MOLECULAR APPROACHES TO THE EPIDEMIOLOGY AND DIAGNOSIS OF OVINE TOXOPLASMOSIS

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Martin Richard Owen

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ABSTRACT

Toxoplasma gondii is a protozoan parasite with a world-wide distribution which is capable of infecting all warm-blooded animals, including man. Clinical toxoplasmosis is an important disease in man and an important cause of reproductive disease in sheep. Under experimental conditions, eighteen ewes were orally infected with 1500 *Toxoplasma* oocysts between 80 and 90 days of gestation. A simple protocol of tissue preparation, not requiring DNA extraction was developed and *T. gondii* was detected in tissues from aborted foetuses and placentae from live, infected lambs by nested PCR amplification of the B1 gene and by mouse inoculation (MI). Placental cotyledon was the optimum tissue for examination by PCR, could be tested even when unfit for MI and was as sensitive as MI. 16/19 placentae were PCR positive compared with 13/18 by MI. Following the experimental infection of 15 pregnant ewes at 80-90 days gestation with 2000 sporulated *Toxoplasma* oocysts, *per os*, the *T. gondii* infection status of placental cotyledons from 11 'acute phase' (less than 14 days post infection) abortions was investigated by B1 gene PCR and MI. That there was no evidence of infection by PCR or MI confirms that these fatalities occur before invasion of the placenta or the foetus by *T. gondii.* Captive-bred *Mus musculus* and *Apodemus sylvaticus* were orally infected with *T. gondii* oocysts and infection in them and their offspring was assessed by B1 gene PCR and serology. A dose of 50 oocysts established chronic infection with minimal mortality. By PCR, in offspring from chronically infected *Apodemus* and *Mus* respectively, vertical transmission was demonstrated in 82.7% (n=83) and 85.0% (n=207) of all pups. By prospective serological surveys, the incidence of *Toxoplasma* infection in, initially seronegative, cohorts of 70-158 sheep was studied for three farms over periods from 9-18 months. Antibody prevalence increased throughout the periods studied on all farms, and seroconversions occurred when sheep received no supplementary feed, indicating acquisition of infection by ingestion of oocysts contaminating pasture and deposited their directly by cats. PCR-RFLP analysis of *T. gondii* directly from tissues from 15 animals (13 sheep, 2 wild rodents) with naturally acquired infections identified the type II lineage of *T.gondii* in all the infections. The results demonstrate the feasibility of direct analysis of *T.gondii* genotype from either clinical or non-clinically affected, but infected tissues. Sequence analysis of the published rDNA unit for 3 strains (P, RH and SAILI) indicates 12 polymorphisms in the NTS and ETS regions, of which 8 affect sites for restriction endonucleases. PCR-RFLP analysis of the 2.5kb NTS/ETS amplicon, produced by *T. gondii* specific primers for the 18S and 28S genes, demonstrated polymorphisms in 5 strains and 2 new *T. gondii* isolates - potential additional genotype markers. Four restriction enzymes (Mae III, Rsa I, Scr I and Spe I) differentiated strains RH and T561 (which show identical RFLPs) from strains C56, M1, ME49 and the 2 isolates. The identical RFLPs obtained for T561 and RH *T. gondii* strains does not correlate with their genotypes according to the distinct lineages recently described (Howe and Sibley, 1995).

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cm^3	centimetre cubed
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide tris-phosphate
dpi	days post infection
FAT	direct fluorescence antibody test
FCS	foetal calf serum
h	hour
Ig	immunoglobulin
i. u.	international units
ITS	internal transcribed spacer
MAT	modified direct agglutination test
mg	milligram
ml	millilitre
mM	millimolar
µg	microgram
µl	microlitre
µM	micromolar
NC-liv	Liverpool isolate of ***Neospora caninum***
ng	nanogram
pg	picogram
PBS	phosphate buffered saline
PCR	Polymerase Chain Reaction
rDNA	ribosomal DNA
RNA	ribonucleic acid
S	Svedberg unit

Toxoplasma gondii

Toxoplasma gondii (Nicolle & Manceaux, 1908) is a coccidian parasite of felids with a world-wide distribution and a wide range of intermediate hosts including all warm-blooded animals and man. Owing to its zoonotic potential, *T. gondii* is of both medical and veterinary importance. The majority of cases in animals and in man are symptomless. However, for non-immunocompetent individuals and for some species, such as the hare, (*Lepus spp*), primary infection can be fatal (Gustafsson *et al.* 1988). During pregnancy in both man and animals, infection can result in foetal death, and hence abortion or, if the foetus survives, congenital infection often with associated malformations (Frenkel, 1989).

The life cycle of *T. gondii* comprises an asexual multiplication in intermediate hosts and a sexual cycle which takes place in the small intestine of non-immune cats, the definitive host. Sexual replication in the cat occurs in epithelial cells in the small intestine by a process of schizogony and leads to the formation of oocysts which are spherical, 10-12μm in diameter and which are shed in faeces (Dubey, 1970). Oocysts sporulate over one to five days, forming two sporocysts, each containing four sporozoites. The sporulated oocysts are infective if ingested and can survive for many months (Beverley, 1976), constituting an infection source for potential intermediate hosts and other non-immune cats.

