, 17 of the 183 samples (9.3%) originating from the eastern part of Switzerland were positive by PCR and showed the typical histological features while 10 cases could not be evaluated due to autolysis (n=7) or showed no pathological changes (n=3). For the central part of Switzerland ten of 52 cases (19.2%) fulfill the requirements for chlamydial abortion; here only one case had to be rejected due to autolysis. For the western part of Switzerland all three cases positive by PCR of all the collected 108 cases (2.8%) fulfill the requirements for abortion caused by *C. abortus*. In a former study by Borel et al. (2006) a low prevalence of only 3.8% was found for chlamydial abortion in the eastern part of Switzerland. These results conflict with the findings in this study, where more cases positive for *C. abortus* were found in relation to the sample size. Strikingly, confirmed positive cases originated mainly of the eastern and the central region of Switzerland. Nevertheless the eastern part is also the same Swiss region where the highest seroprevalence (43%) for *C. abortus* in sheep was found (Borel et al., 2004). From this region origins also the first confirmed case of a bovine abortion due to *C. abortus* (Pospischil et al., 2002b) and the first confirmed
Swiss case of a zoonotic infection of a pregnant woman followed by abortion
(Pospischil et al., 2002a). A difference between the mountainous region compared to
other areas is the type of housing for animals. Traditionally livestock is brought to
summer pastures (transhumenance) where different flocks can get in contact and
also contact between cattle and sheep, goats or wild ruminants is possible. The study
by Borel et al. (2004) showed that summer pasturing is not the only reason for higher
prevalences in sheep in mountainous regions as the seroprevalence for sheep in the
another mountainous region, where the type of housing is similar to the one in
Grisons, had lower seroprevalences. According to the study by Holzwarth et al.
(2011) C. abortus is not a common infectious agent in Swiss Alpine ibex. Further
investigations are needed to clarify the role of C. abortus in other wild ruminants as
an infection source or reservoir.

The high amount of cases positive for C. abortus in the central region of Switzerland
was surprising. Informations on the prevalence of C. abortus in cattle are only
available for the eastern part of Switzerland (Borel et al., 2006). This is the first time
that bovine abortion samples from other regions of Switzerland were investigated for
C. abortus. The available amount of samples for this study was too small to make a
statistical evaluation and therefore a final conclusion on the prevalence of C. abortus
in cattle in the different regions is not possible. Further surveys on the subject are
necessary. Compared to the results achieved for the eastern part in the study of
Borel et al. (2006), the percentage of cases with a positive result from the western
part of Switzerland in this study is similar.

No correlation was found between positive cases by PCR or immunohistochemistry
and serology as all investigated sera samples of the dam were negative for
antibodies against C. abortus. Similar non-correlating findings between serology and
PCR-results in male genital organs and semen of ruminants have been reported by Teankum et al. (2007).

In one of the 50 cases (2.0%) positive for *Chlamydiaceae* by real-time PCR *C. pecorum* could be identified by ArrayTube Microarray. In another four cases (8.0%) *C. suis* was found by ArrayTube Microarray, including three cases of mixed infection with *C. suis* and *C. abortus* which has not yet been described to our knowledge. The development of new PCR-technologies such as the ArrayTube Microarray allow the detection of different *Chlamydiaceae*-species and therefore also mixed infections that could formerly not be detected (Sachse et al., 2009). *C. pecorum* is known as causing agent of conjunctivitis, arthritis, pulmonary inflammation and fertility disorders in ruminants and could also be detected in ovine placenta possibly leading to abortion (Berri et al., 2009). On the other hand, *C. pecorum* occurs in inapparent gastrointestinal infections of ruminants thus fecal contamination of the placental samples by *C. pecorum* investigated in this study can not be excluded. *C. suis* is commonly known to cause fertility problems in pigs. In Switzerland, where agriculture is intense and proximate, *C. suis* was also found in frogs (Blumer et al., 2007). An infection through manure is discussed and thus a fecal contamination of the samples in this study can not be excluded, especially since there was no information available about possible contact between pigs and cattle.

In conclusion, the relevance of *C. suis* and *C. pecorum* in bovine abortion cases with purulent or necrotizing placentitis remains yet unclear and needs further investigations.

In this study mixed infections of *C. abortus* with the BVDV was found in one case as well as with *N. caninum* in two cases. Henning et al. (2002) isolated *N. caninum* and *Waddlia* from an aborted bovine fetus but to our knowledge the combination of these known abortive agents is not described yet. Furthermore there were the three cases
of mixed infection of *C. abortus* and *Parachlamydia* described above. Again it remains unclear if both or only one agent caused the abortion.

**Conclusion**

By the investigations of the present study (i) there is evidence that different members of *Chlamydiales* are of importance in different geographical regions of Switzerland, but further studies on the prevalence of the agents are needed, (ii) *W. chondrophila* does not seem to be of much importance as abortigenic agent in cattle but could be relevant because of its possible zoonotic potential, (iii) co-infections with other abortogenic agents such as different *Chlamydia* and *Chlamydia*-related species, *N. caninum* or bovine virus diarrhea virus are possible and (iv) there is no correlation between placental infection and serological status on *C. abortus* in bovine.

