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Molecular and immunodiagnostic investigations on bovine neosporosis in Switzerland

B. Gottstein,* B. Hentrich, a R. Wyss, a B. Thür, b A. Busato, c K.D.C. Stärk d and N. Müller a

aInstitute of Parasitology, University of Berne, Länggass-Strasse 122, CH-3001 Berne, Switzerland
bInstitute of Veterinary Pathology, University of Zürich, Winterthurerstrasse 265, CH-8057 Zürich, Switzerland
cInstitute of Animal Breeding, University of Berne, Länggass-Strasse 109a, CH-3001 Berne, Switzerland
dInstitute of Virology and Immunoprophylaxis, CH-3147 Mittelhausern, Switzerland

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Abstract

Neospora caninum has gained considerable attention through its role in the aetiology of bovine abortion. Due to its close phylogenetic relationship with Toxoplasma gondii, respective unequivocal differential diagnosis deserves special consideration. In order to evaluate the diagnostic performance of molecular and immunodiagnostic techniques and to provide insights into the epidemiological significance of bovine neosporosis in Switzerland, we conducted a study on 83 cases of bovine abortion: of these, 24 (29%) foetal brains were positive by Neospora-PCR, six of these foetuses were simultaneously seropositive in Neospora-IFAT and/or somatic antigen-ELISA. Conversely, four (5%) foetal brains were considered positive by Toxoplasma-PCR, two of which were also seropositive in the Toxoplasma-P30-ELISA and/or direct agglutination test. The seroprevalence in 1689 cattle sera obtained from 113 dairy farms was 11.3% (95% confidence interval: 9.2-13.8) by Neospora-somatic antigen-ELISA and 10.7% (95% confidence interval: 8.3-12.6) by Toxoplasma-P30-ELISA. From the same samples, 1.1%, less than statistically expected, were positive in both ELISA. Within selected groups of cow-calf farms, the seroprevalence determined using the Neospora-somatic antigen-ELISA was 14% (95% confidence interval: 5.0-23.0) for dams and 15% (95% confidence interval: 3.0-28.0) for offspring calves. Seroprevalences determined by Toxoplasma-P30-ELISA were 8% (95% confidence interval: 4.0-12.0) for dams and 3% (95% confidence interval: 0.3-6.0) for calves. None of the sera gave a positive reaction in both ELISA. Our data indicated that prenatal neosporosis appears as an important cause of bovine abortion in Switzerland. © 1998 Australian Society for Parasitology. Published by Elsevier Science Ltd.

Key words: Neospora caninum; Neosporosis; Toxoplasma gondii; Toxoplasmosis; Abortion; Bovines; ELISA; PCR; IFAT

1. Introduction

Neospora caninum is an apicomplexan parasite causing neuromuscular diseases in various host animals, and is a significant causative agent of abortion and stillbirth in cattle [1] and, occasionally, in other mammals of veterinary importance (reviewed by [2]). The parasite is morphologically similar to Toxoplasma gondii, but distinct in its ultrastructure, immunogenicity and host-related pathogenicity [3]. The parasite has received recent attention since neosporosis was recognised as a major cause of abor-
tion in cattle in the U.S.A. and other countries [4, 5]. In contrast to *N. caninum*, *T. gondii* causes prenatal infection and consecutive abortion or foetal malformations in non-immune sheep and humans [6]. Both diseases occur world-wide [1]; however, there is a paucity of information available on the epidemiological situation of neosporosis in Switzerland. Neosporosis was described as an important cause of economic and reproductive loss to the livestock industry [7, 8]. Outbreaks of confirmed *Neospora*-related abortions as well as the prevalence of neosporosis-associated abortions reported from several countries (reviewed by [2]) justify an epidemiological pilot study in Switzerland. For an accurate determination of the prevalence of infection and disease, reliable techniques for the direct or indirect demonstration of the two parasites, of respective specific host immune reactions and of the damage induced by infection, are required.

Conventional diagnosis of neosporosis in cattle has been based largely on the post mortem demonstration of the organisms in clinical material, e.g., by histology or immunohistology performed on tissue sections of the brain and other organs. Histopathological examination may reveal minimal histologic lesions, such as focal gliosis in the CNS, [9] as well as severe neural lesions, such as non-suppurative encephalomyelitis characterised by multifocal non-suppurative infiltration, with or without multifocal necrosis and multifocal to diffuse non-suppurative leukocytic infiltration of the meninges [2], and occasionally calcifications. Diagnosis may also be achieved by the cultivation of the parasites in experimental animals or cell cultures. Moreover, demonstration of parasite antigens in tissue sections, e.g., by means of immunohistochemistry [10], is a valuable adjunct diagnostic approach. An indirect method refers to the assessment of the host's humoral immune response by the demonstration of parasite-specific antibodies in serum or other body fluids, such as by means of the IFAT [9, 11] or the ELISA [12, 13]. These tests deserve special attention since they are required to discriminate between infection with different apicomplexan parasites [14]. Immunodiagnostic tools have been used to determine epidemic parameters associated with *Neospora*-induced abortion in cows and to study prenatal and postnatal transmission [15].