When ingested by non-immune intermediate hosts, the oocyst wall breaks down, sporocysts excyst and sporozoites penetrate intestinal epithelial cells. In the lamina propria and mesenteric lymph nodes, the parasites undergo rapid asexual replication, by endodyogeny. This proliferative form of *T. gondii*, the tachyzoite, is 4-6μm in length and crescent-shaped. Tachyzoites replicate intracellularly causing host cell lysis and spread to nearby cells and throughout the body via the bloodstream and lymphatic system. Replication continues for up to two weeks when the developing host immunity coincides with the establishment of chronic infection, characterised by the formation of tissue cysts which may contain from a few to several hundred slowly dividing bradyzoites. The cysts are predominantly located in the central nervous system and muscle, but are also found in other organs such as the liver, lungs and kidneys (Garnham and Lainson, 1960). In most animal species and in man, tissue cysts remain viable for the life-time of the host. If tissues from infected animals are ingested, the cyst wall is digested by enzymes in the stomach and small intestine and bradyzoites are released, which penetrate intestinal cells and commence replication. In non-felids, only extra-intestinal replication occurs, leading to the formation of tissue cysts in the new host. In cats, however, concurrent with extra-intestinal replication and the formation of tissue cysts, intestinal replication leads to the production and shedding of further oocysts, completing the parasites life-cycle. Following primary infection, cats and intermediate hosts acquire a solid immunity to further infection, though if immunocompromised, reactivation of infection can occur, which

in cats, can result in oocyst-shedding (Dubey and Frenkel, 1974) and in intermediate hosts such as man, can result in potentially fatal toxoplasmic encephalitis (Luft and Remington, 1992).

In addition to infection by ingestion of oocysts or tissue cysts, *T. gondii* infection can also be transmitted vertically from mother to offspring during pregnancy. In most animal species and in man, it is generally believed that vertical transmission only occurs when a primary infection is contracted during pregnancy (Dubey and Shen, 1991). In contrast, in selected strains of some laboratory rodent species, vertical transmission of *T. gondii* occurs from chronically infected dams to pups in successive litters (Beverley, 1959; De Roever-Bonnet, 1969). Hence it is suggested that vertical transmission of *Toxoplasma* in wild rodents could potentially maintain an environmental reservoir of *Toxoplasma* infection for susceptible cats (Beverley, 1976).

(For references concerning the taxonomy, life cycle and cell biology of *T. gondii* see Dubey and Beattie (1988)).

Toxoplasmosis, an important zoonosis

Many human infections are acquired not by vertical transmission but by ingestion of sporulated *Toxoplasma* oocysts or viable tissue cysts in meat from infected animals (Fayer, 1981). The proportion of infections caused by ingestion of oocysts relative to tissue cysts is unknown and may be

expected to vary according to exposure to oocysts in the environment and to some extent on climatic conditions, since the latter affect longevity of oocysts in the environment (Frenkel and Dubey, 1972). The importance of tissue cysts as a source of infection depends on cultural food habits, since ingestion of under-cooked, infected meat imposes a significant infection risk (Desmonts *et al.* 1965). In the United Kingdom, of the meat producing animals, the high prevalence of toxoplasmosis in sheep would suggest that sheep meat potentially poses the greatest infection risk to man.

Ovine toxoplasmosis

Toxoplasmosis was demonstrated to be a cause of abortion in the 1950's, (Hartley & Marshall, 1957) and since then it has been shown to be one of the most significant causes of ovine abortion in the UK (Linklater, 1979). Sheep are particularly susceptible to toxoplasmosis and can become infected following the ingestion of as few as 20 oocysts (Buxton *et al.* 1991). Epidemiological evidence and clinical case studies suggest that sheep in the UK are often maintained in an environment contaminated with oocysts (Blewett, 1983; Faull *et al.* 1986), with contaminated pasture possibly being the most common source of infection (Buxton, 1990). Most infections in sheep are not accompanied by serious clinical signs (Blewett *et al.* 1982), though the acute phase of infection is usually associated with a pyrexia and there may be an increase in respiratory rate (Buxton *et al.* 1991). As immunity develops, parasite proliferation is suppressed and through the slow replication of bradyzoites, tissue cysts are formed

throughout the body. However, when sheep are infected during pregnancy, the systemic proliferation of tachyzoites leads to infection of the placenta and subsequently, the foetus (Buxton & Finlayson, 1986). Whilst the development of immunity prevents tachyzoite proliferation in most body tissues, local suppression of immunity in the maternal placenta and the immaturity of the immune system in the early conceptus enable parasite replication to go unchecked (Buxton & Finlayson, 1986). The outcome of infection is dependant upon the stage of pregnancy during which it occurs since the ability of the foetus to mount an immune response to pathogens begins in the first half of gestation and continues to develop throughout pregnancy, with immunocompetence being complete at birth (Buxton, 1990). Classically, when ewes are infected during early gestation, the usual outcome is embryonic death, since there is no foetal immune response. Embryonic resorption generally follows, hence affected ewes can be mistakenly considered to be barren (Johnson, 1988). In mid-gestation (50-120 days), infection can still prove fatal for the developing foetus although in the case of multiparous pregnancies, it is not uncommon for weakly or still-born lambs to be born together with a mummified foetus or a foetus which has clearly died at a later stage of pregnancy. Typically, infections in gestation beyond 120 days are resisted by the developing immunocompetence of the foetus and such lambs are generally born alive and healthy, though they are infected and immune (Blewett & Watson, 1983; Watson & Beverley, 1971).

