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Comparison of Immunological and Molecular Methods for the Diagnosis of Infections with Pathogenic *Sarcocystis* species in Sheep

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Sheep may be infected by four species of *Sarcocystis*: *Sarcocystis tenella* and *Sarcocystis arieticanis* are pathogenic, *Sarcocystis gigantea* and *Sarcocystis medusiformis* are non-pathogenic. The two pathogenic species may cause abortion or acute disease during the early phase of infection and chronic disease during the late phase of infection. Thus far, diagnosis of sarcocystiosis has been limited, because traditional diagnostic tests based on the detection of *Sarcocystis*-specific antibodies are only genus-specific and, thus, cannot differentiate between pathogenic and non-pathogenic *Sarcocystis* species. In addition, most of these tests can only detect chronic sarcocystiosis. Therefore, diagnosis of acute sarcocystiosis or *Sarcocystis*-induced abortion has been based mainly on post-mortem examination, i. e. after the animal had succumbed to the disease. Recently, we have established species-specific PCR assays based on unique ribosomal RNA gene sequences of *S. tenella* and *S. arieticanis*. These assays enable the diagnosis and differentiation of infections with *S. tenella* and *S. arieticanis* in sheep intra vitam during the acute phase of the disease and, therefore, facilitate for the first time comprehensive studies on the epidemiology and importance of infections with pathogenic *Sarcocystis* species in sheep.

Keywords : *Sarcocystis tenella*, *Sarcocystis arieticanis*, Diagnosis, Ribosomal RNA, Nested-PCR

INTRODUCTION

Sarcocystis species are obligatorily intracellular protozoa with a typical coccidian life cycle consisting of merogony, gamogony and sporogony. They are also obligatorily heteroxenous, i.e. both an intermediate host and a definitive host need to be present for the continuation of the life cycle [7, 48]. Multiplication by merogony and cyst formation take place in the intermediate host, and gamogony and sporogony in the definitive host. Sheep are intermediate hosts of four *Sarcocystis* species: *Sarcocystis tenella* (synonym *Sarcocystis ovicanis*) and *Sarcocystis gigantea* (synonym *Sarcocystis ovifelis*) are distributed world-wide. *Sarcocystis arieticanis* has been found in Europe, the USA, Australia and New Zealand, while infections with *Sarcocystis medusiformis* have been reported only from Australia and New Zealand [7, 47]. *S. gigantea* and *S. medusiformis* are transmitted by felids, develop macroscopically visible cysts and are non-pathogenic. *S. tenella* and *S. arieticanis* are transmitted by canids, develop microscopically visible cysts and are pathogenic (Tab. 1). A primary infection of sheep with one of the pathogenic species may lead to acute disease or abortion during the early phase of infection and chronic disease during the late phase of infection.

Sheep become infected with *S. tenella* or *S. arieticanis* by ingesting sporocysts with contaminated food or water (Fig. 1). The asexual development of both species consists of two generations of endopolygeny in vascular endothelial cells [8, 18-20, 29, 37]. First generation meronts of both *S. tenella* and *S. arieticanis* occur in arterioles of mesenteric lymphnodes. First generation meronts of *S. tenella* also develop in vascular endothelial cells of the heart, skeletal muscles, liver and kidney. Second generation meronts of both species develop in capillaries of almost all

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Table 1 Location, morphology, pathogenicity and geographic distribution of *Sarcocystis* species in sheep

Species	Localisation of cysts	Size of cysts	Morphology of cyst wall	Degree of pathogenicity	Definitive host	Distribution
S. tenella	all striated muscles, CNS, Purkinje fibres	≤ 700 μm	thick (1-3 μm), striated, with villar protrusions (3.5 × 0.5 μm)	high	canids	world-wide
S. arieticanis	probably all striated muscles	≤ 900 μm	thin (< 1 μm), with hair-like protrusions (5-9 μm)	high	dog	probably world-wide
S. gigantea	predominantly oesophageal, laryngeal and lingual muscles	≤ 10 mm	thin (< 2 μm), smooth, with secondary wall of connective tissue	non-pathogenic	cat	world-wide
S. medusiformis	diaphragmic, abdominal and skeletal muscles	≤ 8 mm	thin (< 2 μm), smooth, no secondary wall	non-pathogenic	cat	Australia, New Zealand