With respect to the molecular identification of *Neospora*, a substantial improvement in the diagnostic sensitivity was achieved by designing oligonucleotide primers suitable for the PCR amplification of *N. caninum*-specific genomic sequences isolated from clinical samples [16–19]. Subsequently, the PCR established in our laboratory [18, 19] was developed further to achieve routine practicability by the introduction of the uracil DNA glycosidase (UDG) system [20] and a rapid and simple amplification-detection system known as DNA-Hybridisation Immunooassay (DIA) [21].

In the present study, we have compared the use of diagnostic methods for the demonstration of infection and disease occurrence of *N. caninum* and *T. gondii* in a series of abortions in Swiss dairy cattle. Methodologically, we employed two highly sensitive PCR assays and indirect serological tools. Based upon the evaluation of these primary diagnostic parameters, the relative seroprevalence was determined in Swiss dairy cattle and in animals of cow–calf farms.

### 2. Materials and methods

**Experimental design.** (1) Different diagnostic tools were established to demonstrate infection with *Neospora* and/or *Toxoplasma* in tissues obtained from clinical cases of bovine abortion. These tools were (a) in-vitro isolation of parasites from tissues, (b) histological demonstration of parasites or parasite-induced lesions in tissues, (c) molecular identification of parasite DNA in tissues by PCR, and (d) detection of host immune responses to infection by IFAT, ELISA and direct agglutination (DA). (2) Serological tools suitable for mass screening were used to determine the seroprevalence of *Neospora* and of *Toxoplasma* by ELISA in Swiss dairy cattle and in animals of cow–calf farms.

**Parasites.** An original isolate of *N. caninum* (NC-1 strain [3]) was obtained from Dr J.P. Dubey, USDA, Beltsville, MD, U.S.A. Parasites were grown in Vero cells as described previously [22,
All reagents for cultivation were obtained from Gibco-BRL. Tachyzoites were separated from host cells by passing the trypsinised cells through a 23-gauge needle, followed by purification on Sephadex G-25M (PD-10* columns, Pharmacia). Purified parasites were stored as pellets at -80°C until required.

**Diagnostic in-vitro isolation of parasites.** For in-vitro isolation of parasites from host tissue (foetal brain, heart, maternal placenta), 20 ml of homogenised tissue of each organ was incubated with 50 ml trypsin at 37°C for 1 h. The samples were subsequently washed several times with sterile PBS (pH 7.2) by centrifugation at 1200 g for 10 min at 4°C until the supernatant was translucent. After discarding the supernatant, 10 ml of each pellet was incubated on a 24-h-old Vero cell monolayer in a 25 cm² tissue culture flask in 10 ml RPMI 1640 containing 10% FCS and 1% amphotericin B. The cultures were incubated at 37°C and the medium replaced 12–16 h after inoculation. The cell cultures were monitored microscopically, and the cultivation medium was exchanged daily. Fourteen days after inoculation, the cultures were considered "negative" for Neospora growth unless parasites were detected microscopically [23].

**PCR.** Further processing of diagnostic tissue samples from brain, musculature and maternal placenta for PCR were carried out as described previously [21], with the exception that a High Pure PCR Template Preparation Kit (Boehringer, Mannheim) was used according to the manufacturer's recommendations for the preparation of DNA. Molecular diagnosis by Neospora- or Toxoplasma-specific PCR and identification of amplification products were also according to our previous description [21]. Putative false-negative results, which may have been caused by inhibitory compounds in the PCR tests, were excluded by performing a parallel inhibition control reaction (data not shown) on the samples in the presence of N. caninum or T. gondii DNA equivalents from about five parasites per reaction (exact performance data in [21]).

**Serological assays.** For the Neospora-IFAT, Neospora tachyzoites were obtained from cell cultures as described above and subsequently coated onto 12-spot IFAT glass slides and air dried. Immediately after drying, the slides were fixed in acetone and stored at -20°C. For the purposes of the investigation, diagnostic bovine sera were diluted 1:200 in PBS-Tween-BSA. The slides were washed in PBS to remove unbound antibodies and subsequently incubated for 1 h at 37°C with FITC-conjugated goat-anti-rabbit IgG (The Binding Site, Birmingham, U.K.) at a dilution of 1:200 in PBS-Tween-BSA. The preparations were washed three times for 5 min in PBS, mounted in Fluoprep (BioMérieux Suisse S.A.) and finally examined using a Leitz Laborlux S fluorescence microscope. The specificity of the two hyper-immune sera was initially confirmed by the absence of immunoreactivity on heterologous T. gondii or N. caninum tachyzoites, respectively [22].
plasma in the negative control sera was confirmed using the same specific tests as employed subsequent to infection. The diluted test and control sera were incubated on the slides for 30 min at 37°C in a moist chamber. The slides were subsequently washed three times with PBS, and the last washing step was followed by a rinse in distilled water. The slides were then incubated for 30 min at 37°C in a moist chamber with a FITC-conjugated monoclonal mouse-anti-bovine IgG antibody (Sigma Immunochemicals, Clone BG-18) at a dilution of 1:300 in PBS. After washing the slides twice in PBS for 5 min, they were mounted in FluorPrep (BioMerieux) and examined on a Leitz Laborlux S fluorescence microscope. In the current study, infection with N. caninum was identified when foetal antibody titres at a dilution of >1/80 were detected. For cows, the antibody-binding activity against Neospora was determined to be “positive” at a serum dilution of ≥1:160.

