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Co-infection with bovine viral diarrhoea virus and *Anaplasma marginale* in a dairy cattle herd may lead to acute bovine anaplasmosis

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ABSTRACT: This report describes an acute exacerbation of subclinical anaplasmosis manifesting itself in clinical signs in a large number of animals after infection with bovine viral diarrhoea virus (BVDV). The simultaneous transmission of BVDV and *Anaplasma* was unintended and most likely the result of a vaccination operation in a large Holstein-Friesian dairy cattle herd in Hungary. From Day 35 after the first vaccination, a total of 33 cows developed fever, depression, general weakness, lack of appetite, a sudden drop of milk production, anaemia, icterus, and tachypnoea on exercise. In addition, a total of seven abortions and three stillbirths occurred. Between Days 30 and 35 after the second vaccination four cows showed clinical signs typical of anaplasmosis, and two stillbirths occurred. The presence of *Anaplasma marginale* infection was demonstrated by haematological, biochemical, PCR and haemocytological examinations of blood samples collected from animals showing clinical signs as well as by necropsy. To assess the prevalence of infection in the herd, a specified number of animals belonging to different age groups were subjected to serological tests. The rate of seropositive animals was substantially higher (50%) in the older (3- to 4-year-old and more than 4-year-old) age groups than in the younger cows (10–30%). This study has demonstrated for the first time that if bovine animals susceptible to both *A. marginale* and BVDV are infected by the two pathogens roughly at the same time, the immunosuppressive effect of BVDV will support the progression of *A. marginale* infection and manifestation of the disease resulting in acute clinical signs.

Keywords: BVDV; mixed infection; epidemiological investigation; immunosuppression; outbreak

List of abbreviations

BVD = bovine viral diarrhoea, **BVDV** = bovine viral diarrhoea virus, **EBL** = enzootic bovine leucosis, **ELISA** = enzyme-linked immunosorbent assay, **EM** = electron microscope, **IBR** = infectious bovine rhinotracheitis, **PCR** = polymerase chain reaction, **PCR-RFLP** = polymerase chain reaction-restriction fragment length polymorphism, **PI** = persistently infected, **qRT-PCR** = quantitative real time reverse transcription polymerase chain reaction, **RNA** = ribonucleic acid

Anaplasma marginale, the causative agent of bovine anaplasmosis is a Gram-negative bacterium belonging to the family Anaplasmataceae of the order Rickettsiales. Members of the genus *Anaplasma* are arthropod vector-borne obligate intracellular bacteria. Of them, *A. marginale* is primarily a pathogen of bovine erythrocytes (Rymaszewska and Grenda 2008). Bovine anaplasmosis is present and causes substantial losses primarily in tropical and subtropical regions; however, the presence and spread of *A. mar-*

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ginale are increasingly being reported also from other areas including Hungary and Austria (Schlerka and Baumgartner 1991; Baumgartner et al. 1992; Hotter et al. 1995; Hornok et al. 2007; Kocan et al 2010).

The membrane-associated inclusion bodies present in bovine red blood cells contain four to eight rickettsiae. During acute infection, more than 70% of the red blood cells may be infected. Depending on the severity of infection, the incubation period may vary within wide ranges (7–60 days) and its average duration is 28 days (Rymaszewska and Grenda 2008). The clinical signs that are characteristic of anaplasmosis reflect the infection and destruction of red blood cells. Infected red blood cells are phagocytosed by cells of the bovine reticuloendothelial system. Subsequently, as a result of extravascular haemolysis, anaemia and icterus develop to varying degrees, depending on the severity of infection, without signs of haemoglobinaemia and haemoglobinuria. Other typical features include high fever developing in the early phase of the disease, which is followed by lack of appetite, weight loss, depression, general weakness, pale mucous membranes, icterus, laboured breathing, and abortion due to compromised oxygen supply or hypoxia (Kocan et al. 2003). The severity of the clinical signs is correlated to the age of the affected animal: while anaplasmosis is usually subclinical and asymptomatic in less than 1-year-old animals and moderately severe in the 1- to 2-year-old age group, cattle older than two years typically develop severe anaplasmosis which may lead to even 30–50% mortality in the absence of treatment or if treatment is started too late (Birdane et al. 2006). Animals that survive the acute stage of the disease and no longer exhibit clinical signs, as well as animals less than one year of age that have passed through subclinical anaplasmosis will develop lifelong persistent infection. Persistently infected or 'carrier' animals are characterised by a low-level but continuous rickettsaemia, in the course of which these animals act as bacterium reservoirs and maintain the infection in the herd; however, such animals develop active immunity and, thus, will be protected against a future exacerbation of the disease (Kocan et al. 2003).

Persistently infected animals play a critical role in the mechanical transmission of the pathogen with infected red blood cells and in its entry into biological vectors (*Ixodes* sp., *Dermacentor* sp.; Rymaszewska and Grenda 2008). If the pathogen is present in persistently infected animals in a herd without adequate disease prevention and control measures, the presence of biological vectors is not absolutely necessary (Baumgartner et al. 1993), as the mechanical transmission of *A. marginale* is also possible. Mechanical transmission most often takes place in connection with surgical interventions and iatrogenic effects such as dehorning, ear-tagging, castration and transmission with injection needles; however, the transmitting role of mechanical vectors, most prominently biting flies (e.g. the stable fly *Stomoxys calcitrans* and horse flies of the Tabanidae family) is also not negligible. If the biological vector is not present in the place where the herd is kept or if an outbreak occurs in the autumn or winter, i.e. in a season when the vector is not active, this indicates the presence of one or several carrier animal(s) in the herd. Acute outbreaks are primarily triggered by stress factors or immunosuppressive effects in susceptible animals (Kocan et al. 2010). Currently the vertical transmission of the pathogen is not fully clear yet; however, in certain regions the transplacental transmission of *A. marginale* has a profound impact on the epidemiological status of the pathogen (Aubry and Geale 2011). Calves usually do not show clinical signs after an intrauterine infection; however, there has been a report that in four calves that acquired transplacental infection during their intrauterine life the disease subsequently manifested itself in severe clinical signs after birth leading to fatal outcomes (Pypers et al. 2011). In these cases, a non-specified immunosuppressive effect was suspected as the underlying cause (Wandera and Munyua 1971).