CNS, central nervous system

internal organs. Endozoites of the terminal generation of endopolygeny initiate the formation of cysts in various striated muscles. Cysts of *S. tenella* may also be formed in cells of the central nervous system or in Purkinje fibres of the heart (Table 1). In immature cysts further asexual reproduction takes place by repeated endodyogeny of metrocytes. Mature cysts contain several hundred thousands of cystozoites, which do not divide further and are the terminal life cycle stage in sheep. If these cysts are ingested by a canine definitive host, the cystozoites initiate the sexual phase of the life cycle (gamogony), which takes place in cells of the small intestine and leads to the formation of oocysts [3, 7]. Oocysts sporulate in the *lamina propria* of the small intestine. Sporulated oocysts are the terminal life cycle stage in canids. As the oocyst wall is thin it often ruptures and free sporocysts are released into the intestinal lumen and passed into the environment with the faeces.

Diagnosis of disease or abortion caused by *S. tenella* and *S. arieticanis* in sheep has been complicated by the fact that currently used immunological tests are only genus-specific and, therefore, cannot differentiate infections with pathogenic *Sarcocystis* species from infections with non-pathogenic species. In addition, most serological tests can only detect antibodies during the late phase of infection. Thus far, diagnosis of infections with pathogenic *Sarcocystis* species has been based mainly on histological detection of meronts at post mortems. Although some clinical symptoms can be indicative of sarcocystiosis, there is no clinical symptom that is specific for sarcocystiosis. For these reasons, a molecular biological test that enables early and species-specific detection of *S. tenella* and *S. arieticanis* molecules in sheep is a great advance for the diagnosis of acute sarcocystiosis and *Sarcocystis*-induced abortions, and will facilitate epidemiological studies on these parasites that have not been possible so far.

CLINICAL SYMPTOMS

The severity of clinical symptoms caused by *S. tenella* and *S. arieticanis* depends on the dose of ingested sporocysts and the immune status of the host. Clinical symptoms during the early merogonic phases in vascular endothelial cells of internal organs are usually more severe than those observed during the formation and development of cysts in muscle or nervous tissue [3, 7, 9, 41, 51].

The uptake of a high dose of sporocysts of *S. tenella* or *S. arieticanis* can lead to acute sarcocystiosis in previously uninfected sheep

that have not yet developed immunity to the respective species. In addition to several non-specific symptoms, such as fever, anorexia, tachypnoe, tachycardy and anaemia, signs of acute sarcocystiosis include central nervous symptoms caused by encephalitis and encephalomyelitis, and haemorrhagic diathesis which can cause the death of the animal [6, 14, 18-20, 24, 29]. In pregnant sheep, acute sarcocystiosis frequently results in foetal death, abortion or premature birth of the offspring [11, 24, 30, 34]. Animals surviving a primary *Sarcocystis* infection usually acquire immunity that protects them against acute disease after challenge with the homologous species, but not after infection with the heterologous pathogenic species [41, 51].

Chronic sarcocystiosis can result from the ingestion of a low dose of sporocysts of *S. tenella* or *S. arieticanis* and causes economic losses due to reduced quality and quantity of meat, milk or wool [10, 29, 35, 36, 41]. Additional economic losses in the sheep industry are caused by the macroscopic cysts of *S. medusiformis* and *S. gigantea* which result in condemnation of whole carcasses or affected parts after slaughter [4, 38, 41].

PATHOLOGY AND PATHOHISTOLOGY

The most dominant signs of acute sarcocystiosis observed at post mortems are capillary haemorrhages in all organs, particularly in the tongue, heart and skeletal muscles. Encephalitis with haemorrhages and focal gliosis can be found in some cases. Histologically meronts are predominantly found in the brain, tongue, heart and kidneys. However, the meronts of *S. tenella* and *S. arieticanis* cannot be differentiated morphologically. In addition, meronts may already have disappeared by the time the animal succumbs to the disease [6, 7, 42, 51]. In *Sarcocystis*-induced abortions meronts associated with haemorrhages and focal necrosis can be found in maternal tissues of the placentome, but are not consistently found in foetal membranes or tissues [51].