Toxoplasma abortions generally occur during the last month of the gestation period, by which time the continued replication of parasites in the placentomes causes multiple and extensive foci of necrosis, visible macroscopically as white, often calcified, spots in the cotyledons of shed placentae (Beverley *et al.* 1971). Initially, the parasite-induced damage to the placentome and the resultant inflammatory reactions are associated with the release of prostaglandin $F_{2\alpha}$ and a temporary fall in blood progesterone levels (Fredriksson *et al.* 1990a; Fredriksson *et al.* 1990b). However, the pathological changes finally responsible for abortion are thought to be related to placental insufficiency caused by tachyzoite replication in placentomes, which eventually leads to the anoxia-induced lesions of focal leukoencephalomalacia frequently observed in the brains of aborted lambs (Buxton *et al.* 1982). Other characteristic brain lesions consist of lymphoid meningitis and glial foci surrounding areas of necrosis, the latter being a combination of the damage caused by parasitic replication with the immune response of the foetus (Buxton, 1990). In experimental *Toxoplasma* infections, abortions can also occur within seven to fourteen days of infection (Dubey, 1984; Dubey *et al.* 1987; McColgan *et al.* 1988; Trees *et al.* 1989, Buxton *et al.* 1991), in the absence of evidence of infection or characteristic pathological lesions associated with *Toxoplasma*-induced abortion. The mechanism by which these early abortions occur is poorly understood, but in humans abortion can occur following pyrexia (Kline *et al.* 1985) and may be involved in the aetiopathogenesis of acute phase *Toxoplasma* abortions in sheep (McColgan *et al.* 1988). Studies in

pregnant mice have demonstrated the release of abortion-inducing cytokines in response to the administration of a pyrogenic lipopolysaccharide (Gendron *et al.* 1990), hence it is possible that the triggering of similar immuno-regulatory mechanisms, including the release of prostaglandin $F_{2\alpha}$ (Fredriksson *et al.* 1990), in ovine toxoplasmosis leads, in some cases, to early abortion. The extent to which these 'acute phase' abortions occur following natural infections is unknown but their frequency in the UK may well be under-estimated (Trees *et al.* 1989), since most sheep at this stage of pregnancy are kept at grass, where the expulsion of a small foetus and the mild clinical signs of infection in the ewe would easily go unnoticed.

The macroscopic lesions of classical *Toxoplasma* abortions often enable their identification as such when adequate abortion tissues are presented, in good condition, for examination. In the absence of macroscopic lesions, *Toxoplasma* organisms may be detected by histopathology but the often sparse distribution of parasites in infected tissue makes immunohistopathology (Buxton *et al.* 1981) a preferable diagnostic technique. However, tissues from abortions are frequently presented in poor condition, often autolysed, making detection of *T. gondii* by routine histopathology or immunohistopathology difficult.

Diagnosis of toxoplasmosis in man and animals

Parasites may be detected in infected tissues, taken either by biopsy or at *post mortem* examination. However, histological examination of serial sections of potentially infected tissue is a laborious technique which may fail to detect *Toxoplasma* organisms. Inoculation of suspect tissue into *Toxoplasma* susceptible mice by sub-cuticular or intra-peritoneal injection (Abbas, 1967) followed by the detection of specific antibody and of *Toxoplasma* tissue cysts in mouse brains is a more sensitive diagnostic technique but infected mice may not develop cysts for 30 days (Derouin *et al.* 1987) or detectable *Toxoplasma* specific antibody for up to eight weeks (Dubey *et al.* 1995). The undesirable delay in diagnosis and the large numbers of mice required make mouse inoculation (MI) unsuitable for routine diagnosis and it tends to be used more as a research tool. Furthermore, only viable *Toxoplasma* organisms are detected and tissues inoculated must not be heavily contaminated with bacteria, to prevent iatrogenic death of mice, hence tissues from ovine abortion cases are often unsuitable for MI. Human congenital *T. gondii* infections have been diagnosed by inoculation of tissue culture cells (Derouin *et al.* 1987) but the technique requires aseptic collection of foetal samples for inoculation which is rarely, if ever, feasible in clinical cases of ovine toxoplasmosis. Furthermore, even under ideal conditions in human infections, *T. gondii* is only detected in approximately 50% of cases (Derouin *et al.* 1988). For diagnostic purposes, the failings in traditional techniques of parasite detection and the delay imposed by MI make antibody detection assays a

favourable diagnostic alternative in most cases of infection in man and in animals, when tissue samples are not available for examination.

Antibody detection assays

In immunocompetent individuals, there is both a cell-mediated and a humoral immune response to *T. gondii* infection (Frenkel, 1989). Hence the detection of specific antibody is indicative of exposure to *Toxoplasma* infection, though unless paired sera are available, a titre may give little information as to exactly when an infection was contracted since in man and in many animals, antibody titres can remain high for years following infection (Dubey and Beattie, 1988. Chapter 1, page 37). Many serological tests have been developed for diagnosis of *Toxoplasma* infection, which can be classified in terms of those using whole intact tachyzoites (the dye test, the direct agglutination test, the indirect fluorescent antibody test and the immunosorbent agglutination assay), and those which utilise disrupted parasites or recombinant proteins as an antigen source (the indirect haemagglutination test, the complement fixation test, the latex agglutination test and the enzyme linked immuno-assay).