For ELISA, the following procedures were used. Neospora tachyzoites obtained and purified from in-vitro cultures as described above were washed in sterile PBS. The pellet was resuspended in PBS containing 0.01% NaN₃ and homogenised. Subsequent treatment by three freezing-thawing cycles (−50°C/+37°C) and final processing by ultrasonication (3 x 20 s at 65 W) at 1°C yielded the extract solution. The solution was sedimented at 10 000 g at 4°C for 30 min. The soluble supernatant was used as somatic ELISA-antigen (hereinafter referred to as Neospora-SA-ELISA) or stored at −80°C until use. Coating Dynatech polystyrene plates was done at a concentration of 5 µg protein per ml carbonate buffer (pH 9.6) at 4°C for 12 h. For the detection of antibodies against T. gondii, ELISA plates were coated in a similar manner with 1 µg protein per ml of an affinity purified P30 surface antigen (SR2B, Arville, France, cat. No. TXP30B), hereinafter referred to as Toxoplasma-P30-ELISA. All protein concentrations were assessed by the Bio-Rad protein assay using bovine albumin as the standard. Neospora- and Toxoplasma-ELISA plates were subsequently processed as described for other ELISAs performed in our laboratories [24]. In brief, the washing, blocking and serum dilution solution was PBS containing 0.3% Tween 20 (PBS–TWEEN). The test and control sera were diluted 1:100 in PBS–Tween. Serum incubations were for 2 h at 37°C. The second antibody was an alkaline phosphatase-conjugated rabbit-anti-bovine antibody (Sigma Immunochemicals, cat. No. A 0705) diluted at 1:500 in PBS–TWEEN. The conjugate was incubated for 1 h at 37°C. Antibody reactivity was detected by adding 4-nitrophenylphosphate at a concentration of 1 mg ml⁻¹ 0.1 M-diethanolamine, pH 9.8. The reaction was stopped after 15 min with 3 N-NaOH, and absorbance values were read at A₄₀₅ using a Dynatech MR7000 reader coupled to a Macintosh Performa 6300 computer with Biocalc® software (Dynatech).

Positive and negative control sera for the Neospora-SA-ELISA were the same as used for the IFAT. For the Toxoplasma-P30-ELISA, the same negative control serum was used as for the Neospora serology. The Toxoplasma-positive control serum was a gift from Dr David Buxton (Moredun Research Institute, Edinburgh, U.K.). The basic test parameters for the Neospora and Toxoplasma antigens were established by investigating 50 sera of animals (no history of abortion; anamnestic lack of clinical or epidemiological signs indicative for neosporosis or toxoplasmosis) negative in Neospora-IFAT and Toxoplasma-DA (see below) using the respective ELISA mean value +4 SD. as the cut-off value. Any value higher than this cut-off value was considered to be “positive”; lower values were considered as “negative”. Threshold values discriminating between diagnostically positive and negative reactions were thus determined for both antigens listed above. Reproducibility of ELISA results was monitored by including a low reactive bovine control serum in triplicate, this in addition to the negative and positive standard sera, both also tested in triplicate.

In addition to the tests listed above, antibody concentration against T. gondii was determined in parallel by the commercially available agglutination test Toxo-Screen DA (BioMérieux). Test performance was determined according to the manufacturer’s instructions, which corresponds to the United States Department of Agriculture (USDA) procedure described elsewhere [14].

Tissue collection. Organs and blood samples from 83 naturally aborted foetuses (all originating from
different farms in Switzerland) were collected during 1993–1994 and were examined pathologically at the Institute of Veterinary Pathology of the University of Zurich, using conventional macroscopic and histopathologic examination techniques. At the same institute, investigation for the presence of bovine viral diarrhea virus (BVDV) by immunohistochemistry (LSAB: labelled Streptavidin-Biotin) was performed. Other conventional microbiological investigations representing the spectrum of micro-organisms recommended in the survey programmes of Swiss veterinary health offices (Rotavirus, IBR/IPV, Listeria, Coxiella, Brucella, Actinomyces) were done in routine diagnostic laboratories.

At necropsy, the following samples were obtained from the foetuses and processed separately for parasitological analyses: (i) a foetal heart blood or body-cavity fluid sample; (ii) the skull was opened under aseptic conditions and a sample of the cerebrum was transferred into a sterile 50-ml Falcon tube prefilled with 20 ml sterile PBS containing 200 U ml⁻¹ of penicillin, 200 µg ml⁻¹ streptomycin and 0.5 µg ml⁻¹ fungizone; (iii) heart tissue was processed as described above for brain tissue; (iv) placental tissue was obtained in 32 cases; (v) blood samples were collected from 12 cows after abortion. Classical histopathological examinations of the organs, in particular the foetal brain and the maternal placenta, were performed at the Institute of Veterinary Pathology, University of Zurich. Precolostral-IgG determination was carried out using a radioimmunodiffusion IgG-Determination-kit (RID-Kit; The Binding Site), results being expressed in mg IgG per litre.