In chronic sarcocystiosis cysts can be found microscopically in skeletal or heart muscles. In some cases, mature cysts of *S. tenella* or *S. arieticanis* can be identified in stained histological sections or squash preparations based on the morphology of the cyst wall (Table 1). However, in many cases a species-specific diagnosis of *Sarcocystis* cysts requires immunohistochemical or electron microscopic methods. As only a small section of muscle tissue can be examined by these methods, they lack sensitivity and also may not detect mixed infections with both species [7, 51].

Several epidemiological studies on *Sarcocystis* infections in sheep have been carried out by enzymatic digestion of muscle samples derived from animals at the time of slaughter and subsequent microscopic examination of the muscle digest for cystozoites. However, this method does not allow species differentiation, because the cyst walls are digested and the cystozoites of different *Sarcocystis* species are too similar morphologically to be differentiated from each other [7, 44]. Therefore, this method is not suitable for collection of accurate epidemiological data on infections with pathogenic *Sarcocystis* species in sheep.

IMMUNOHISTOCHEMISTRY

Several immunohistochemical methods have been developed to improve the sensitivity and specificity of the histological detection of life cycle stages of *Sarcocystis* species [25, 46, 51]. However, these methods have been complicated by the fact that polyclonal antisera derived from sheep that have been infected experimentally with any species of *Sarcocystis* show a high cross-reactivity with antigens of heterologous *Sarcocystis* species. In addition, immune sera of rabbits that have been immunised with traditionally derived *Sarcocystis* antigens may also cross-react with antigens of other cyst-forming coccidia infecting sheep, such as *Toxoplasma gondii* [25, 51]. Therefore, a species-specific diagnosis of *Sarcocystis* stages can only be made when specific monoclonal antibodies are used for immunohistochemistry [51]. However, the repertoire of monoclonal antibodies that are directed against *Sarcocystis* species of sheep is limited. Thus far, species-specific monoclonal antibodies have only been developed for *S. tenella* and *S. gigantea* [40, 41, 47].

SEROLOGY

Several immunological tests have been developed for serological diagnosis of *Sarcocystis* infections in sheep [7, 51]. The most commonly used tests are the enzyme-linked immunosorbent assay (ELISA) and

the indirect fluorescent antibody test [41, 44, 51]. These tests show a high sensitivity for the late phase of infection. However, they are not species-specific because traditional antigen preparations derived from cystozoites or merozoites of any *Sarcocystis* species are highly cross-reactive with antibodies directed against heterologous *Sarcocystis* species [13, 39, 43, 45-47, 50, 53]. The high cross-reactivity among different *Sarcocystis* species has long hampered the development of species-specific diagnostic methods for *Sarcocystis* infections in sheep. The only species-specific serological test which is currently available is an ELISA that utilises a recombinant polypeptide of *S. tenella* as antigen [33]. However, this test shows only a low sensitivity that is not sufficient for diagnostic purposes. Therefore, it is currently not possible to differentiate between infections with pathogenic and non-pathogenic *Sarcocystis* species in sheep intra vitam.

In addition, the immunological diagnosis of acute sarcocystiosis or *Sarcocystis*-induced abortion is complicated by the fact that humoral antibody levels measured with traditional antigen preparations are usually very low during the early phase of infection. Therefore, it is often not possible to detect *Sarcocystis*-specific antibodies at the time clinical disease becomes obvious [41, 47, 51].

MOLECULAR BIOLOGICAL METHODS

In recent years, the advent of new molecular biological techniques has provided new diagnostic means for parasitic infections. For example, direct diagnostic tests based on the detection of *Sarcocystis* molecules by specific DNA probes provide a valuable alternative to indirect tests based on the detection of *Sarcocystis*-specific antibodies, and may be able to overcome the diagnostic drawbacks caused by delayed seroconversion at the time of acute sarcocystiosis. Thus far, only few *Sarcocystis* genes have been sequenced, and DNA probes with diagnostic potential are only known for *S. tenella* [26]. However, a very successful strategy in the development of new diagnostic methods for *Sarcocystis* species has been the analysis of variable regions of ribosomal RNA (rRNA) to identify species-specific nucleotide sequences that can be targeted by corresponding synthetic oligonucleotides in hybridisation assays or polymerase chain reactions (PCR) [12, 17, 22, 49].