The Dye Test (DT)

The dye test is the most commonly accepted reference assay for the measurement of *Toxoplasma* antibody in man (Sabin & Feldman, 1948), and it shows superior sensitivity to many other antibody detection assays in animals (Dubey *et al.* 1995). The test is principally a complement

mediated neutralising antigen-antibody reaction (Fleck & Kwantes, 1980). Since its description in 1948, the dye test has been standardised (Beverley & Beattie, 1958), and adapted to a microscale (Balfour *et al.* 1982). The test is quantitative for immunoglobulin G (IgG) (though immunoglobulin M (IgM) is also bound) and it is highly sensitive and specific. Specific antibody is detectable with the dye test within 2 weeks of infection, reaching a peak in humans in approximately 2 months. Antibody levels do, however, decline gradually over months or years, with the duration of antibody titres varying between animal species. Cattle, for example, have detectable antibody for a relatively short period of time (Dubey *et al.* 1985). The test is difficult to perform, requiring live parasites and a supply of accessory factor (a factor like complement) obtained from seronegative human blood donors. Consequently, the test is often restricted to specialist laboratories. The test may give false positive results with mice infected with *Hammondia hammondii* (Christie & Dubey, 1977) and in normal ruminant sera, though this may be avoided by heat inactivation of the causative agents in the serum (Dubey and Beattie 1988. Chapter 1, page 37).

The Direct and Modified Agglutination Tests (DAT) and (MAT)

The direct agglutination test, originally developed by Fulton & Turk (1959), (later modified by Desmonts & Remington (1980) by treatment of test sera with 2 mercapto-ethanol to eliminate non-specific reactions caused by IgM), shows good correlations of sensitivity and specificity with the dye test for

human (Desmonts & Remington, 1980) and animal sera (Dubey, 1985). The test is considerably cheaper than the dye test, and safer, since it does not use live tachyzoites. Furthermore, for the detection of *T. gondii* antibodies in foetal fluid from aborted lambs, it shows superior sensitivity to most other tests, including the latex agglutination test (see below), (Dubey *et al.* 1987). Despite its expense and its non-adaptability to automation, because of its sensitivity and its high correlation with the DT, the DAT is currently used extensively to detect *Toxoplasma* infection in animals (Pinckney *et al.* 1994; Davis & Dubey, 1995; Dubey *et al.* 1995).

The Direct and Indirect Fluorescent Antibody Test (FAT and IFAT)

Like the DAT, these tests have the advantage of not requiring live *Toxoplasma* tachyzoites. Whole, killed tachyzoites, fixed onto slides, are incubated with serially diluted test serum and antibody is detected using appropriate anti-species IgG fluorescent labelled conjugates and viewed by fluorescence microscopy. The IFAT may be used in the diagnosis of toxoplasmosis in animals (Frenkel, 1971), though it appears to be less sensitive than the DT (Hunter *et al.* 1980), whilst in humans titres normally correspond well with those of the DT (Walton *et al.* 1966)

The Immunosorbent Agglutination Assay (ISAGA)

Essentially used to detect human congenital toxoplasmosis and acute acquired infections, the ISAGA (Desmonts *et al.* 1981), detects IgM antibodies, and hence detects infection earlier than IgG based tests. Test

sera is added to microtitre plate wells, coated with anti-human IgM antibodies and serum IgM is bound but still capable of agglutinating *Toxoplasma* antigens, which are added in the form of a suspension of whole tachyzoites. The test is simple and rapid and technically easier than the IgM ELISA. However, the large number of tachyzoites required makes the test expensive. IgM IGASA tests have a high degree of sensitivity compared with other tests (Skinner *et al.* 1989). This, combined with their high specificity, makes them the assays of choice for IgM detection in the diagnosis of toxoplasmic encephalitis in AIDS patients (Holliman, 1991) and for congenitally infected infants (Holliman & Johnson, 1989). An IgA based IGASA test provides even more accurate diagnosis of human congenital toxoplasmosis (Bessieres *et al.* 1991), but has little, if any, application in animals.

The Complement Fixation Test (CFT)

Developed by Warren & Russ (1948), using antigen produced from *Toxoplasma*-inoculated eggs, the sensitivity of the test is low compared with the dye test and despite later modifications to improve its sensitivity (Fleck & Payne, 1963), it not widely used because of the complexity of procedures involved in the test and the lack of standardization of antigen and reagents (Dubey and Beattie, 1988. Chapter 1, page 37).

The Indirect Haemagglutination Test (IHA)

Less complex to perform than the CFT, pyruvic aldehyde-treated sheep erythrocytes are coated with soluble *T. gondii* tachyzoite antigen and the sensitized cells agglutinate in the presence of *Toxoplasma*-specific antibody in test serum (Jacobs & Lunde, 1957). Following an infection, antibodies are detected later by IHA than by IFAT (Botros, 1976) and consequently acute infections may not be not detected. Whilst non-specific false positive results are obtained in bovine sera (Dubey *et al.* 1985), the test has been used extensively in the investigation of ovine toxoplasmosis (Blewett *et al.* 1983).

