Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

ELSEVIER International Journal for Parasitology 28 (1998) 679–691

Molecular and immunodiagnostic investigations on bovine neosporosis in Switzerland

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

[a]*Institute of Parasitology, University of Berne, Länggass-Strasse 122, CH-3001 Berne, Switzerland*
[b]*Institute of Veterinary Pathology, University of Zürich, Winterthurerstrasse 265, CH-8057 Zürich, Switzerland*
[c]*Institute of Animal Breedings, University of Berne, Länggass-Strasse 109a, CH-3001 Berne, Switzerland*
[d]*Institute of Virology and Immunoprophylaxis, CH-3147 Mittelhäusern, Switzerland*

Received 15 September 1997; received in revised form 15 December 1997; accepted 15 December 1997

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 11.5% (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-

*Corresponding author. Tel: +41-31-631-24-18; Fax: +41-31-631-26-22; e-mail: bruno.gottstein@ipa.unibe.ch.

S0020-7519/98 $19.00+0.00 © 1998 Australian Society for Parasitology. Published by Elsevier Science Ltd. Printed in Great Britain
PII: S0020-7519(98)00006-X

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 Immunoassay (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,

19]. 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-cm^3 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 to be "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]).

Histological and immuno-histological examinations. One portion of each tissue sample obtained at necropsy was fixed in 4% formalin and processed for routine paraffin embedding. Paraffin-embedded sections of 3–4 μm thickness were mounted on poly-L-lysin precoated glass slides, deparaffinised in xylene and stained with H & E or for immunohistology, slides were rehydrated in descending concentrations of methanol, rinsed in H_2O, and non-specific binding sites were blocked with PBS containing 0.05% Tween and 3% BSA (Fluka AG) (designated PBS–Tween–BSA) for 1 h. Slides were then incubated with the respective rabbit hyperimmune sera [22] for 1 h at 37°C. The anti-*N. caninum* and the anti-*T. gondii* hyperimmune rabbit sera, and the respective pre-immune sera (as controls) 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 (BioMerieux Suisse S.A.) and finally examined using a Leitz Laborlux S fluorescence microscope. The specificity of the two hyperimmune sera was initially confirmed by the absence of immunoreactivity on heterologous *T. gondii* or *N. caninum* tachyzoites, respectively [22].

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 1:40 in sterile PBS with subsequent two-fold titration. Positive and negative control sera were each diluted at 1:40 and 1:80. The positive control serum was obtained from a pregnant cow experimentally infected with live *N. caninum* tachyzoites (Hentrich et al., unpublished) according to the procedure described by others [9]. Infection in this cow was proven by PCR-based detection of *Neospora*-DNA in the foetal brain using the same technique as described previously for mice [21]. The negative control serum was obtained from the same cow (first gestation) prior to experimental infection. The absence of antibodies against *Neospora* and *Toxo-*

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 FluoPrep (Bio-Merieux) 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 $\geqslant 1/80$ were detected. For cows, the antibody-binding activity against *Neospora* was determined to be "positive" at a serum dilution of $\geq$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% NaN_3 and homogenised. Subsequent treatment by three freezing–thawing cycles ($-50°/+37°C$) and final processing by ultrasonication (3 × 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 Immmunochemicals, 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 A_{405nm} 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 S.D. 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 diarrhoea 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 $\mu g\, ml^{-1}$ streptomycin and 0.5 $\mu g\, ml^{-1}$ 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 Zürich. Precolostral-IgG determination was carried out by 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 $-80^{\circ}C$.

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 placentas 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[a]

Animal ID	*Neospora* serology	*Neospora*-PCR	*Toxoplasma* serology	*Toxoplasma*-PCR	Foetal age (months)	Pathology (macro- and microscopical criteria)[b]
1	POS	POS	neg	neg	8	Brain lesions; iivo
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; iivo
5	POS	POS	neg	neg	6	Hyperplasia of cerebellum; [BVDV+]
6	POS	POS	neg	neg	+1[c]	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	Granulocytostasis in brain; pneumonia
12	neg	POS	neg	neg	+1[c]	Enteritis with necrotic lesions in Peyer's patches; [BVDV+]
13	neg	POS	neg	neg	5	nsp
14	neg	POS	neg	neg	5	iivo; 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; iivo
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; iivo; necrotic lesions in brain, liver, placentitis
26	neg	neg	neg	POS	9	iivo; conjunctivitis, calcifications in placenta
27	neg	neg	neg	POS	7	Conjunctivitis; [*Aeromonas hydrophila*]
28	POS	neg	POS	neg	+3[c]	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)[d]	See Results section