The first species-specific oligonucleotides for *S. tenella* were derived from variable sequences of its small subunit (SSU) rRNA gene and were used for detection of *S. tenella* by a RNA hybridisation assay [22]. rRNA molecules are the most abundant macromolecules in cellular organisms. Growing cells contain 10-50 times more RNA than DNA. rRNA constitutes about 90-95 % of the total cellular RNA, and about 30 % of this is SSU rRNA. Therefore, some authors have suggested that diagnostic assays targeting naturally abundant rRNA should be much more sensitive and accurate than assays targeting rRNA genes [12, 52].

However, the differentiation of *Sarcocystis* species in sheep under natural conditions requires a diagnostic test that is applicable to the examination of a broad range of different specimens. For example, diagnosis of abortion or of fatal acute disease caused by *S. tenella* or *S. arieticanis* requires their detection in tissue samples taken from the placenta or internal organs at post mortems. By contrast, epidemiological studies on infections with *Sarcocystis* species require a test that is suited for examination of a large number of samples, such as blood or buffy coats, derived from sheep intra vitam. Such samples are usually several hours to days old when they arrive in the laboratory and, thus, have already been subject to autolysis before they can be examined. Therefore, it may not be feasible to base a diagnostic test designed for the examination of field samples, or for the examination of large numbers of samples for epidemiological studies, on rRNA which is not a stable target and is easily degraded by enzymatic digestion. In addition, standardisation of RNA hybridisation assays is difficult [12, 32], but would be a pre-requisite for accurate comparison of epidemiological data collected at different times and places. Another disadvantage of hybridisation assays is that it is usually necessary to use radioactively labelled oligonucleotide probes to obtain reasonable sensitivities for diagnostic purposes [12, 22, 32]. This restricts the use of these tests to a limited number of specialised laboratories and is impractical for many diagnostic applications and for prolonged epidemiological studies.

For these reasons, we used a different rationale to develop a molecular biological

test for species-specific diagnosis and differentiation of *S. tenella* and *S. arieticanis* in sheep. We designed a series of oligonucleotides that can be used as primers for species-specific amplification of SSU rRNA gene fragments from genomic DNA templates of the homologous parasite species by PCR [17, 49]. Such DNA templates can be expected to be stable targets for PCR amplification, even when diagnostic samples have been stored at ambient temperature for some time. In the genome of *T. gondii* the ribosomal transcription unit is present in 110 copies [15]. Therefore, it can be assumed that a similar high number of copies exists in the genome of closely related cyst-forming coccidia [48] and that a PCR assay based on rRNA genes of *Sarcocystis* species has a sufficient sensitivity for diagnostic applications.

DEVELOPMENT OF ST-NESTED-PCR AND SA-NESTED-PCR

To develop diagnostic PCR assays for *S. tenella* and *S. arieticanis*, their SSU rRNA gene sequences were aligned with those of three other cyst-forming coccidia occurring in sheep in Europe, i.e. *S. gigantea*, *T. gondii* and *Neospora caninum*, and analysed with respect to variable regions that may have potential for the development of species-specific oligonucleotides [47, 49]. Although nucleotide differences between the SSU rRNA gene sequences of these five cyst-forming coccidia are low (0.45-4.5 %) in comparison with other protozoa [48], this analysis showed that most differences are located in six short regions with a length of 15-72 bp [47, 49]. Based on information derived from these regions, we developed several species-specific oligonucleotides for *S. tenella* and *S. arieticanis* [49]. The evaluation of these oligonucleotides as primers for PCR showed that only one species-specific oligonucleotide is required for species-specific amplification of SSU rRNA gene fragments from genomic DNA templates of *S. tenella* or *S. arieticanis* [17, 49].