Tissue samples not processed for histological, immunological or molecular diagnosis were stored at −80°C.

**Bovine sera.** A total of 1689 bovine sera (adult cows) from 113 dairy farms collected for a different project [25] were subsequently investigated in the present study. The animals and farms had been selected as representative of the Swiss dairy cattle population in 1994. Baseline information on health and fertility problems was available for all farms. The samples were investigated for the presence of antibodies against Neospora sp. and *T. gondii* using the respective ELISA (see above). Sera from 85 dams and from 102 corresponding offspring calves were obtained from 36 different cow–calf farms. The age of the calves at the time of blood-sample collection was between 8 and 10 months [26].

**Statistical analyses**

The association between serological *Neospora* and *Toxoplasma* results in dairy and cow–calf serum samples, and between seropositivity of dams and offspring calves, was assessed by the Fisher's Exact test (two-tailed) using EpiInfo v. 6.04 (CDC, Atlanta). Confidence intervals (CI) for clustered data were set at 95% and calculated as described elsewhere [27].

3. Results

3.1. Diagnostic in-vitro isolation of parasites

From the overall number of 83 abortion cases, brain material from 27 foetuses and five corresponding placenta could be processed for in-vitro isolation of *Neospora* sp. (and putatively *T. gondii*). For 56 cases, the in-vitro isolation could not be performed because either the biological samples were of too poor condition, the size of the sample was too small, or cultivation led to premature microbial contamination of cultures. The 27 samples investigated included two groups: (i) 15 cases negative for any diagnostic *Neospora* or *Toxoplasma* test (serology and PCR) as listed below; (ii) 12 cases positive in *Neospora* tests as follows: four cases *Neospora*-seropositive and positive in *Neospora*-PCR (case Nos 3–6, see Table 1), and eight cases *Neospora*-seronegative but positive in *Neospora*-PCR (case Nos 7–14, see Table 1). Isolation was also attempted from the placental material obtained from two cases (Nos 2, 4) sero- and PCR-positive in *Neospora* assays and from three cases (Nos 8, 10, 25) only *Neospora*-PCR-positive. The diagnostic in-vitro isolation of the parasite failed in all cases, including especially also those cases where the mother-cow had been serologically “*Neospora*-positive” or where the *Neospora*-PCR had been positive with foetal brain material.
Table 1
Brains of 83 aborted bovine foetuses were investigated by PCR for the presence of *Neospora* and *Toxoplasma* DNA*

| Animal ID | Neospora serology | Neospora PCR | Toxoplasma serology | Toxoplasma PCR | Foetal age (months) | Pathology (macro- and microscopical criteria) |
|-----------|-------------------|--------------|---------------------|---------------|-------------------|-----------------------------------------------|
| 1         | POS               | POS          | neg                 | neg           | 8                 | Brain lesions; ivivo                           |
| 2         | POS               | POS          | neg                 | neg           | 8                 | Placental hyperaemia; conjunctivitis; afls     |
| 3         | POS               | POS          | neg                 | neg           | 9                 | Conjunctivitis, pneumonia                     |
| 4         | POS               | POS          | neg                 | neg           | 7                 | Calcifications and necrotic lesions in placenta; afls; ivivo |
| 5         | POS               | POS          | neg                 | neg           | 6                 | Hyperplasia of cerebellum; [BVDV +]           |
| 6         | POS               | POS          | neg                 | neg           | +1*               | Hydrocephalus internus; haemorrhage in kidney  |
| 7         | neg               | POS          | neg                 | neg           | 5                 | Necrotic brain lesion; encephalitis; pneumonia |
| 8         | neg               | POS          | neg                 | neg           | 7                 | nsP                                           |
| 9         | neg               | POS          | neg                 | neg           | 7                 | nsP                                           |
| 10        | neg               | POS          | neg                 | neg           | 6                 | nsP                                           |
| 11        | neg               | POS          | neg                 | neg           | 8                 | Granulocytosis in brain; pneumonia            |
| 12        | neg               | POS          | neg                 | neg           | +1*               | Enteritis with necrotic lesions in Peyer's patches; [BVDV +] |
| 13        | neg               | POS          | neg                 | neg           | 5                 | nsP                                           |
| 14        | neg               | POS          | neg                 | neg           | 5                 | ivivo; necrotic lesions in cerebellum; meningitis |
| 15        | neg               | POS          | neg                 | neg           | 6                 | Necrotic lesions in brain; conjunctivitis [Streptococcus] |
| 16        | neg               | POS          | neg                 | neg           | 5                 | Calcifications in placenta                    |
| 17        | neg               | POS          | neg                 | neg           | 5                 | Necrotic lesions in brain and placenta, epicarditis; ivivo |
| 18        | neg               | POS          | neg                 | neg           | 6                 | Necrotic lesions and calcifications in brain   |
| 19        | neg               | POS          | neg                 | neg           | 6                 | Hyperplasia of cerebellum, conjunctivitis      |
| 20        | neg               | POS          | neg                 | neg           | 6                 | Haemorrhage in cerebellum                     |
| 21        | neg               | POS          | neg                 | neg           | 7                 | nsP                                           |
| 22        | neg               | POS          | neg                 | neg           | 6                 | Haemorrhage in cerebellum                     |
| 23        | neg               | POS          | neg                 | neg           | 7                 | nsP                                           |
| 24        | neg               | POS          | neg                 | POS           | 7                 | Haemorrhage in heart; calcifications in placenta with fungi |
| 25        | neg               | neg          | neg                 | POS           | 7                 | afls; ivivo; necrotic lesions in brain, liver, placentitis |
| 26        | neg               | neg          | neg                 | POS           | 9                 | ivivo; conjunctivitis, calcifications in placenta |
| 27        | neg               | neg          | neg                 | POS           | 7                 | Conjunctivitis; [Aeromonas hydrophila]         |
| 28        | POS               | neg          | POS                 | neg           | +3*               | Haemorrhage in kidney, enteritis, abomasitis [Rotavirus] |
| 29        | neg               | neg          | POS                 | neg           | 11                | Atresia ani and malformation in diverse urogenital organs |
| 30-83     | neg               | neg          | neg                 | neg           | (8)*              | See Results section                           |