The Latex Agglutination Test (LAT)

Using antigens from disrupted tachyzoites, fixed to latex beads and originally developed as a slide test (Siim & Lind, 1960), the test was relatively insensitive and inconsistency was evident between batches (Kwantes *et al.* 1972). Tsubota *et al.* (1977) described a LAT for the microtitre system allowing qualitative and quantitative assessment of antibody titres. Agglutination is observed within 12 hours of incubation when test sera contains specific antibody (Payne *et al.* 1984). In humans, when compared to the DT, the LAT shows a sensitivity of 98.7% and specificity of 95.8% (Johnson *et al.* 1989). In experimental *Toxoplasma* infections in ewes, the LAT is a sensitive, reliable and rapidly responsive serological test, which compares favourably with the IFAT and is superior

to at least one (discontinued) commercial indirect haemagglutination assay (IHA) (Trees *et al.* 1989).

The Enzyme Linked Immunosorbent Assay (ELISA) and the Enzyme Linked Immunosorbent Agglutination Assay

Voller *et al.* (1976) described an ELISA which detects *Toxoplasma*-specific IgG, in which soluble antigen from disrupted tachyzoites is coated onto micro-titre plate wells. Antigen-antibody binding is amplified using a secondary enzyme-linked antibody conjugate, after which addition of a colourigenic substrate enables the reaction to be assessed quantitatively by colorimetry, a process which lends itself to automation. Consequently, ELISAs are widely used in veterinary science (Buxton *et al.* 1988, Tenter *et al.* 1992, Wastling *et al.* 1993), and though generally favourably comparable in sensitivity and specificity to the DT, there is some species-associated variation, despite using recombinant antigens (Tenter *et al.* 1994).

The assay has been developed to detect IgM antibodies, (Payne *et al.* 1982), which enables detection of infection in the 'acute phase' in immunocompetent adults and of infection in the human foetus, by prenatal sampling. More recently developed ELISAs quantitatively measure antigen binding, or 'avidity' of *Toxoplasma*-specific IgG (Suzuki *et al.* 1990) and acute infections in man can be diagnosed by detection of low avidity antibodies.

Molecular Assays

DNA detection assays hold several advantages over serological assays and conventional parasite detection assays. Firstly, unlike serological tests, molecular assays detect the presence of parasitic material. In contrast, serological tests detect the host immune response to infection, hence depend upon host immune competence. Non-immunocompetant patients (AIDS cases, chemotherapy patients, early conceptuses) can give false negatives in serological assays. Secondly, DNA detection techniques potentially have a far greater sensitivity than conventional parasite detection techniques. Thirdly, many DNA detection assays are rapid, simple and cheap and offer the potential to analyse many samples simultaneously. Finally, that DNA is resistant to degradation relative to the integrity of biopsied tissue and *post mortem* tissues from clinical cases and relative to the longevity of antibodies, makes it possible to diagnose *T. gondii* infection in tissue that would otherwise be too autolysed for histopathological examination, for mouse inoculation and when antibodies would no longer be detectable. The resistance of DNA to degradation makes its detection particularly useful to diagnose infections in tissues from aborted ovine foetuses which are frequently autolysed or even mummified when presented for examination. Furthermore, DNA extraction and detection is possible from formalin-fixed, paraffin embedded tissues (Cooper *et al. 1997)*

Advances in molecular biology in the last decade have led to the development of several diagnostic assays which detect *T. gondii* nucleic acid in host tissue or body fluid samples. The identification of DNA probes (Boothroyd *et al.* 1987; Savva, 1989) has facilitated dot blot assays (Angel *et al.* 1992; Blanco *et al.* 1992) and the application of the polymerase chain reaction to amplify otherwise undetectable amounts of *Toxoplasma* DNA in human (Burg *et al.* 1989) and animal clinical samples (Stiles *et al.* 1996).

The Dot Blot Assay

In mock infections, Blanco *et al.* (1992) were able to detect as little as 80 pg of *T. gondii* DNA, (equivalent to approximately 10^3 parasites), in the presence of 1.5×10^6 human and mouse leucocytes, using a radiolabelled DNA probe to target a highly repetitive DNA sequence (ABGTg 4, in the *Toxoplasma* genome. In cerebrospinal fluid from human clinical cases of toxoplasmic encephalitis, using a non-radioactive label as few as 5×10^4 parasites are detectable (Angel *et al.* 1992).

The Polymerase Chain Reaction (PCR)

Exquisite and greater sensitivity of detection of DNA can be achieved by polymerase chain reaction amplification of a specific 'target' DNA of interest. This technique has been applied to the detection of *Toxoplasma* DNA for research purposes and to identify infections in clinical cases in man and animals, using PCR primers which specifically amplify a number of *T. gondii* DNA sequences, such as the P30 (SAG1) gene (Savva *et al.* 1990;

Weiss *et al*), 1991, the B1 gene (Burg *et al.* 1989; Grover *et al.* 1990; van de Ven *et al.* 1991; Parmley *et al.* 1992; Joss *et al.* 1993; Wastling *et al.* 1993; Stiles *et al.* 1996) and to amplify ribosomal DNA (rDNA) sequences (Cassenave *et al.* 1991; Guay *et al.* 1993; MacPherson & Gajadhar, 1993; Schoondermark-van de Ven *et al.* 1993).

The sensitivity of the detection of *Toxoplasma* by PCR varies according to the copy number of the target DNA sequence in the genome. Sensitivities of detection equivalent to or better than 1 parasite in 1 x 10^5 host cells can be achieved (Burg *et al.* 1989). In experimental infections in sheep, Wastling *et al.* (1993) found amplification of the multicopy B1 gene to be more sensitive than that of the single copy P30 (SAG1) gene in the detection of *Toxoplasma* DNA. Ribosomal DNA sequence based assays promise to be ultrasensitive since there are 110 copies of the rDNA unit is in each *T. gondii* organism (Guay *et al.* 1992), enabling the detection of the equivalent to or less than one parasite (Cassenave *et al.* 1991; Guay *et al.* 1993).