[a]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.
[b]iivo = inflammatory infiltrates in various organs; afls = activated fetal lymph system; nsp = no specific pathology; [ ] = additional pathogens identified.
[c]+1 and +3 were calves which died 1 and 3 days after birth, respectively.
[d]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 precolostral IgG. The mean concentration of pre-colostral 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, hydronephrosis, 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.

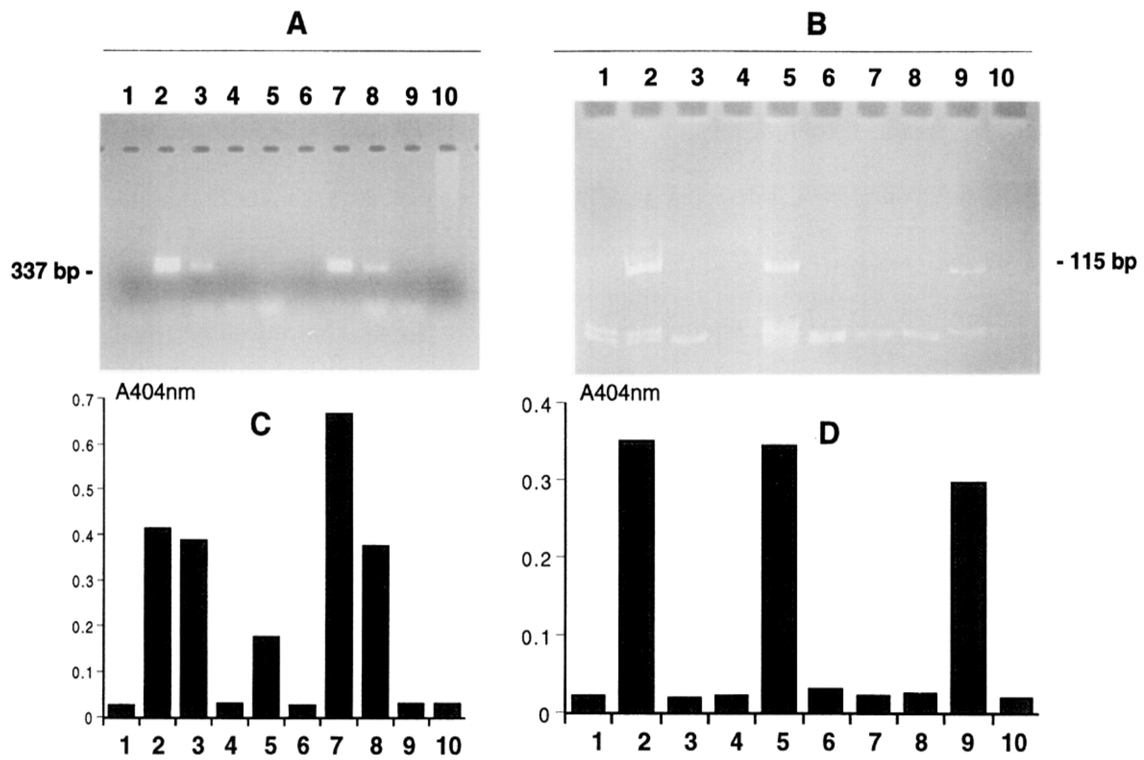

Fig. 1. Gel electrophoresis of *Neospora*- (A) and *Toxoplasma*-specific (B) amplification products (2% agarose gel for *Neospora*-PCR; 10% polyacrylamide for *T. gondii*-PCR). Comparative DIA for specific PCR-based diagnosis is shown in (C) for *Neospora* and in (D) for *Toxoplasma*. Lane 1: negative control (= no DNA); lane 2: positive control; lane 3: diagnostic foetal template DNA from case No. 2 (see Table 1); lane 4: corresponding maternal placenta template DNA from case No. 2; lane 5: foetus, case No. 24; lane 6: placenta, case No. 24; lane 7: foetus, case No. 17; lane 8: placenta, case No. 17; lane 9: foetus, case No. 25; lane 10: placenta, case No. 25. Lower bands refer to resolved primer molecules.