To obtain optimal sensitivity for diagnostic applications, we developed a Nested-PCR for *S. tenella* (ST-Nested-PCR) which consisted of two consecutive amplifications each of which employed a combination of a species-specific and a universal oligonucleotide, which was originally designed for SSU rRNA gene sequences of eucaryotes, as primer pair for PCR [17]. Optimal results were obtained using the oligonucleotides ST1 (5'- GGA TCG CAT TAT GGT CAT - 3') and AP2 (5'- CCC GGG ATC CAA GCT TGA TCC TTC TGC AGG TTC ACC TAC - 3') as external primers and the oligonucleotides 8 (5'- TTT GAC TCA ACA CGG G - 3') and ST3 (5'- CGT TGC CGC GCG TTA A - 3') as nested primers. The SSU rRNA gene fragment of *S. tenella* amplified by the ST-Nested-PCR has a size of 529 bp. Likewise, a Nested-PCR for *S. arieticanis* (SA-Nested-PCR) was developed using the oligonucleotides STA (5'- TTT CGC AAG GAA GAG GA - 3') and SA2 (5'- TGA AAC GGC GCG TAG A - 3') as external primers and the oligonucleotides 2 (5'- AGG GTT CGA TTC CGG AG - 3') and SA1 (5'- GCG GGA AGA GGA GAA T - 3') as nested primers. The SSU rRNA gene fragment of *S. arieticanis* amplified by the SA-Nested-PCR has a size of 374 bp [17].

Under laboratory conditions, the ST-Nested-PCR and SA-Nested-PCR could detect 100 fg and 10 fg of genomic DNA templates of the homologous *Sarcocystis* species, respectively [17]. Thus, assuming an average DNA content of about 221-254 fg for haploid life cycle stages of *Sarcocystis* species [31], both Nested-PCRs can detect amounts of genomic DNA that are equivalent to less than one parasite. Ideally, a diagnostic test for acute sarcocystiosis in sheep should enable the detection of endozoites or circulating DNA of *S. tenella* and *S. arieticanis* in blood samples derived from sheep intra vitam. However, blood samples have been described to contain several factors that may inhibit a PCR, such as erythrocytes, haemoglobin or anticoagulants [1, 21, 23, 27]. Therefore, we evaluated the effect of various anticoagulants on the ST-Nested-PCR. Blood samples derived from a coccidia-free sheep containing either EDTA, sodium citrate, lithium heparin or sodium heparin as anticoagulant were spiked with a defined number of *S. tenella* cystozoites. DNA was extracted from buffy coat preparations in which the parasites were enriched after centrifugation of the blood samples [2]. In this experiment, optimal amplification of *S. tenella* SSU rRNA gene fragments was obtained from blood samples that contained either sodium or lithium heparin as anticoagulant [16, 17]. Therefore, all further experiments were car-

ried out using heparinised blood samples. In such blood samples, one cystozoite per 100 μl of blood could be detected by the Nested-PCRs. To evaluate the specificity of the ST-Nested-PCR and SA-Nested-PCR, buffy coat preparations derived from a coccidia-free sheep were spiked with defined amounts of genomic DNA templates of *S. tenella*, *S. arieticanis*, *S. gigantea*, *T. gondii*, *N. caninum* or sheep. In this experiment, the ST-Nested-PCR and SA-Nested-PCR specifically amplified only the homologous SSU rRNA gene fragment of *S. tenella* or *S. arieticanis*, respectively [17].