In uncontrolled field studies, amplification of the SAG1 gene has been used to detect *T. gondii* in equine toxoplasmosis (Turner & Savva, 1990, 1991) and in the organs and tissues of lambs aborted apparently following ovine toxoplasmosis (Greig *et al.* 1993; Turner & Savva, 1992; Wheeler *et al.* 1990). B1 gene PCR has been investigated for suitability for detection of *Toxoplasma* in feline and canine simulated infections (Stiles *et al.* 1996)

and *T. gondii* rDNA targets have been used in PCRs in mock infections in animals (MacPherson & Gajadhar, 1993).

In addition to facilitating the detection of *Toxoplasma*, molecular biological techniques enable investigation into the epidemiology of *T. gondii,* by differentiation between strains and isolates on the basis of identification of DNA polymorphisms. Heterogeneity in the *Toxoplasma* population has been demonstrated by techniques such as riboprinting (Brindley *et al.* 1993), restriction fragment polymorphism (RFLP) (Cristina *et al.* 1991), random amplified polymorphic DNA-PCR (Guo & Johnson, 1995) and PCR-RFLP (Howe & Sibley, 1994; Sibley & Boothroyd, 1992).

AIMS OF THE RESEARCH

The work presented deals with the epidemiology and diagnosis of *Toxoplasma gondii*, with particular reference to toxoplasmosis in sheep. In addition to being a significant cause of reproductive loss in sheep, *T. gondii* infections in sheep pose a zoonotic threat to man.

The first part of this study (Manuscript I), examines the potential of using molecular biological technology to diagnose *Toxoplasma* infection in aborted ovine foetal tissues, initially from experimentally infected ewes. A simple protocol for tissue preparation for PCR amplification of *Toxoplasma gondii* DNA from infected tissues was developed and applied to abortion tissues from experimental, and later, from naturally acquired infections. Using the protocol developed, the aetiopathogenesis of 'early' ovine *Toxoplasma* abortions, from experimental infections, was investigated by examination of foetal tissues for *T. gondii* infection by PCR (Manuscript II).

Most ovine *Toxoplasma* infections occur as a result of the ingestion of oocysts, probably in a contaminated environment. An environmental reservoir of *Toxoplasma* infection, capable of infecting cats which then shed oocysts, may be maintained in an area, in wild rodents by vertical transmission of toxoplasmosis. Transmission of *T. gondii* from chronically

infected dams to their offspring was investigated by PCR in colonies of wild, outbred *Mus musculus* and *Apodemus sylvaticus* (Manuscript III).

Evidence from epidemiological studies and from limited clinical case studies suggests that sheep acquire toxoplasmosis whilst at pasture. In Manuscript IV, the incidence and cumulative prevalence of *T. gondii* infection in grazing sheep was studied on three farms over periods from nine to eighteen months.

In human cases of toxoplasmosis, different *Toxoplasma* genotypes are now recognised, which appear to be correlated with disease. Little is known about the epidemiology of *T. gondii* in animals, but animal infections provide the opportunity to study and compare *Toxoplasma* types in both healthy individuals and in cases of disease. Hence naturally acquired *Toxoplasma*-infected tissues from abattoir lambs, from aborted ovine foetuses and from wild rodents (the latter both collected from farms), were used in PCR-RFLP studies to investigate the molecular epidemiology of *T. gondii* infecting animals (Manuscript V).

Finally, with a view to finding additional markers for genotype analysis of *T. gondii* that may enable greater sensitivity of parasite detection from infected tissues than the polymorphic single-copy genetic markers currently available, PCR-RFLP studies, with genomic DNA, derived from five strains

and two new isolates of *T. gondii* were applied to ribosomal DNA (rDNA) (Manuscript VI).

MANUSCRIPT I

DIAGNOSIS OF OVINE *TOXOPLASMA* ABORTION BY POLYMERASE CHAIN REACTION

M. R. OWEN, M. J. CLARKSON* and A. J. TREES

Veterinary Parasitology, Liverpool School of Tropical Medicine/Faculty of Veterinary Science, University of Liverpool, Pembroke Place, Liverpool. L3 5QA.

*Veterinary Clinical Science and Animal Husbandry, Veterinary Teaching Hospital, Leahurst, University of Liverpool, Chester High Road, Neston, S. Wirral. L64 7TE.

SUMMARY

Eighteen oestrous synchronised ewes were experimentally infected with 1500 sporulated oocysts of *Toxoplasma gondii* between 80 and 90 days of gestation. Infection induced a pyrexia and specific antibody in all ewes. One ewe resorbed its foetus, 5 ewes aborted and 12 delivered live, congenitally infected lambs (antibody positive pre-colostral serum). Tissues from aborted foetuses and placentae from live lambs were examined for *Toxoplasma* infection by polymerase chain reaction (PCR) amplification of the B1 gene and by mouse inoculation (MI). Using a simple protocol of tissue preparation without DNA extraction and a nested PCR format, PCR was as sensitive as MI. Placental cotyledon was the optimum tissue for examination (higher sensitivity of detection than for brain, lung or liver) and 16/19 placentae were PCR positive compared with 13/18 by MI. In mock infected tissues, as few as 10 tachyzoites could be detected. PCR could be applied to tissues unfit for MI.