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.

Table 2
Serodiagnostic association between seropositivity in the *Neospora*-SA-ELISA and the *Toxoplasma*-P30-ELISA with regard to animals kept in conventional dairy cattle farms

		Neospora-SA-ELISA		Accumulative totals
		+	−	
Toxoplasma-P30-ELISA	+	19	161	180
	−	175	1334	1509
Accumulative totals		194	1495	1689

Seroprevalence for *Neospora*: 194/1689 = 0.115; seroprevalence for *Toxoplasma*: 180/1689 = 0.107.

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 $\geq 1/160$. 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 aetiological 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.

Acknowledgements—We thank Dr H. Kaufmann and O. Flechtner for their technical and logistic support as well as for fruitful discussions, and are indebted to Dr A. Hemphill for stimulating discussions and critical comments on the manuscript. Many thanks to Dr J.P. Dubey (United States Department of Agriculture, Beltsville, U.S.A.), and Dr D. Buxton (Moredun Research Institute, Edinburgh, U.K.) for providing parasites and reagents and to Dr Bernadette Connolly for critical reading of the manuscript. This work has been supported by a research grant from the "Bundesamt für Veterinärwesen" (grant No. 012.4.93.4) and by the "Bundesamt für Bildung und Wissenschaft" (COST-820 project BBW C96.0068).

References

[1] Dubey JP, Lindsay DS. Neosporosis. Parasitol Today 1993;9:452–458.

[2] Dubey JP, Lindsay DS. A review of *Neospora caninum* and neosporosis. Vet Parasitol 1996;9:452–458.

[3] Dubey JP, Carpenter JL, Speer CA, Topper MJ, Uggla A. Newly recognized fatal protozoan disease of dogs. J Am Vet Med Assoc 1988;192:1269–1285.

[4] Anderson ML, Blanchard PC, Barr BC, Dubey JP, Hoffman RL, Conrad PA. *Neospora*-like protozoan infection as a major cause of abortion in California dairy cattle. J Am Vet Med Assoc 1991;198:241–244.

[5] Paré J, Thurmond MC, Hietala SK. Congenital *Neospora caninum* infection in dairy cattle and associated calfhood mortality. Can J Vet Res 1996;60:133–139.

[6] Soulsby EJL. Helminths, arthropods and protozoa of domesticated animals, 7th ed. London: Baillière Tindall, 1982.

[7] Ellis J, Luton K, Baverstock PR, Brindley PJ, Nimmo KA, Johnson AM. The phylogeny of *Neospora caninum*. Mol Biochem Parasitol 1994;64:303–311.

[8] Hoar BR, Ribble CS, Spitzer CC, Spitzer PG, Janzen ED. Investigation of pregnancy losses in beef cattle herds associated with *Neospora* sp. infection. Can Vet J 1996;37:364–366.

[9] Conrad PA, Sverlow K, Anderson M., et al. Detection of serum antibody responses in cattle with natural or experimental *Neospora* infections. J Vet Diagn Invest 1993;5:572–578.

[10] Cole RA, Lindsay DS, Dubey JP, Blagburn BL. Detection of *Neospora caninum* in tissue sections using a murine monoclonal antibody. J Vet Diagn Invest 1993;5:579–584.

[11] Trees AJ, Guy F, Balfour AH, Dubey JP. Prevalence of antibodies to *Neospora caninum* in a population of urban dogs in England. Vet Rec 1993;132:125–126.

[12] Björkman C, Liunden A, Holmdahl J, Barber J, Trees AJ, Uggla A. *Neospora caninum* in dogs: detecton of antibodies by ELISA using an iscom antigen. Parasit Immunol 1994;16:643–648.

[13] Paré J, Hietala SK, Thurmond MC. An enzyme-linked immunosorbent assay (ELISA) for serological diagnosis of *Neospora* sp. infection in cattle. J Vet Diagn Invest 1996;7:352–359.