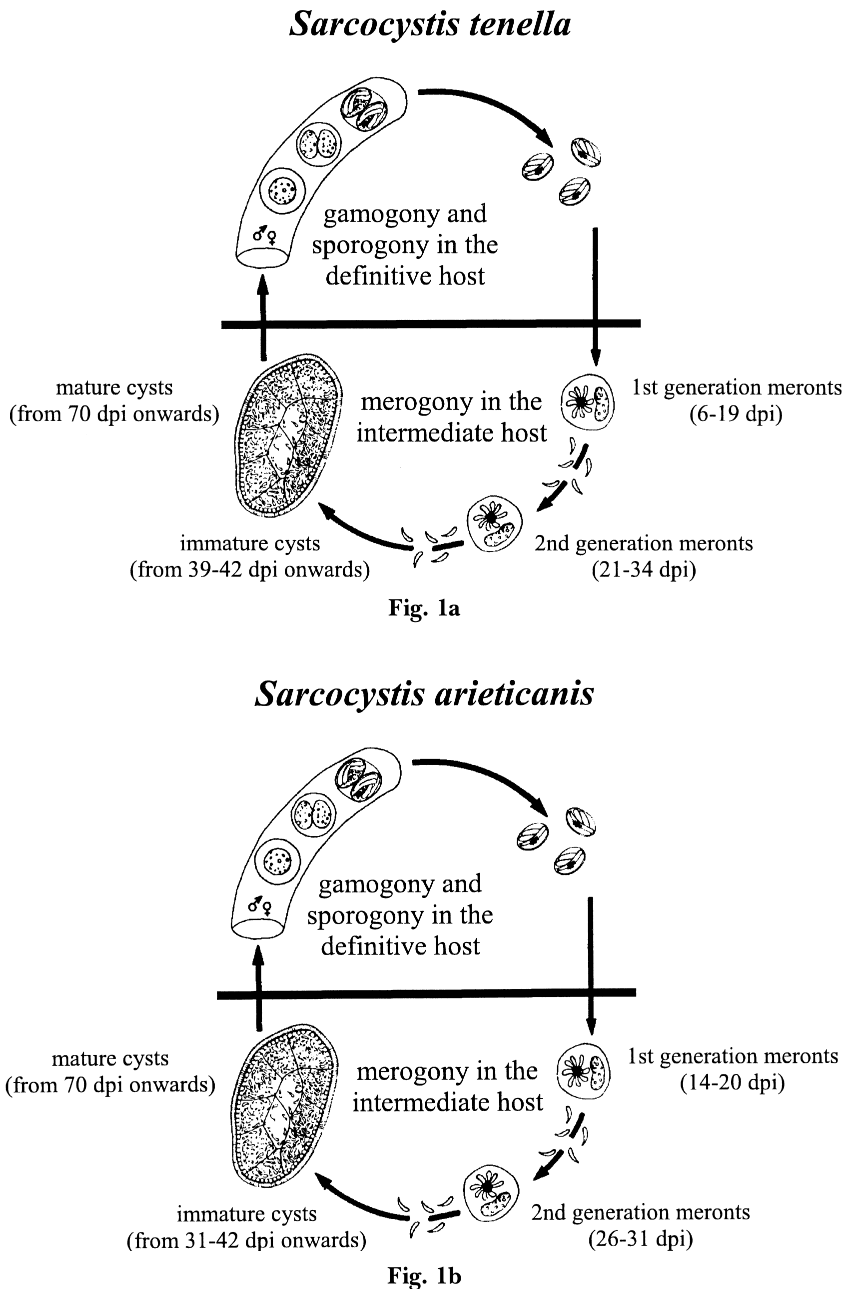

Fig. 1 The life cycles of *S. tenella* (a) and *S. arieticanis* (b). Data were taken from references 8, 18-20, 37.

EVALUATION OF ST-NESTED-PCR AND SA-NESTED-PCR WITH DIAGNOSTIC SAMPLES FROM EXPERIMENTALLY INFECTED SHEEP

To evaluate the diagnostic potential of the ST-Nested-PCR and SA-Nested-PCR coccidia-free sheep were infected experimentally with *S. tenella* or *S. arieticanis* [16,17]. Heparinised blood samples were taken daily to determine the packed cell volume (PCV) and prepare buffy coat preparations for examination by both Nested-PCRs. Rectal temperature was measured and serum samples were examined for the presence of anti-*Sarcocystis* antibodies by ELISAs using traditionally derived antigens of *S. tenella* or *S. arieticanis* cystozoites.

Five sheep infected with 20000 or 50000 sporocysts of *S. tenella* suffered from acute sarcocystiosis between 23 and 35 days after infection (dpi) [16, 17]. Rectal temperature rose up to 41.5℃ and PCV decreased to values between 20 and 25%. First positive results were obtained by the ST-Nested-PCR on 23 or 24 dpi. The ST-Nested-PCR showed continuously positive results for 24-30 days, until about day 50 pi. It then showed intermittently positive results during another 26-56 days and became negative between 76 and 107 dpi. The positive results in the ST-Nested-PCR correlated with the phase of acute disease as well as with the second phase of endopolygeny in vascular endothelial cells described for *S. tenella* (Fig. 1a). By contrast, *Sarcocystis*-specific antibodies were detected only from 60 dpi onwards. The SA-Nested-PCR remained negative for these animals over the whole period of examination (120 days).