*Comparatively, foetal fluids were assessed for the presence of antibodies against *Neospora* (accumulative IFAT and/or SA-ELISA-positivity) or *Toxoplasma* (accumulative P30-ELISA- and/or DA-positivity). Pathological examinations were performed under routine diagnostic procedures prior to parasitological examinations.

**iivo** = inflammatory infiltrates in various organs; afls = activated fetal lymph system; nsP = no specific pathology; [ ] = additional pathogens identified.

*+1 and +3 were calves which died 1 and 3 days after birth, respectively.

For the 54 cases all negative in each test, the mean age of the foetuses was 8.1 months.
3.2. Neospora- and Toxoplasma-PCR and histopathological examinations

All brain samples obtained from the 83 abortion cases were investigated by PCR for the detection of parasite-specific DNA. Neospora was detected in 24 (29%) cases and Toxoplasma in four (5%) cases. In one of these cases (No. 24), the simultaneous presence of both parasites was demonstrated (Table 1). Additional results of pathological findings are also included in Table 1. Histological examination of the 24 Neospora-PCR-positive samples revealed that 18 samples exhibited pathologic findings compatible with neosporosis. For samples obtained from either the brain and/or the placenta, seven cases included multifocal necrotising encephalitis, two cases hypoplasia of the cerebellum, one case hydrocephalus internus and five cases multifocal necrotising placentitis or calcification within the placenta, among other features (for details see Table 1), which were not indicative of a protozoal infection in most cases. In most of the 24 Neospora-PCR-positive cases, no other infectious agents were found. Exceptions were two foetal BVDV cases and one case of Streptococcus detection in the placenta. From the three foetal brain samples which were only positive by Toxoplasma-PCR (but Neospora-PCR-negative), the histological examinations, performed prior to PCR investigations, had already provided pathologic findings indicative of a protozoal infection (random multifocal necrotic lesions in the brain, calcified microlesions in the placenta and conjunctivitis). The single case positive for both Neospora- and Toxoplasma-PCR showed also multifocal calcifications in the placenta, but no specific pathology in the foetal tissues could be found. The mean age of the Neospora-PCR-positive aborted foetuses was 6.8 months (95%CI: 6.1–7.5), with 18 cases (75%) being 4–7 months of age. Another parameter which significantly (P = 0.95) discriminated between the group of Neospora-PCR-positive cases and respective negative cases concerned the pre-colstral IgG. The mean concentration of pre-colstral IgG in 21 Neospora-PCR-positive cases suitable for respective analysis was 1195 mg ml⁻¹ versus 512 mg ml⁻¹ in 45 Neospora-PCR-negative cases.

For the 56 cases which were negative by both Neospora- and Toxoplasma-PCRs, characteristic pathologic findings indicating neosporosis were considerably less frequent for cerebral involvement (subsequent data refer to case Nos 30–83 in Table 1). While cerebral modifications were macro- or microscopically detectable in 52% of Neospora-PCR-positive cases, similar findings were obtained only in 21% of PCR-negative cases. Furthermore, other infectious agents were found at a relatively high frequency in the PCR-negative specimens: seven cases with viral infections, including BVDV (four cases) and Rota/Corona viruses; nine cases with bacterial infections, including the genera Mycoplasma, Streptococcus, Staphylococcus and Pasteurella; 16 cases with other pathologies, including myocarditis, hydrothorax, hydropsphrosis, degeneration or malformation of different organs, hepatitis, microencephaly, tumours, cerebral thrombosis, oedema in the lungs, calcified neurons, haemorrhages in different organs, pneumonia, conjunctivitis, placentitis with and without necrosis and calcifications, asphyxia, encephalitis.