BVDV is a positive-sense single-stranded RNA virus, which has two genotypes (BVDV-1, BVDV-2) belonging to the genus *Pestivirus* of the family Flaviviridae. Based on the cytopathic effects they exert in cell cultures, both known genotypes can be divided into cytopathic and non-cytopathic biotypes. In endemic areas, the non-cytopathic biotype occurs more frequently and can induce persistent infection which ensures the survival of BVDV and its circulation in the herd (Gamlen et al. 2010). BVD usually takes a subclinical course in seronegative, immunocompetent cattle. In the case of acute infection, transient viraemia lasting 10–14 days is accompanied by leukopenia, lymphopenia and/or thrombocytopenia of short duration, apoptosis in the cells of the thymus, postulated immunosuppression, fever and, possibly, diarrhoea.

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In the course of viraemia lasting a few days, approximately between Days 3 and 14 after infection the virus can be detected from the blood or nasal secretion (Pedrera et al. 2011). From Week 2–3 after infection, the levels of specific antibodies produced against BVDV rise continuously and reach their peak in Weeks 10–12 after infection, resulting in lifelong seropositivity. The immunosuppressive effect of BVDV plays a key role in facilitating the colonisation and propagation of other facultative pathogens affecting cattle, and in the development of the diseases caused by them (Ridpath et al. 2007; Lanyon et al. 2014). This postulated immunosuppression is partly due to the direct toxic effect exerted by BVDV on T- and B-cells circulating in the bloodstream and partly the result of lymphocyte apoptosis occurring in the gut-associated lymphoid tissue (Pedrera et al. 2012). Due to the strong affinity of the virus for immunocompetent cells, the cells participating in the immune response are killed by the pathogenic effect of the virus or damaged to an extent that prevents them from fulfilling their normal functions. BVDV decreases immunoglobulin and interferon production, inhibits the growth of lymphocytes participating in mounting the immune response and the chemotaxis of monocytes. As a result of the deleterious effect exerted on the cell-mediated immune response, the rate of the humoral immune response will also be lower (Blanchard et al. 2010; Lanyon et al. 2014).

Infection of the foetus with BVDV may result in the development of various clinical signs depending on the stage of gestation at the time of infection, i.e. the existence of the maternal-embryonic relationship and the maturity of the foetal immune system. From the epizootiological point of view, infection of a seronegative, immunocompetent dam with non-cytopathic BVDV from conception up to Day 125 of gestation has particular importance (Hamers et al. 1998). In such cases, no developmental anomalies will occur in the foetus: a persistently infected (PI) calf of average developmental status or a weak, slightly retarded PI calf will be born at term (Houe 1995). PI animals are usually seronegative, but because of the high antigenicity of BVDV the PI calves given colostrum of high antibody content may become transiently seropositive in the first 3–4 months of their life. This may give rise to false negative results in a disease control programme.

In addition to transplacental transmission, horizontal spread of BVDV within a herd is also possible. In this latter case, BVDV is transmitted from animal to animal by direct contact, with different secretions. With regard to iatrogenic transmission, the transmission of BVDV from animal to animal with virus-contaminated injection needles, the inadequate cleaning and disinfection of fomites, and the presence of blood-sucking parasites represent the most important modes of infection (Nettleton 2013).

The aim of the present study was to demonstrate that the immunosuppressive effect of BVDV may cause the exacerbation of the subclinical, asymptomatic anaplasmosis induced by *A. marginale* in cattle susceptible to both pathogens.

MATERIAL AND METHODS

The studies were conducted in a dairy farm of 800 cows and 1600 cattle in total, located in the southern part of the Great Hungarian Plain. The majority of the cows had more than 95% Holstein-Friesian genotype but there were also 50 Holstein-Friesian × Jersey crossbreds with 12–25% Jersey gene ratio.

Epidemiology and the diagnostic methods for BVD. Based upon the serological tests performed, the herd had been free of BVD at least since 2002. In the summer of 2012, a certain proportion of the immunocompetent, susceptible pregnant heifers contracted BVDV infection on the pasture. The infection was diagnosed by the Department of Virology of the National Food Chain Safety Office, Veterinary Diagnostic Directorate.

In order to determine the prevalence of infection, between February and April 2013 serological tests (virus neutralisation) for BVD were performed. In March, 38 animals of the 5- to 6-month-old age group were subjected to serological testing. In April, these preliminary tests were extended to a representative number of animals from other age groups, and five 1.5-month-old calves kept in pens, five 4-month-old calves kept in the calf-rearing unit, 10 dairy cows of the 3- to 6-year-old age group, five 20- to 22-month-old pregnant heifers and five 8- to 11-month-old young heifers were tested serologically.