Six sheep infected with 5000, 50000 or 100000 sporocysts of *S. arieticanis* suffered from acute sarcocystiosis between 5 and 33 dpi [16, 17]. During this time, up to three phases of fever up to 42.0℃ were observed. The PCV decreased to values below 25 %. Two sheep succumbed to the disease on 28 and 29 dpi. The SA-Nested-PCR showed positive results in up to three phases. One sheep was positive on 5 dpi, five sheep were positive between 13 and 20 dpi and all six sheep were positive between 21 and 35 dpi (Fig. 2). These phases correlated with the fever phases and with the two phases of endopolygeny in vascular endothelial cells described for *S. arieticanis* (Fig. 1b). The SA-Nested-PCR showed intermittently positive results between 35 and 45 dpi and became negative thereafter. *Sarcocystis*-specific antibodies were detected only in the four surviving sheep which seroconverted between 18 and 46 dpi. The ST-Nested-PCR remained negative for these animals over the whole period of examination (120 days).

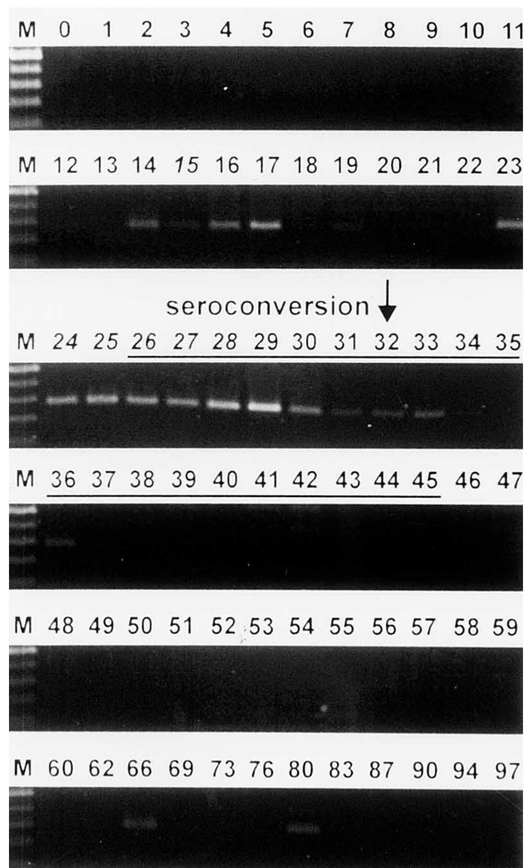

Fig. 2 Reactions of a sheep infected experimentally with *S. arieticanis* in the SA-Nested-PCR. Numbers above the lanes show the days after infection. Numbers in italics indicate the days when fever was observed; numbers underlined indicate the days when PCV was below 25 %. The arrow indicates the time of seroconversion observed in an ELISA with cystozoite-derived antigen of *S. arieticanis*. M, 100 bp marker showing a pronounced band at 600 bp.

CONCLUSIONS

Under natural conditions sheep may be infected with different pathogenic and non-pathogenic *Sarcocystis* species at the same time. Diagnosis of abortion and acute disease caused by *S. tenella* and *S. arieticanis* has been limited by the low specificity and sensitivity of traditional diagnostic methods. The ST-Nested-PCR and SA-Nested-PCR described here based on the detection of the SSU rRNA genes of *S. tenella* or *S. arieticanis* in blood samples of infected sheep are the first tests for species-specific diagnosis and differentiation of the two pathogenic *Sarcocystis* species infecting sheep. Therefore, it will now be possible for the first time to collect accurate epidemiological data on the prevalence, incidence and transmission dynamics of infections with pathogenic *Sarcocystis* species as well as the importance of mixed infections with different cyst-forming coccidia in sheep. In future, it will be possible to identify geographic areas in which infections with *S. tenella* or *S. arieticanis* are endemic as well as areas with endemic instability in which animals are at risk from acute sarcocystiosis or *Sarcocystis*-induced abortion.

Another advantage of the PCR assays described above is their potential to be adaptable to the examination of parasite stages from canine definitive hosts. The collection of epidemiological data on *Sarcocystis* infections in definitive hosts has been complicated by the fact that definitive hosts of *Sarcocystis* species do not develop parasite-specific antibodies. *Sarcocystis* sporocysts can be found in faecal samples of canids. However, the sporocysts of different *Sarcocystis* species are too similar morphologically to be differentiated by coproscopical methods. Therefore, the Nested-PCRs described here can be employed in various ways in epidemiological studies which have not been possible with traditional diagnostic methods. In future, these PCR assays will be invaluable for the detection and identification of *S. tenella* and *S. arieticanis* in many clinical and epidemiological investigations.

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