Among these 56 PCR-negative cases, two were serologically positive for Toxoplasma, and one of them was also positive by Neospora-SA-ELISA. In these two cases, the seropositive results could not be associated with any characteristic pathologic finding (for details, see Table 1). The mean age of aborted foetuses negative by all Neospora and Toxoplasma tests (PCR and serology) was 8.1 months (95%CI: 7.5–8.8). From these 54 cases, 20 (37%) were 4–7 months of age. In 32 cases, a placental tissue sample was available in parallel to the foetus and was thus used for subsequent PCR analysis. The comparative results between PCR performed with foetal brain and placenta were the following: 23 cases were negative by both Neospora- and Toxoplasma-PCR in the two tissue samples; seven cases were Neospora-PCR-positive only in the foetal brain (negative by the Toxoplasma-PCR) and negative in respective placental PCR investigations; one case was positive only by Neospora-PCR for both foetal brain and placenta and one case was Toxoplasma-PCR-positive only in the foetal brain but negative in the corresponding placental PCR investigation. Representative PCR results with a selection of cases of abortion associated with Neospora or Toxoplasma are shown in Fig. 1.
Immunohistological examination of fixed foetal brain samples using anti-Neospora or anti-Toxoplasma hyper-immune sera provided results which did not allow a specific and unambiguous identification of parasites for the first 10 cases tested. Consequently, and due to the time-consuming aspects of the technological procedure, we decided that this approach was not practicable for large-scale rapid routine diagnosis.

3.3. Neospora and Toxoplasma serology

Serum from foetal heart blood or body cavity fluid samples from all 83 abortion cases was tested for the presence of antibodies against *Neospora* and *Toxoplasma* using IFAT, ELISA or DA. The results are shown in Table 1. For *Neospora*, four out of seven IFAT-positive sera reacted positively by SA-ELISA (case Nos 4, 5, 6, 28). One serum (case No. 28) proved double-positive by both *Neospora* and *Toxoplasma* serology, but it was negative by either of the two PCR tests. For *Toxoplasma*, one out of two DA-positive sera reacted positively by P30-ELISA. Both *Toxoplasma*-seropositive foetuses were PCR-negative (for both parasites).

In 12 abortion cases, it was possible to determine the serological parameters in association with a maternal serum sample drawn on the day of abortion. With respect to *Neospora*, seven out of 12 cases were negative for both dam and foetus using all immunodiagnostic and molecular tools. There was one single case with a seropositive dam and a foetus being also positive by *Neospora* serology (SA-ELISA and IFAT) and *Neospora*-PCR (case No. 4, Table 1). There were three cases where the dams were *Neospora*-seropositive (P30-ELISA and
IFAT) and the foetuses Neospora-PCR-positive, but simultaneously these offspring were negative by Neospora serology. Finally, there was one seropositive (SA-ELISA and IFAT) dam with a seronegative foetus which had no Neospora DNA detectable by PCR. With respect to Toxoplasma, there were seven cases where all serological and molecular tests were negative. Then, there were five cases with the dams being Toxoplasma-seropositive (five by P30-ELISA, three by DA) but with the foetuses being negative by both Toxoplasma-PCR and Toxoplasma serology.

3.4. Seroprevalence in dairy cattle

From 1689 dairy cattle sera investigated, 194 sera (prevalence = 11.5%; 95%CI 9.2–13.8) were positive by Neospora-SA-ELISA and 180 (prevalence = 10.7%; 95%CI 8.3–12.6) were positive by Toxoplasma-P30-ELISA (Table 2). From those 194 Neospora-positive sera, 19 also contained antibodies reactive in the Toxoplasma-P30-ELISA, thus, the overall rate of double-positivity was 1.1%. The double-positivity rate with respect to reactive sera only (19 double-positives per 374 positives by Neospora and/or Toxoplasma serology) was 5.1%. There was no statistically significant association between seropositivity for Neospora and seropositivity for Toxoplasma (P = 0.80). The seroprevalence concerning anti-Neospora antibodies in Swiss dairy cattle was plotted against the incidence of (a) fertility problems, (b) return to oestrus problems, (c) cumulative incidence of abortions (results not shown) and (d) herd/farm size. Again, no statistically significant correlation was observed.

3.5. Seroprevalence in cow-calf farms

Serological results of this group of cows and calves were as follows. For Neospora: 10 out of 73 sera of dams and 12 out of 78 corresponding offspring calves tested positive by Neospora-ELISA, resulting in a seroprevalence of 14% (95%CI: 5.0–23.0) and of 15% (95%CI: 3.0–28.0), respectively. For Toxoplasma: seven out of 85 dams and three out of 95 corresponding offspring calves tested positive by Toxoplasma-P30-ELISA, resulting in a seroprevalence of 8% (95%CI: 4.0–12.0) and of 3% (95%CI: 3.0–6.0), respectively. None of the Neospora-positive sera were positive by Toxoplasma-P30-ELISA and vice versa; thus, within this animal population, specificity was 100%. The association between serological Neospora or Toxoplasma results was assessed by the Fisher’s exact two-tail test. Hence, there was no statistically significant correlation between seropositivity against Neospora versus seropositivity against Toxoplasma, neither at herd level, nor for dams or calves. A significant association, however, was found between dam and offspring calf seropositivity at the individually paired level.