Based upon the results of the serological tests, testing of the whole herd was initiated in order to detect BVDV and identify PI animals. In June, blood samples from a total of 1494 animals were tested using qRT-PCR, for which test 'pooled' blood

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samples were used, each pooled sample representing 30 individual animals. Blood samples from 99, 76 and 53 calves in July, August and September, respectively, were tested using qRT-PCR as pooled samples of 10 calves each. Finally, ear notch (cartilage) samples taken first from 84 and then from 30 animals in September and from 40 and 14 animals in October were tested using qRT-PCR as pooled samples of five animals each. All pooled samples giving positive results were identified individually. In the case of pooled samples testing positive in qRT-PCR, individual animals present in the herd at the time of repeated testing were tested individually using the virus neutralisation test three weeks later. If that virus neutralisation test was negative or demonstrated low antibody titres, an antigen-ELISA (ag-ELISA) test was carried out to differentiate persistently and transiently infected individuals. In order to survey the spread of BVDV infection in the herd, in September 10 young, 14- to15-month-old heifers and 20 cows, and in October, 60 cows, were tested serologically. The eight cases of abortion and one stillbirth that occurred in the period of study were also investigated in laboratory examinations. In the case of the single stillbirth that occurred, the dam was seronegative. In the case of the abortions and the stillbirth, the possible aetiological role of brucellosis, EBL, IBR, Listeria and Schmallenberg virus was ruled out by laboratory examination of the organs of foetuses and blood samples from the dams.

The virus neutralisation test was carried out following the method described in the World Organization for Animal Health (OIE) Terrestrial Manual BVDV (chapter 2.4.8) using the 100-300 $TCID_{50}$ BVDV 1 NADL cytopathogenic strain (EU reference laboratory, Hannover, Germany) as an antigen. Sera were incubated for 1 h at 37 °C with the standard amount of virus before bovine testicular cell suspension was added to the complex. A cytopathic effect was assessed after 4–5 days incubation in 5% CO_2 atmosphere at 37 °C. The viral nucleic acid was detected using qRT-PCR and the presence of the viral antigen was demonstrated using the BVDV Antigen Test Kit/Serum Plus (IDEXX Laboratories, Inc., Liebefeld-Bern, Switzerland), also in the Hungarian reference laboratory. The qRT-PCR (5' exonuclease assay, TaqMan) was developed by the Virology Laboratory of VDD. Primers and probe were designed to bind to the 5'UTR part of the genome, a conserved region, which allows detection of a wide range of BVD viruses. Primers and probe sequences are listed in Table 1. The antibody ELISA was performed according to the manufacturer's instructions.

In order to identify PI animals, the farm veterinarian tested ear notch (cartilage) samples from all calves born on the farm from October 2013 onwards using the BVDV Ag Point-of-Care (POC) Test (IDEXX Laboratories, Inc., Liebefeld-Bern, Switzerland).

Vaccination. To reduce virus shedding as well as to prevent the further intensive spread of BVDV within the herd and the infection of foetuses, primary immunisation of the entire herd with an inactivated vaccine (Bovilis® BVD, MSD Animal Health) was carried out in October 2013 (first vaccination) and four weeks later (second vaccination).

Epidemiology of *A. marginale*. Blood samples from the clinically ill cattle after the first vaccination were subjected to haematological, biochemical, haemocytological (Giemsa staining) and PCR tests, while animals that died were necropsied and subjected to toxicological examination.

In the period following vaccination against BVDV there were also seven abortions and five stillbirths; the aetiological role of brucellosis, enzootic bovine leukaemia virus, infectious bovine rhinotracheitis virus, BVDV, *Listeria monocytogenes* and Schmallenberg virus was ruled out in the laboratory of the National Food Chain Safety Office, Veterinary Diagnostic Directorate by examination of the foetal organs and by testing blood samples from the dams.

Table 1. Positions refer to the genome of NADL BVDV strain (NC001461)

Primers for BVDV qRT-PCR	Sequence 5'-3'	Location in the genome	Position in the genome
Sense	GCCATRCCYTTAGTAGGACKAGC	UTR	105
Antisense	CAACTCCATGTGCCATGTACAG	UTR-Npro	394
Probe (sense)	56FAM/CCCTGAGTA/ZEN/ CAGGGKAGTCGTCARTGGTTC/31ABkFQ	UTR	175

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The possibility of copper poisoning was excluded by toxicological examination of the liver using atomic absorption spectrometry for the determination of copper.

The examined haematological parameters from whole-blood (K_2EDTA blood collection tube) were white blood cell count, red blood cell count, haemoglobin, haematocrit, mean cell volume, mean cell haemoglobin, mean cell haemoglobin concentration and platelet count. The biochemical indicators, mainly liver and kidney function and ions were examined from serum.

Light microscopic examination of Giemsa-stained blood smears from animals showing clinical signs demonstrated an infection suggestive of anaplasmosis. The light microscopic examination of Giemsa-stained blood smears was performed at the Department of Pathology of Faculty of Veterinary Science, Szent Istvan University, where extensive *A. marginale* infection of the red blood cells was demonstrated. By PCR and sequencing, *A. marginale* DNA was identified in the blood samples at the Department of Parasitology and Zoology of the Faculty of Veterinary Science, Szent Istvan University. For preliminary assessment of the samples EHR 16SD (5'-GGT ACC YAC AGA AGA AGT CC-3') and EHR 16SR (5'-TAG CAC TCA TCG TTT ACA GC-3') primers were used, which amplify an approximately 345 bp fragment from the 5' region of the 16S rRNA gene from various members of the family Anaplasmataceae and closely related rickettsial agents (Brown et al. 2001). After purification with Wizard® SV gel and PCR clean-up system (Promega, Madison, WI, USA), ABI Prism® Big Dye Terminator v3.1 Cycle Sequencing Kit (PerkinElmer, Applied Biosystems Division, Foster City, CA, USA) was used for DNA sequencing reactions.