**Conflict of interest statement**

All authors declare there is no financial or personal relationships with other people or organizations that could have inappropriately influenced their work.

**Acknowledgements**

We thank the laboratory staff of the Institute Galli-Valerio, Lausanne, of the Cantonal Laboratory of Veterinary Bacteriology, Grisons and of the Institute of Veterinary Pathology, namely Carmen Kaiser and Belinda Senn, University of Zurich. We are also grateful to Sebastien Aeby and Geneviève Goy from the Microbiology Institute of the University of Lausanne for technical help.

**References**


Teankum, K., Pospischil, A., Janett, F., Brugnera, E., Hoelzle, L.E., Hoelzle, K.,
Prevalence of chlamydiae in semen and genital tracts of bulls, rams and bucks.
Theriogenology. 67 (2), 303-310.

Chlamydophila psittaci in wild birds—potential risk for domestic poultry, pet birds, and
Figures

Fig. 1 a. Placenta; cow, case no. 12. Histopathology of a case positive for *Parachlamydia* by real-time PCR (Ct-value 36.3) and immunohistochemistry showing necrotizing placentitis (arrow). Hematoxylin and eosin staining. b. Placenta; cow, case no. 12. Immunohistochemistry with the anti-Parachlamydia antibody. Presence of positive granular reaction. AEC/peroxidase method, hematoxylin counterstain.

Fig. 2 a. Placenta; cow, case no. 74. Histopathology of a case positive for *Chlamydiaceae* by real-time PCR (Ct-value 23.0) and immunohistochemistry showing purulent to necrotizing placentitis (asterisk) and vasculitis (arrow). Hematoxylin and eosin staining. b. Placenta; cow, case no. 74. Immunohistochemistry with the anti-chlamydial LPS antibody. Presence of positive granular reaction. AEC/peroxidase method, hematoxylin counterstain.
**Table 1.** Details of cases (n = 3) positive by real-time PCR for *Waddlia*

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Placentitis</th>
<th>Vasculitis</th>
<th>real-time PCR</th>
<th>IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(^1)</td>
<td>necrotizing</td>
<td>no</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>2(^1)</td>
<td>purulent-necrotizing</td>
<td>yes</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>3</td>
<td>purulent-necrotizing</td>
<td>yes</td>
<td>pos</td>
<td>neg</td>
</tr>
</tbody>
</table>

pos = positive  
neg = negative  

\(^1\) mixed infection: *Parachlamydia*
Table 2. Details of cases (n = 46) positive by real-time PCR for *Parachlamydia*

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Placentitis</th>
<th>Vasculitis</th>
<th>real-time PCR</th>
<th>IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>necrotizing</td>
<td>no</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>4 - 16</td>
<td>necrotizing</td>
<td>no</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>17</td>
<td>necrotizing</td>
<td>no</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>18</td>
<td>necrotizing</td>
<td>yes</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>19 - 29</td>
<td>necrotizing</td>
<td>no</td>
<td>pos</td>
<td>neg</td>
</tr>
<tr>
<td>30</td>
<td>necrotizing</td>
<td>no</td>
<td>pos</td>
<td>neg</td>
</tr>
<tr>
<td>31</td>
<td>necrotizing</td>
<td>no</td>
<td>pos</td>
<td>neg</td>
</tr>
<tr>
<td>2</td>
<td>purulent-necrotizing</td>
<td>yes</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>32</td>
<td>purulent-necrotizing</td>
<td>yes</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>33</td>
<td>purulent-necrotizing</td>
<td>no</td>
<td>pos</td>
<td>pos</td>
</tr>
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<td>34</td>
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<td>no</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>35</td>
<td>purulent-necrotizing</td>
<td>no</td>
<td>pos</td>
<td>neg</td>
</tr>
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<td>36</td>
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<td>pos</td>
<td>pos</td>
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<td>37</td>
<td>purulent</td>
<td>no</td>
<td>pos</td>
<td>neg</td>
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<tr>
<td>38 - 40</td>
<td>autolytic</td>
<td>no</td>
<td>pos</td>
<td>pos</td>
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<tr>
<td>41</td>
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<td>pos</td>
<td>pos</td>
</tr>
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<td>42 – 46</td>
<td>autolytic</td>
<td>n.e.</td>
<td>pos</td>
<td>neg</td>
</tr>
<tr>
<td>47</td>
<td>no pathologic changes</td>
<td>n.e.</td>
<td>pos</td>
<td>neg</td>
</tr>
</tbody>
</table>

pos = positive
neg = negative
n.e. = not evaluated due to autolysis

1 mixed infection: *Waddlia*

2 mixed infection: *C. abortus*

3 mixed infection: *N. caninum*
Table 3. Details of cases (n = 50) positive or questionable positive by real-time PCR for *Chlamydiaceae*