4. Discussion

The present study was designed to assess the performance of diagnostic tools for the identification of Neospora infection and, comparatively, Toxoplasma infection in 83 aborted foetuses, and to determine preliminarily the potential occurrence and thus the importance of Neospora in bovines of Switzerland.

By using PCR, we observed an unexpectedly high percentage (29%) of foetuses in which Neospora DNA was detected in the damaged brain tissue. This percentage appeared to be high insofar as the other infectious organisms searched for within these foetuses occurred at only a very low rate. Unexpectedly, Toxoplasma DNA was also detected, but
in only a few brains (5%). According to conventional knowledge, *T. gondii* is principally not considered to be a cause of abortion in bovines [28]. Therefore, our respective results need to be interpreted with caution. Our findings showed the presence of *Toxoplasma* DNA in the damaged foetal brain, which does not prove a causative correlation between infection and abortion by itself. Nevertheless, histopathologic findings unambiguously supported a protozoal infection and induced tissue damage in all three cases.

As the seroprevalence was similar for both parasite species in adult cattle, but the PCR findings were significantly higher for *Neospora* in foetuses, the comparison between seroprevalence and PCR findings consequently indicates a higher prenatal transmission rate for *Neospora* to the foetus than for *Toxoplasma*. The diaplacental transmission seems to be epidemiologically more important for *Neospora*, whereas postnatal infection seems to be more important for *Toxoplasma*. Beside the pathologic findings depicted in prenatal neosporosis, another parameter, that of elevated pre-colostral IgG, indirectly pointed at the pathologic significance of a *Neospora* infection in the foetus.

In contrast to PCR-based diagnosis, demonstration of the parasites by in-vitro culturing, or demonstration of parasite antigens by immunohistochemistry, were unsuitable as diagnostic tools. Although the two latter techniques may be considered as gold-standard methods to diagnose neosporosis, they both lack methodical and diagnostic sensitivity, as already discussed by several authors [2, 9]. This lack of technical sensitivity may also be one explanation why, in previous attempts, demonstration of *T. gondii* in bovine foetuses has failed in many cases [28]. The unavailability of sensitive techniques may have considerably hampered previous studies on the reliability of prevalence assessment [15]. In these studies, conventional histopathology criteria, beside immunohistochemistry, were also included to select positive cases of bovine neosporosis related to abortion. In a preliminary study (not included in the present study) based on experimental infection of pregnant cows, we observed a considerable difference between the technical sensitivity of DNA detection by PCR and aetiological diagnosis by in-vitro cultivation or immunohistochemistry. However, more data are needed to significantly evaluate and elucidate the differences between PCR and immunohistochemistry. With regard to diagnostic in-vitro cultivation, we selected for our study an incubation period of 2 weeks to assess in-vitro parasite growth or growth absence from diagnostic brain samples. This period was selected based upon another report [23] and on local practicabilities in our routine laboratory process. Retrospectively, diagnostic experiences made by others [29] have shown that a minimal cultivation time of 8 weeks would have provided a much higher potential to obtain in-vitro growth of parasites, a fact which will have to be considered in future studies.

The application of different threshold dilutions to discriminate between positive and negative serological reactions in IFAT for foetuses, calves and cows has been discussed by several authors [30, 31]. Although applying two different cut-off titres in our IFAT for foetuses and calves/cows, the results obtained indicated that this would not have been necessary in that antibody titres of all animals were either at dilutions < 1/80 or > 1/160. For ELISA, it had been shown previously that the use of different cut-off absorbance values for foetal or adult cattle sera may lead to false-positive results [31]. Due to the lack of appropriate experiences, we had decided not to apply different threshold ELISA values indicative for *N. caninum* infection in foetuses. The full characterisation of operating characteristics of our ELISA still requires further parametric investigations with special emphasis on the problem in question, and we will have to consider the determination of different threshold values for different groups of animals in future investigations. Furthermore, new developments in the purification or biotechnologic synthesis of *Neospora* antigens with improved immunodiagnostic potential will have to be considered in future studies [12, 13, 32]. Our thus relatively preliminary serological results have shown that only 25% of the foetuses with a positive cerebral *Neospora*-PCR had detectable antibodies against *Neospora* at the time of abortion. It has been shown in previous work that the seroprevalence in foetuses increased with gestational age [30]. The same authors had found anti-*N. caninum* antibodies in 37 of 74 foetuses with
suspected or confirmed neosporosis-associated abortion; 31 of these 37 seropositive foetuses were 6 months of gestational age or older. In our study, all except one Neospora-seropositive foetus were 7–9 months of gestational age or older, whereas the larger part of seronegative foetuses was significantly younger. Thus the failure to find anti-N. caninum antibodies indicated that most of our foetuses may not yet have reached the appropriate humoral immunocompetence during the acute phase of infection until abortion. In addition, we have studied in 12 cases the association between the serological results of the foetus and the corresponding dam in parallel. Only one of the foetuses was seropositive, whereas five of the dams were seropositive. From the seronegative foetuses, four were positive by Neospora-PCR performed on brain tissue. Although the data are based upon only a few cases, we could thus obtain an indication that prenatal neosporosis seems to be related predominantly to seropositive dams, but that most of the foetuses were not able to seroconvert during infection prior to abortion. This information indicates that serological screening in foetuses is of relatively little diagnostic value, whereas serological pre-screening in dams may provide some indication for a potential prenatal transmission of the parasite. However, more field data are needed in order to provide clear statistical evidence for these observations.