The clinically ill animals were administered 30 mg per kg oxytetracycline *i.m.* once or twice. Injectable products supporting metabolism, liver function and haematopoiesis were used as symptomatic adjunctive therapy: infusion of Ringer's saline with 5% glucose (1000 ml/animal) administered intravenously once or twice, 30 ml/animal vitamin ADE *i.m.* once and 30 ml/animal vitamin B complex and vitamin C subcutaneously four times.

In February 2014, after precise diagnosis of *A. marginale* infection, a specified number of animals belonging to different age groups were tested serologically using an indirect ELISA for the detection of antibodies against *A. marginale* (Svanovir *A. marginale*-Ab, Boehringer Ingelheim Svanova, Uppsala, Sweden) in order to assess the prevalence of infection in the herd. The antibody ELISA was performed according to the manufacturer's instructions. When selecting the individuals, three animals that had shown clinical signs in October (one 2-year-old and two 3-year-old animals) and 140 asymptomatic animals were chosen as controls.

RESULTS

Epidemiological situation of BVD

To enable easier orientation, the results of laboratory tests conducted to survey BVDV infection are presented in Table 2. This table shows the dates of the tests performed, the number and characteristics of the animals tested (age group, repeated testing), the test method requested by the client, in the case of the qRT-PCR tests also the number of samples tested (pooled) at any given time, the types of samples processed for the tests, and the results of the tests. Comparing the results of the serological surveys conducted in the spring (March, April) and autumn (October), it can be observed that the seropositivity rate increased from 10% to 40–45%. In the laboratory tests a total of nine PI animals were identified; these animals were removed from the herd.

With the help of the rapid test used on the farm, in October and November 2013 three PI calves were identified and removed from the farm. Around Day 280 after the first vaccination against BVDV a further six PI calves were born in the herd. Out of the eight cases of abortion that took place in the period of study, seven dams were seropositive for BVD (Case 1: 1 : 320, Case 2: the titre was not determined; Case 3: 1 : 1280; Case 4: 1 : 210; Case 5: 1 : 450; Cases 6 and 7: the titre was not determined), while the eighth cow was seronegative. The aborted, seronegative cow tested negative by ag-ELISA as well.

Clinical signs and diagnostic investigation of bovine anaplasmosis

In Weeks 5–6 after the first vaccination, 33 cattle (12 non pregnant heifers and 21 milking cows) showed fever (39.5–41 °C), depression, general weakness, lack of appetite, a sudden drop of milk

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Table 2. Samples tested by the National Food Chain Safety Office, Veterinary Diagnostic Directorate and the results

Date of test	Number of tested cattle (pcs)	Age group/individuals	Method ("pool")	Sample	Results (pcs)	
					positive (titre of VN)	negative
March 1	38	5–6 months	VN	blood	37	1
April 12	5	1.5 month	VN	blood	1	4
	5	4 months	VN	blood	5	0
	10	3–6 years	VN	blood	1	9
	5	20–22 months	VN	blood	1	4
	10	8–11 months	VN	blood	0	10
June 14–24	1494	herd	PCR (30)	blood	0	1494
July 16	99	calf	PCR (10)	blood	7	92
August 24 (control of July 16)	1	PCR-positive calf	ag-ELISA	blood	1 (PI)	0
			VN	blood	0	1
August 24	76	calf	PCR (10)	blood	10	66
September 13 (control of August 24)	1	PCR-positive calf	VN	blood	0	1
			ag-ELISA	blood	0	1
	1	PCR-positive calf	VN	blood	1 (1 : 1280)	0
	1	PCR-positive calf	VN	blood	1 (1 : 1280)	0
	1	PCR-positive calf	VN	blood	1 (1 : 10)	0
			ag-ELISA	blood	0	1
	1	PCR-positive calf	VN	blood	1 (1 : 10)	0
			ag-ELISA	blood	0	1
September 13	53	calf	PCR (10)	blood	every pool	0
September 20	84	53 (control of September 13) + 31 new calves	PCR (5)	ear notch	24	60
October 7 (control of September 20)	24	PCR-positive calf	VN	blood	13 (5 cattle < 1 : 80)	11
October 7 (control of VN)	16	calves with negative result or low titre of VN	ag-ELISA	blood	6 (PI)	10
September 30	30	calf	PCR (5)	ear notch	4	26
October 25 (control of September 30)	1	PCR-positive calf	VN	blood	1	0
	1	PCR-positive calf	VN	blood	1	0
	1	PCR-positive calf	VN	blood	0	1
			ag-ELISA	blood	0	1
	1	PCR-positive calf	VN	blood	0	1
			ag-ELISA	blood	1 (PI)	0
October 7	54	calf	PCR (5)	ear notch	2	52
October 25 (control of October 7)	1	PCR-positive calf	VN	blood	1	0
	1	PCR-positive calf	VN	blood	0	1
			ag-ELISA	blood	1 (PI)	0
September–October	10	14–15 months	VN	blood	0	10
	80	cow	VN	blood	43	37

ag-ELISA = antigen-enzyme-linked immunosorbent assay, PI = persistently infected, VN = virus neutralisation

production, then anaemia and in some cases icterus, with tachypnoea on exercise, and sudden death in a few cases. Eighteen of the affected cattle that had clinically recovered following the administration of oxytetracycline at a dose of 30 mg/kg body weight as well as 15 cattle died. Furthermore, in Week 7, 10