Toxoplasmosis, caused by infection by the protozoan parasite *Toxoplasma gondii,* is an important zoonosis and is a major cause of disease in sheep. Whilst infection in most healthy individuals is generally not associated with serious clinical signs, in immunocompromised individuals and for developing foetuses, infection can be life-threatening or fatal. In sheep, foetal resorption, abortion or perinatal mortality of lambs, occur when ewes suffer a primary infection during pregnancy (Dubey and Towle 1986). Abortion diagnosis has traditionally relied on histopathological examination and serological tests and on the isolation of *Toxoplasma* by mouse inoculation, which is both time-consuming and hazardous. Molecular techniques such as the polymerase chain reaction (PCR) enable the detection of small quantities of DNA, and potentially offer an alternative sensitive diagnostic tool. Sequences in the P30 gene (Savva and others 1990), the B1 gene (Burg and others 1989) and the 18S ribosomal RNA gene (MacPherson and Gajadhar 1993) have been used to detect *Toxoplasma* in various human and animal clinical samples. B1 gene amplification has been used to detect the parasite in lymph and peripheral blood samples from experimentally infected sheep (Wastling and others 1993) and there are reports of the use of the P30 gene to detect parasite DNA in naturally aborted ovine foetuses following presumed maternal toxoplasmosis (Turner and others 1991). However, the use of PCR to detect toxoplasmosis in aborted ovine tissues from experimental infections has not been evaluated. This study aimed to

assess PCR in comparison with mouse inoculation (MI) for the diagnosis of ovine toxoplasmosis from tissue samples and to develop a field diagnostic method.

MATERIALS AND METHODS

Twenty-one Welsh half-bred sheep, judged susceptible to toxoplasmosis on the basis of antibody titres in the latex agglutination test (LAT) (Eiken Chemical Company, Japan) of less than 1/64 (Trees and others 1989), were oestrous-synchronised, mated and confirmed pregnant at 60-70 days gestation by ultrasound examination. Sporulated *Toxoplasma* oocysts of the M1 strain were obtained from the faeces of a cat experimentally infected with *T. gondii*, as described previously (Buxton and others 1988). Between days 80-90 of gestation, ewes each received 500 sporulated oocysts by stomach tube on alternate days on three occasions, giving a total infective dose of 1500 oocysts per ewe. Three uninfected, pregnant sentinel sheep were kept with the infected group to detect adventitious *Toxoplasma* infection. Rectal temperatures were measured from 3 days pre-infection to 12 days post-infection. Tissues from abortions and foetal membranes from live-born lambs were collected and tested for *Toxoplasma* infection by MI and PCR. Thoracic fluid from aborted foetuses and pre-colostral sera from live lambs were tested for *Toxoplasma* specific antibody.

Serology

In sheep and in live lambs *Toxoplasma* antibody was assayed using the latex agglutination test (LAT) (Eiken Chemical Company, Japan), (Trees and others 1989), using a screening serum dilution of 1/64. For mice and aborted lambs antibody was detected with the modified agglutination test (MAT), (Biomerieux, Marcy L' Etoile, France), which shows superior sensitivity to the LAT for aborted lambs (Dubey and others 1987). For the MAT, sera were assayed using a screening dilution of 1/40 and also at 1/4000 to prevent the false negative results which can occur at low dilutions using sera with high antibody titres.

Tissue collection

Tissues were collected with single-use disposable gloves and implements in order to prevent cross-contamination. From each abortion, samples of placental cotyledon, foetal brain, lung and liver were collected. Placental samples were collected from live-born lambs and approximately 1cm cubed of each tissue was collected for both PCR and MI. Samples for PCR were stored in sterile bijoux bottles at -20°C. Samples for mouse inoculation were stored at 4°C, prepared and injected within 8 hours of collection.

Mouse inoculation

Each tissue sample was chopped in a petri dish using a sterile scalpel blade, and homogenised with 3ml of sterile phosphate buffered saline (PBS), pH 7.2, supplemented with 100 i.u./ml penicillin G and 100μg/ml

dihydrostreptomycin (Gibco, Paisley, UK) using a sterile syringe and a series of hypodermic needles of descending gauge (19-23 gauge). Homogenised samples were allowed to stand at room temperature for 1 hour to combat susceptible bacteria. Two Balb/c mice without detectable *Toxoplasma* antibody using the modified agglutination assay (MAT), (Biomerieux, Marcy L' Etoile, France) were each inoculated with 0.5ml of tissue homogenate by intraperitoneal injection. Mice were tested for *Toxoplasma* antibodies at 8 weeks post-injection by MAT using screening dilutions of 1/40 and 1/4000 (to test for false negatives obtained when high antibody titres are tested at 1/40). A positive titre at either dilution in either mouse was taken as evidence of infection in the sample tested.