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Placentitis</th>
<th>Vasculitis</th>
<th>Real-time PCR</th>
<th>IHC</th>
<th>ArrayTube / 16S rRNA PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>necrotizing</td>
<td>no</td>
<td>pos</td>
<td>neg</td>
<td>C. abortus / --</td>
</tr>
<tr>
<td>30</td>
<td>necrotizing</td>
<td>no</td>
<td>doubt</td>
<td>neg</td>
<td>C. abortus / --</td>
</tr>
<tr>
<td>31</td>
<td>necrotizing</td>
<td>no</td>
<td>pos</td>
<td>neg</td>
<td>neg / C. abortus</td>
</tr>
<tr>
<td>48 - 56</td>
<td>necrotizing</td>
<td>no</td>
<td>pos</td>
<td>neg</td>
<td>C. abortus / --</td>
</tr>
<tr>
<td>57, 58</td>
<td>necrotizing</td>
<td>no</td>
<td>doubt</td>
<td>neg</td>
<td>C. abortus / --</td>
</tr>
<tr>
<td>59</td>
<td>necrotizing</td>
<td>no</td>
<td>pos</td>
<td>neg</td>
<td>C. abortus, C. suis / --</td>
</tr>
<tr>
<td>60</td>
<td>necrotizing</td>
<td>no</td>
<td>pos</td>
<td>neg</td>
<td>neg / C. abortus</td>
</tr>
<tr>
<td>61</td>
<td>necrotizing</td>
<td>no</td>
<td>pos</td>
<td>neg</td>
<td>C. pecorum / --</td>
</tr>
<tr>
<td>62, 63</td>
<td>necrotizing</td>
<td>no</td>
<td>doubt</td>
<td>neg</td>
<td>neg / neg</td>
</tr>
<tr>
<td>64 – 66</td>
<td>necrotizing</td>
<td>yes</td>
<td>pos</td>
<td>neg</td>
<td>C. abortus / --</td>
</tr>
<tr>
<td>67 - 69</td>
<td>purulent-necrotizing</td>
<td>no</td>
<td>pos</td>
<td>neg</td>
<td>C. abortus / --</td>
</tr>
<tr>
<td>70</td>
<td>purulent-necrotizing</td>
<td>no</td>
<td>pos</td>
<td>neg</td>
<td>C. abortus / --</td>
</tr>
<tr>
<td>71 - 73</td>
<td>purulent-necrotizing</td>
<td>yes</td>
<td>pos</td>
<td>neg</td>
<td>C. abortus / --</td>
</tr>
<tr>
<td>74</td>
<td>purulent-necrotizing</td>
<td>yes</td>
<td>pos</td>
<td>pos</td>
<td>C. abortus / --</td>
</tr>
<tr>
<td>75</td>
<td>purulent-necrotizing</td>
<td>yes</td>
<td>pos</td>
<td>neg</td>
<td>neg / C. abortus</td>
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<tr>
<td></td>
<td>Description</td>
<td>Find</td>
<td>Test</td>
<td>Result</td>
<td>Test</td>
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<tr>
<td>76</td>
<td>purulent-necrotizing</td>
<td>yes</td>
<td>pos</td>
<td>neg</td>
<td>C. suis</td>
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<tr>
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<td>purulent-necrotizing</td>
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<td>pos</td>
<td>neg</td>
<td>neg / neg</td>
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<tr>
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<td>doubt</td>
<td>pos</td>
<td>C. abortus</td>
</tr>
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<td>79</td>
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<td>neg</td>
<td>neg / C. abortus</td>
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<tr>
<td>80 - 83</td>
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<td>neg</td>
<td>C. abortus</td>
</tr>
<tr>
<td>84</td>
<td>autolytic</td>
<td>n.e.</td>
<td>doubt</td>
<td>pos</td>
<td>C. abortus</td>
</tr>
<tr>
<td>85</td>
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<td>no</td>
<td>doubt</td>
<td>pos</td>
<td>C. abortus</td>
</tr>
<tr>
<td>86</td>
<td>autolytic</td>
<td>no</td>
<td>doubt</td>
<td>neg</td>
<td>C. abortus</td>
</tr>
<tr>
<td>87</td>
<td>autolytic</td>
<td>no</td>
<td>pos</td>
<td>neg</td>
<td>C. abortus, C. suis</td>
</tr>
<tr>
<td>88 - 91</td>
<td>autolytic</td>
<td>no</td>
<td>pos</td>
<td>neg</td>
<td>neg / neg</td>
</tr>
<tr>
<td>92</td>
<td>no pathologic changes</td>
<td>no</td>
<td>pos</td>
<td>neg</td>
<td>C. abortus</td>
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<tr>
<td>94</td>
<td>no pathologic changes</td>
<td>no</td>
<td>doubt</td>
<td>neg</td>
<td>C. abortus, C. suis</td>
</tr>
</tbody>
</table>

pos = positive  
doubt = questionable positive  
neg = negative  
n.e. = not evaluated (due to autolysis)

1 mixed infection: Parachlamydia  
2 mixed infection: bovine viral diarrhea virus (BVDV)  
3 mixed infection: N. caninum