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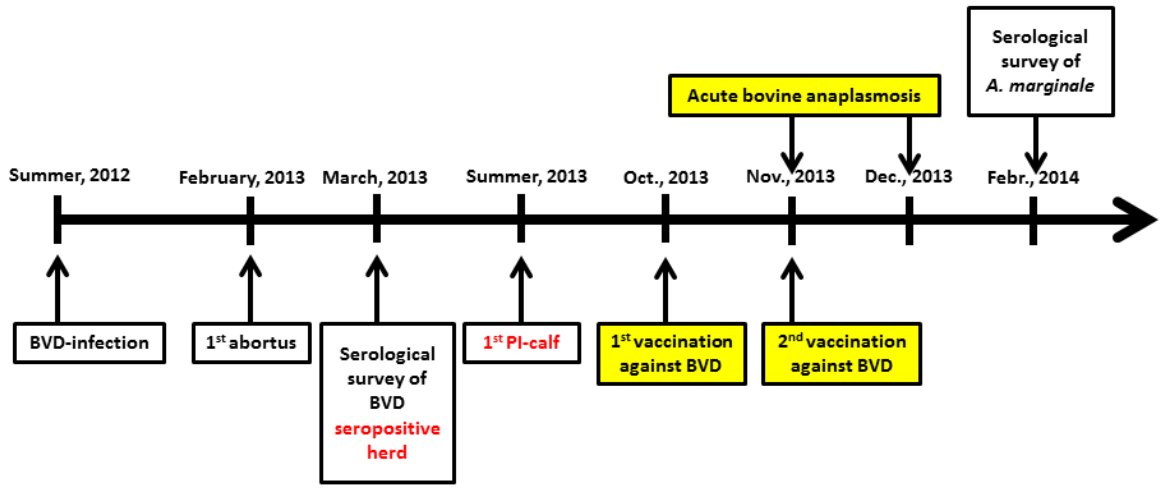

Figure 1. Timeline of BVD-infection and acute bovine anaplasmosis. The 1st PI-calf detected in August of 2013 was six months of age

pregnant cows showed clinical signs, seven of which had abortions and three of which had stillbirths. After the repeated vaccination, four non-pregnant cows developed clinical signs typical of anaplasmosis, which were successfully cured by oxytetracycline treatment, and two stillbirths occurred (Figure 1).

The haematological and biochemical variables of blood samples taken from cows with clinical signs of acute anaplasmosis showed similar changes including a substantial decrease in red blood cell count, haematocrit value, haemoglobin concentration and blood glucose level. Mild hypoalbuminaemia accompanied by a moderate or high increase in AST, ALT, GGT, ALP, CK and LDH activities as well as in total and direct bilirubin levels could also be observed (Table 3).

Pathological and histopathological analysis

Gross pathological lesions (necropsy findings) included (I.) pale, and yellowish discoloured visible mucus membranes (in oral cavity, conjunctiva, preputium, vagina) due to icteroanaemia; (II.) yellowish discoloration of the subcutaneous tissues, visceral serosal membranes due to icterus; (III.) haemolytic, and icteric blood; furthermore, light microscopic examination of Giemsa-stained, *post-mortem* separated blood smears showed cytopathological signs of *A. marginale* infection (parasitised erythrocytes) (Figure 2); (IV.) enlarged haemolymph node; (V.) diffuse enlargement of the darker spleen (splenomegaly); (VI.) hepatomegaly, and pale yellowish discoloration of the liver; (VII.) dilated gall bladder filled with concentrated bile; (VIII.) acute congestion, oedema, interstitial emphysema, and haemorrhages in the lung.

Microscopic pathological lesions (histopathological findings of the H-E-stained slides) included (I.) spleen: the limit between white and red pulp disappeared due to red pulp hyperplasia, and enlargement of the lymphoid follicles; increased plasma cell proliferation; in red pulp proliferation of macrophages characterised by erythrophagocytosis and haemosiderosis (Figure 3A). (II.) Liver: macrovesicular lipidosis/steatosis without nuclear changes; mild sinusoidal stenosis due to hepatocyte swelling; multifocal centrilobular hepatic (coagulative) necrosis; intraductal-, and intercellular cholestasis; mild portal mononuclear cell infiltration, and bile

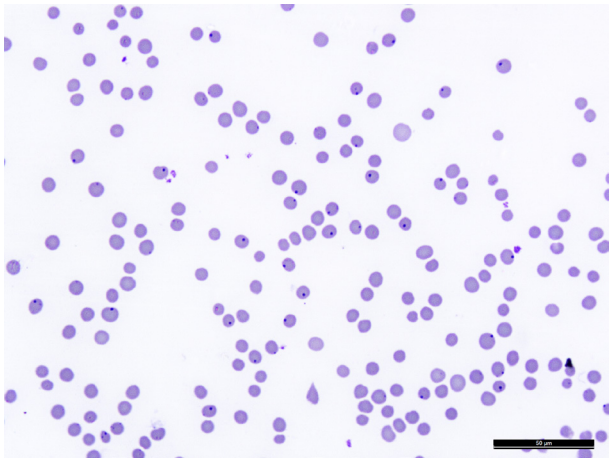

Figure 2. Marginally located, dark blue spherical inclusion bodies of *A. marginale* in infected bovine erythrocytes Giemsa-staining. Magnification × 630. Bar = 50 µm

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Table 3. Haematological and biochemical variables of blood samples from three cattle showing signs of acute anaplasmosis