[14] Dubey JP, Lindsay DS, Adams D.S., et al. Serologic responses of cattle and other animals infected with *Neospora caninum*. Am J Vet Res 1996;57:329–336.

[15] Thurmond MC, Hietala SK, Blanchard PC. Herd-based diagnosis of *Neospora caninum*-induced endemic and epidemic abortion in cows and evidence for congenital and postnatal transmission. J Vet Diagn Invest 1997;9:44–49.

[16] Ho MS, Barr BC, Marsh A.E., et al. Identification of bovine

Neospora parasites by PCR amplification and specific small-subunit rRNA sequence probe hybridization. J Clin Microbiol 1996;34:1203–1208.

[17] Payne S, Ellis J. Detection of *Neospora caninum* DNA by the polymerase chain reaction. Int J Parasitol 1996;26:347–351.

[18] Kaufmann H, Yamage M, Roditi I., et al. Discriminatiom of *Neospora caninum* from *Toxoplasma gondii* and other apicomplexan parasites by hybridisation and PCR. Mol Nucl Prob 1996;10:289–297.

[19] Yamage M, Flechtner O, Gottstein B. *Neospora caninum*: specific oligonucleotide primers for the detection of brain "cyst" DNA of experimentally-infected nude mice by the polymerase chain reaction (PCR). J Parasitol 1996;82:272–279.

[20] Longo MC, Berninger MS, Hartley JL. The use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reaction. Gene 1990;93:125–128.

[21] Müller N, Zimmermann V, Hentrich B, Gottstein B. Diagnosis of *Neospora* sp. and *Toxoplasma gondii* by PCR and DNA-Hybridization Immuno-Assay (DIA). J Clin Microbiol 1996;34:2850–2852.

[22] Hemphill A, Gottstein B, Kaufmann H. Adhesion and invasion of bovine endothelial cells by *Neospora caninum*. Parasitol 1996;112:183–197.

[23] Conrad PA, Barr BC, Sverlow K.W., et al. In vitro isolation and characterization of a *Neospora* sp. from aborted bovine foetuses. Parasitology 1993;106:239–249.

[24] Gottstein B, Pozio E, Connolly B, Gamble HR, Eckert J, Jakob HP. Epidemiological investigation of trichinellosis in Switzerland. Vet Parasitol 1997;72:201–207.

[25] Stärk KDC, Frei-Stäheli C, Frei P.P., et al. Häufigkeit und Kosten von Gesundheitsproblemen bei Schweizer Milchkühen und deren Kälber (1993–1994). Schw Arch Tierheilkd 1997;139:343–353.

[26] Busato A, Steiner L, Gottstein B, Gaillard C. Häufigkeiten und Ursachen von Kälberverlusten und Kälberkrankheiten in Mutterkuhbetrieben. III. Seroprävalenz ausgewählter Krankheiten und Prävalenz von Endoparasiten beim Absetzen. Dtsch Tierärztl Wschr 1997;104:191–195.

[27] Levy PS, Lemeshov S. Sampling of populations, methods and applications. A Wiley-Interscience Publication. New York: John Wiley, 1991.

[28] Dubey JP, Beattie CP. Toxoplasmosis of animals and man. Boca Raton, FL: CRC Press, 1988.

[29] Stenlund S, Björkman C, Holmdahl OJM, Kindahl H, Uggla A. Characterization of a Swedish bovine isolate of *Neospora caninum*. Parasitol Res 1997;83:214–219.

[30] Barr BC, Anderson ML, Sverlow KW, Conrad PA. Diagnosis of bovine fetal *Neospora* infection with an indirect fluorescent antibody test. Vet Rec 1995;137:611–613.

[31] Blanchard PC, Hietala SK, Thurmond MC. Diagnostic interpretation of *Neospora* serology. Abstract Proc. 38th Ann. Meet. Am. Assoc. Vet. Lab. Diagn., Sparks, Nevada, 1995;40.

[32] Lally NC, Jenkins MC, Dubey JP. Evaluation of two *Neospora caninum* recombinant antigens for use in an enzyme-linked immunosorbent assay for the diagnosis of bovine neosporosis. Clin Diagn Lab Immunol 1996;3:275–279.