With regard to the time of abortion with respect to the duration of gestation, our findings fitted a presumed expectation based upon other publications (see [2]). The mean age (6.8 months) of aborted foetuses positive by Neospora-PCR was significantly lower than the mean age (8.1 months) of aborted foetuses negative by any Neospora and Toxoplasma test. Abortion occurred in 18 (75%) of 24 cases positive by Neospora-PCR between the 4th and 7th month of gestation, whereas for the cases negative for any Neospora and Toxoplasma test, only 20 (37%) of 54 cases occurred in the same period. As already discussed by other authors [2, 15], Neospora-induced abortion predisposes for the approximate period of gestation as mentioned above. Later on, infection may not necessarily lead to abortion, but may result in still-birth or weak calves. Neospora-associated abortion does not imply that the parasite has actually caused the foetal damage primarily responsible for abortion [15]. Nevertheless, in those cases where foetal and placental material was tested, the comparative analysis showed that there was no case where the parasite was detectable only in the placenta but not in the foetus. However, seven cases were Neospora-PCR-positive in foetal brain but not in the placenta, and only one case was simultaneously Neospora-PCR-positive for both the foetal brain and the placenta. Thus, it seems that the parasite develops very well in the foetus and thus becomes detectable by PCR, while it is not readily detected in the placenta.

Among the 83 foetuses tested, BVDV was demonstrated by immunohistology in two of 24 Neospora-PCR-positive cases and in four of 59 Neospora-PCR-negative cases. Thus, no statistical association became evident between BVDV and any diagnostic findings relating to Neospora or Toxoplasma.

With regard to the serological investigations, there was an interesting consistency of Neospora results when comparing dairy cattle and cow–calf populations. In both groups, the Neospora seroprevalence of adult cows was very similar (12% and 14%, respectively) and was at an approximately equivalent level (15%) in offspring calves on cow–calf farms. For Toxoplasma, the seroprevalence in adult cattle was not significantly different between dairy cattle and cow–calf populations (11% and 8%, respectively), but clearly lower (3%) for offspring calves. It may be that diaplacental transmission dominates the epidemiology of neosporosis, reflected by the almost identical seroprevalence in dams and calves. On the other hand, peroral infection with T. gondii oocysts may be the predominant mode of infection for cattle. This hypothesis would be supported by the lower seroprevalence in young animals.

Technically, there is still some need for further elaboration of the exact operating characteristics of the ELISA systems compared with the IFAT for neosporosis and DA for toxoplasmosis. For foetal Neospora serology, the IFAT appeared more sensitive (six IFAT-positive foetuses, of which only three were positive by SA-ELISA). Adult cow sera positive by IFAT were always positive by SA-ELISA, although the number of animals tested did
not allow an accurate statistical analysis. Therefore, the seroprevalence data obtained from the two cattle populations should be interpreted with caution. By using a somatic *Neospora*-tachyzoite antigen similar to that employed in our study, an ELISA sensitivity of 88.6% and specificity of 96.5% had been estimated earlier [13]. When compared with the IFAT, the ELISA appeared more sensitive and specific for serodiagnosis of *Neospora* infection [13]. We believe that new technical approaches are needed to improve the diagnostic parameters and especially standardisation of serological tools, such as proposed by others [32]. It has been shown that recombinant antigen ELISAs are capable of distinguishing between sera from *Neospora*-infected cows and sera from non-infected control cows. As the recombinant antigens in question show no evidence of cross-reactivity with sera from animals inoculated with *T. gondii* or some *Sarcocystis* species, this kind of approach may be suitable for routine application.

Our results indicate that PCR may be a valuable diagnostic alternative with a superior performance to other tests such as in vitro isolation or immunohistology. Furthermore, we showed that PCR combined with serology does accurately discriminate between *Neospora* and *Toxoplasma* infection. Finally, the association between specific pathologic findings in the foetal brain, the demonstration of *Neospora* DNA at the same site, and the absence of other aetiologic abortive microorganisms in most cases not only indicate foetal infection, but also support the hypothesis of a causative role of the parasitic infection for abortion in Switzerland. The high rate of *Neospora*-PCR-positivity within abortion events in Swiss cattle justifies, therefore, further studies on the molecular epidemiology and pathogenesis of neosporosis in Switzerland.

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