	Value 1	Value 2	Value 3	Reference range
Haematology				
White blood cells ($\times 10^9$/l)	10.1	8.6	5.67	4.5–11.7
Red cell count ($\times 10^{12}$/l)	1.52	1.08	1.54	5.5–10.0
Haemoglobin (g/l)	30.8	30.1	29.6	60–145
Haematocrit (proportion of 1.0)	9.44	8.69	9.3	28–45
Mean cell volume (fl.)	62.0	80.7	60.2	40–60
Mean cell haemoglobin (pg)	20.2	27.9	19.2	10–17
Mean cell haemoglobin concentration (g/l)	32.6	34.6	31.9	30–36
Platelet count ($\times 10^9$/l)	61.7	125	163	100–800
Biochemistry				
Aspartate aminotransferase (IU/l)	2020	1370	730	10–80
Alanine aminotransferase (IU/l)	106	82	36	10–50
Total bilirubin (μmol/l)	198.4	165.4	76.8	1.5–10.0
Direct bilirubin (μmol/l)	35	35.2	9.8	0.1–2.0
Alkaline phosphatase (IU/l)	395	346	320	10–300
Gamma-glutamyl transferase (IU/l)	95	182	91	10–40
Total serum protein (g/l)	67.3	75.4	68.5	60–80
Albumin (g/l)	24.6	29.3	29.8	30–35
Glucose (mmol/l)	0.6	0.9	4.8	1.94–4.05
Total cholesterol (mmol/l)	0.8	3.0	2.3	2–5
Urea (mmol/l)	10.8	4.2	11.1	3.3–7.0
Phosphorus (mmol/l)	1.6	1.7	2.5	1.6–2.3
Calcium (mmol/l)	1.9	2.0	2.5	2.3–2.8
Iron (μmol/l)	41.5	48.3	46.1	10–35
Creatine kinase (IU/l)	43 560	4 610	2 513	10–250
Lactate dehydrogenase (IU/l)	15 050	9 200	11 270	100–1500

duct hyperplasia (Figure 3B). (III.) Lung: histiolymphocytic interstitial pneumonia, proliferation of type I pneumocytes, emphysema (Figure 3C). (IV.) Heart: multifocal myodegeneration, mild disorientation of the myofibrils (Figure 3D). (V.) Kidney: vascular congestion, without any histopathological signs of the haemoglobinuria.

In the Giemsa-stained slides, *A. marginale* stained intensely dark-blue in the margins of the bovine erythrocytes found in the dilated vasculature of the different necropsy tissue samples (Figures 4–6).

Epidemiological situation of *A. marginale*

The results of the competitive ELISA tests demonstrated that the pathogen was endemic in the herd (Table 4). About 50% of the more than 3-year-old animals and 10–30% of the younger age groups were seropositive. Regarding the perinatal period, we found that on the day of their birth, before the uptake of colostrum all calves were seronegative, but among the 3-day-old calves we could already find seropositive animals.

Table 4. Results of indirect ELISA of *A. marginale* infection in the herd

Age groups	Number of samples (pcs)	Positive (pcs)
0 days (before colostrum drinking)	8	0
3 days	7	2
3 months	15	3
4 months	5	0
5 months	5	0
6 months	7	1
7 months	8	0
9–12 months	15	3
1–2 years	10	1
2–3 years	10	1
3–4 years	15	7
> 4 years	38	19

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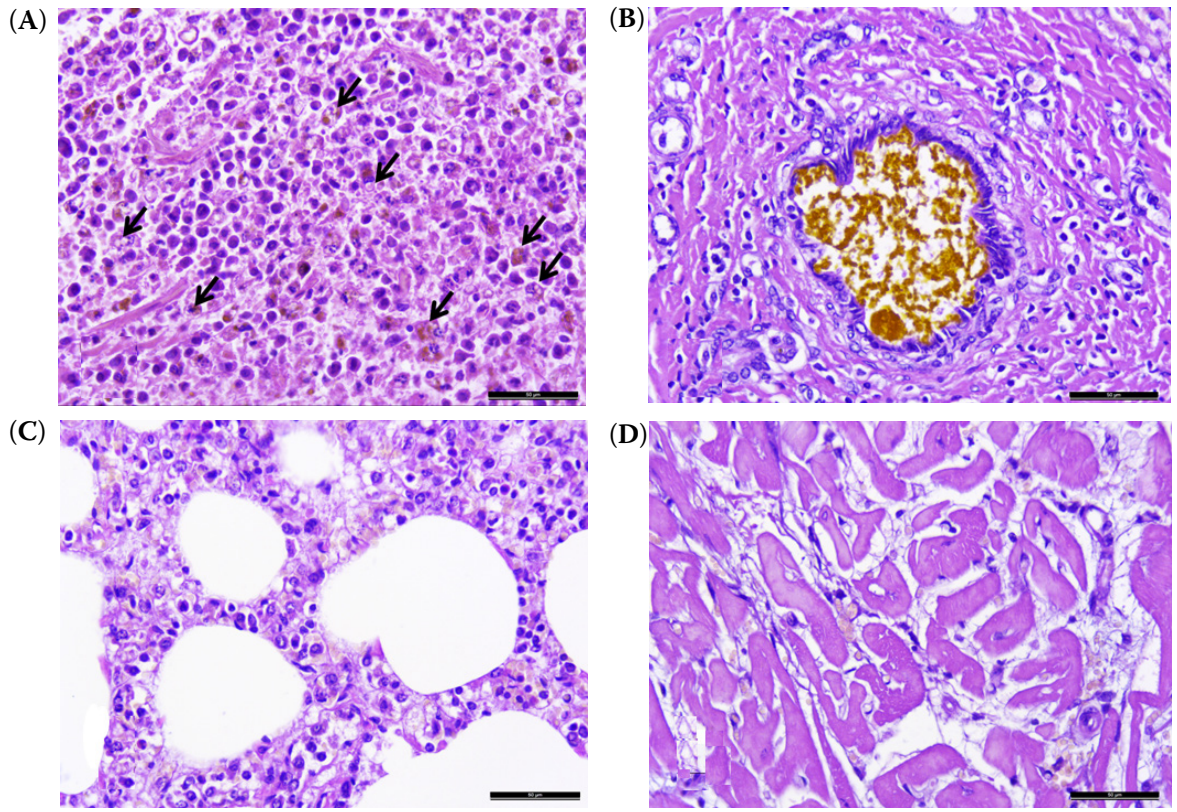

Figure 3. (**A**) Reactive splenitis caused by *A. marginale* infection Severe plasma cell proliferation, and histopathological hallmarks of the erythrocytophagocytosis, siderocytosis (arrows). Haematoxylin and eosin staining. Magnification × 400. Bar = 50 µm. (**B**) Intraductal choletasis, and portal (periductal) mild mononuclear infiltration. Haematoxylin and eosin staining. Magnification × 400. Bar = 50 µm. (**C**) Interstitial pneumonia in bovine lung, caused by *A. marginale.* The alveolar septa are notably thickened by moderate infiltration of histiocytes and lymphocytes. Haematoxylin and eosin staining. Magnification × 400. Bar = 50 µm. (**D**) Multifocal myodegeneration in the heart muscle from a cow infected by *A. marginale*. Haematoxylin and eosin staining. Magnification × 400. Bar = 50 µm

The size of the herd was 1600 animals: 800 cows, 700 heifers and 100 calves, and 210 cows were certified as being pregnant. During the outbreak 49 animals showed clinical signs. Fifteen of 49 diseased cattle died. The morbidity was 3%, the death rate was 0.93% relative to the total herd size and the death rate was 30.6% relative to the number of ill cattle. From the 210 vaccinated, confirmed pregnant animals seven aborted and there were five stillbirths (morbidity was 6.2% in the confirmed pregnant group) and 37 showed clinical signs from 1390 vaccinated, non-pregnant cattle (morbidity was 2.6% in the non-pregnant group).

In all cases, medication was successful only if treatment with oxytetracycline (30 mg/kg body weight) was administered immediately after the appearance of clinical signs. In the majority of cases, causal therapy consisted of single administration of a long-acting injectable product containing oxytetracycline as an active ingredient, but in a few cases a second treatment with a fractional dose (15 mg/kg body weight) was necessary.

DISCUSSION

Based on our study we assume a correlation of the simultaneous infections with BVDV and *Anaplasma marginale* and identify the spread of BVDV due to vaccination as the probable cause. The changes and lesions observed during the gross and histopathological examinations supported the results of the haematological and biochemical tests. Elevated liver enzyme activities and cholestasis were due to hypoxia-induced liver degeneration. Elevated urea levels could be attributed to degeneration of the renal tubular epithelium, which was also due to hypoxia. Anaemia and icterus were at-

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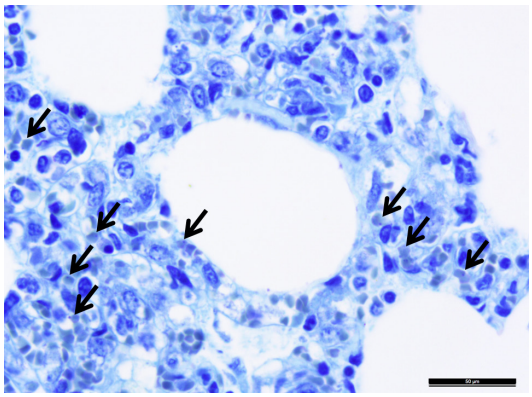

Figure 4. Histopathological picture of the interstitial pneumonia caused by *A. marginale.* Arrows indicate the parasitised bovine erythrocytes in the dilated alveolar capillary. Haemoparasites as a marginal inclusion can be seen at end of the arrows. Giemsa-staining; Magnification × 630. Bar = 50 µm

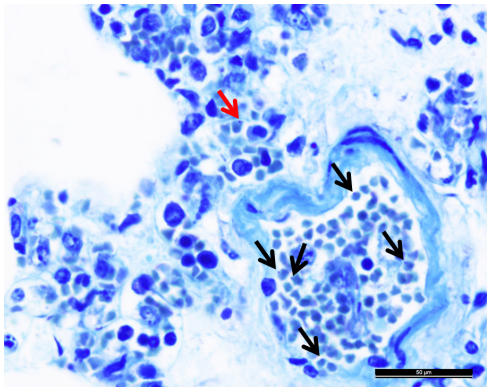

Figure 5. Histopathological picture of the interstitial pneumonia in a cow caused by *A. marginale.* Black arrows indicate the parasitised bovine erythrocytes in the dilated intrapulmonary vein, and the red arrow indicates a parasitised bovine erythrocyte in the dilated alveolar capillary. Giemsa-staining; Magnification × 630. Bar = 50 µm

tributable to red blood cell destruction caused by *A. marginale*. Based upon the laboratory detection of *A. marginale* from the blood and in blood smears (by PCR, Giemsa staining and EM), it can be confirmed that the disease cases accompanied by fever, anaemia, icterus and occasionally abortions, occurring in the herd up to the time of vaccination sporadically and limited to a few animals, were caused by *A. marginale*. The herd was regularly administered anti-ectoparasitic treatment. After identification of *Anaplasma marginale*, the results of the serological tests also support the conclusion that the pathogen had been endemically present in the herd also before the current outbreak. The results of the serological tests are consistent with the data reported in the literature (Baumgartner et al. 1993; Birdane et al. 2006; Aubry and Geale 2011), as the seropositivity rate was substantially higher (50%) in the older animals (3- to 4-year-old, more than 4-year-old) than in the younger age groups (10–30%). Despite the fact that clinically apparent anaplasmosis occurred in the older animals, the younger (3- to 12-month-old) age groups also contained seropositive animals which, however, did not exhibit clinical signs.

The massive acute clinical manifestation of anaplasmosis, which was otherwise present in the herd in subclinical form, was presumably triggered by stress factors or immunosuppressive effects. Because of the acute nature of the outbreak that simultaneously involved 49 cows, the source could be traced back to a specific point in time, which could be roughly determined by taking into account the incubation period of *Anaplasma* infection. The incubation period of *A. marginale* infection varies widely and its average duration is 28 days according to the literature (Kocan et al. 2003). Immunisation against BVD was performed about 35 days before the onset of the outbreak, and in the period between the two time-points no other known herd-level negative effects or changes (e.g. feed change, movement of animals, introduction of new animals into the herd) occurred. Vaccination can be regarded as a stress factor for the animals, and theoretically it could be the factor responsible for the appearance of acute anaplasmosis. However, herd-level vaccinations (e.g. immunisations against IBR every spring) with unsterilised needles had

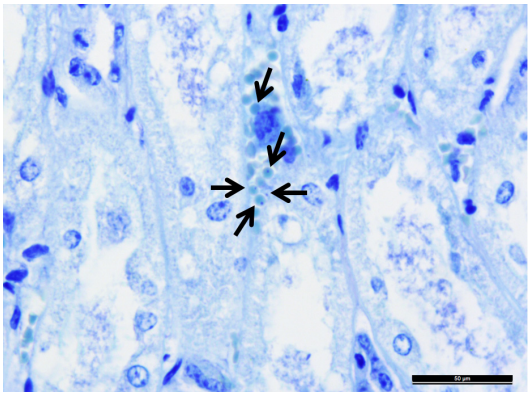

Figure 6. Histopathological picture of the bovine kidney. Arrows indicate parasitised bovine erythrocytes in the intertubular vessel. Giemsa-staining; Magnification × 630. Bar = 50 µm

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been carried out also earlier, when *A. marginale* may already have been present in the herd; nevertheless, to date vaccination or unsterilised needles by themselves have not been described to trigger the massive appearance of clinical signs typical of anaplasmosis. At the same time, sporadic cases accompanied by fever, anaemia and icterus and restricted to one or two animals per year had already been occurring on the farm for several years, but their cause or possible correlation with vaccination could not be established. The use of jet injectors for mass vaccination may be a possible iatrogenic factor related to herd-level vaccination. The use of such injectors could provide an opportunity for the horizontal spread of both BVDV and *A. marginale*, as the still undetected *A. marginale* and the BVDV already diagnosed at the time of outbreak were simultaneously present in the asymptomatic but carrier animals. The harmful effects exerted by BVDV on the immune system and the immunosuppressive properties of the virus probably played acritical role in the acute exacerbation of *A. marginale* infection in the herd. In the case of simultaneous infection with *A. marginale* of cows infected with BVDV either before or at the time of vaccination, the immunosuppressive effects of BVDV can be perceived from Days 3–7 after infection, facilitating the clinical manifestation of anaplasmosis in the susceptible animals. However, BVD virus can no longer be detected in animals affected with anaplasmosis (with the exception of PI animals, but at the time of the study there were no PI animals in the herd), as transient viraemia lasts up to Day 14 after infection while the clinical signs of anaplasmosis can be observed only around day 28 after infection. In the present case, serological testing aimed at the detection of antibodies produced against BVDV did not support the epidemiological monitoring either, as the clinical signs of anaplasmosis appeared in Weeks 5–6 after both the first and the second vaccination. At that time the animals were already seropositive, and as the vaccine against BVD is not a marker vaccine, it cannot be unambiguously determined whether seropositivity resulted from infection with a virulent strain or a vaccine virus. However, PI calves were born around the time of 280 days after vaccination, strong evidence that BVD virus carrier cattle had been in the herd at the time of vaccination.

The results of epidemiological investigations indicated that although the spread of BVD virus in the susceptible herd had been extensive before the vaccination (BVDV seropositivity was 10% in April 2013 and 45–50% in September 2013), no mass disease outbreak suggestive of anaplasmosis had occurred during that period. This is due to the fact that in animals persistently infected by *Anaplasma* the development of active immunity prevents re-exacerbation of the disease, even in the presence of strong immunosuppression caused by BVDV. Namely, in the present case the development of acute bovine anaplasmosis involving many animals was primarily due to the transmission of *A. marginale* from carrier animals to susceptible cows transiently infected with BVDV or to those contracting BVDV infection via the iatrogenic route during vaccination.

All this allows us to conclude that if a bovine animal susceptible to both *A. marginale* and BVDV is infected by the two pathogens roughly at the same time, the immunosuppressive effects of BVDV will support the progression of *A. marginale* infection and manifestation of the disease in acute clinical signs. Our results also call attention to the importance of excluding the presence of BVDV and *A. marginale* in cattle herds to be subjected to herd-level vaccination against any infectious agent, and to avoid the use of mass vaccination devices.

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