Polymerase Chain Reaction

Each sample was finely chopped with a sterile scalpel blade, then powdered, by crushing, under liquid nitrogen using a pestle and mortar. Powdered tissues were incubated at 37°C for 15 minutes in erythrocyte lysis buffer (10mM Tris, 155mM NH_4Cl, pH 7.2) at a ratio of 1 part tissue to 9 volumes buffer, centrifuged at 2000g for 10 minutes, and washed in sterile PBS, pH 7.2 three times, then 500mg of each tissue was digested in 1ml digestion buffer (50mM Tris, 1mM EDTA, 0.5% Tween 20, 200μg/ml proteinase k, pH 8.5) at 55°C for 2 hours and then boiled for 8 minutes to inactivate the proteinase and coagulate proteins. Digested samples were centrifuged for 30 seconds at 11500g and 5μl of supernatent was used in the first of a nested PCR. PCRs were conducted in 100μl of

reaction mixture consisting of 20mM $(NH_4)_2SO_4$, 75mM Tris HCl, pH 9.0, 0.01% Tween 20 (w/v), 2.5mM $MgCl_2$, 0.1mM d-NTPs, 0.2μM each primer, 0.3 units Red Hot Taq polymerase (Advanced Biotechnologies, Leatherhead, UK). The primers used and amplification conditions were as previously described (Wastling and others 1993), except that the denaturing, annealing and extension times used were each 1 minute. In the first PCR, the primers were GGAACTGCATCCGTTCATGAG and TCTTTAAAGCGTTCGTTCGTGGTC. Following successful amplification of a 193 base-pair product, 1μl of each reaction was used in a second PCR, with a new primer pair, TGCATAGGTTGCAGTCACTG and GGCGACCAATCTGCGAATACACC, to produce a 94 base-pair amplicon. PCR products were visualised after electrophoresis of 30μl of reaction mixture on ethidium bromide stained 2% agarose gels. Distilled water and non-infected tissues were used as negative controls and were run with tests to monitor for cross-contamination. Disposable gloves and single use plastic wear were used to prevent contamination of tests.

Southern blotting

Southern blotting was used on occassions to confirm the identity of the B1 PCR product. DNA products were transferred onto Hybond (Amersham, UK) membranes and DNA-DNA hybridisation was performed with ^{32}P - labelled fragment produced from internal PCR primers using a template of *Toxoplasma* tachyzoite derived DNA.

Sensitivity and Specificity of B1 PCR

T. gondii tachyzoites, grown in Vero cells using RPMI-1640 media (Sigma Chemical Co., Poole, Dorset) supplemented with 2% foetal calf serum (GibcoBRL), 50 i. u. penicillin G and 50μg/ml streptomycin (GibcoBRL) were separated from Vero cells by scraping the monolayer with a rubber policeman, homogenizing through a descending series of needles (19-25 gauge), washing three times with PBS, then passing the homogenate through a sepharose G25 minicolumn (Pharmacia, Uppsala, Sweden). A series of mock-infected placental samples, containing from 10^0-10^6 parasites was created by adding purified tachyzoites to 500mg of powdered tissue samples prior to tissue digestion.

To determine the specificity of the PCR test, amplification was attempted with DNA from *Sarcocystis tenella, Sarcocystis gigantea* (gift from Dr John Ellis, UTS, Sydney, Australia) and *Neospora caninum* (NC-Liv isolate). In addition, *Chlamydia psittaci* infected placental samples (diagnosed by indirect immunofluorescence antibody test (gift from Bruce Wilson, Preston Veterinary Investigation Centre), were processed for PCR as above. In no case was a positive result obtained.

Statistical methods

The data were analysed by chi-square test.

Clinical Findings

All ewes dosed with oocysts developed a pyrexia which reached a mean peak of 41.3°C six days after the first infective dose. Pyrexic ewes had increased respiratory rates but showed no other signs of illness and continued to eat normally. There was no pyrexia in the sentinel ewes. In the infected group, one ewe aborted nine days after the first oocyst dose and four ewes aborted between 120 and 140 days of gestation (30-60 days after infection), producing six dead foetuses (one mummified). Twelve ewes delivered 14 live, full-term lambs, in one case with a mummified twin, but small white foci, characteristic for toxoplasmosis (Beverley and others 1971), were visible on the placental cotyledons from eight of these. One ewe resorbed its foetus. There were no visible lesions on placental cotyledons from the sentinel ewes, which delivered three live lambs at full term.

Serology

Twenty-one days after the first inoculum of oocysts, all dosed ewes had developed at least an eight-fold rise in *T. gondii* antibodies, with titres ranging from 1/64-1/1024. Of the seven dead foetuses, two were mummified and had no foetal fluid. Of the samples from the five other foetuses, one had a titre of 1/64. Pre-colostral serum was collected from

live lambs and all had detectable specific *T. gondii* antibody with titres (and numbers) of 1/4096 (4), 1/1024 (8), 1/256 (1) and 1/64 (1).

PCR and Mouse Inoculation

Using mock-infected tissue samples, PCR was capable of detecting as few as 10 parasites in placental cotyledon samples. Southern blotting confirmed the identity of the 94 base pair PCR product. PCR Results are shown in Figure 1.

Samples of foetal brain and lung were available from seven dead foetuses and liver samples from six. Placental cotyledons were examined from all seven dead foetuses and from twelve live, full-term lambs. All tissues collected were tested by PCR but tissues from one foetus were unsuitable for MI (Table 1). The sensitivity of PCR was similar to that of MI for foetal brain, lung and liver and placental cotyledons and the differences were not statistically significant ($p > 0.05$, chi-squared test with Yates' correction). Placental cotyledons and foetal tissues from the ewe which aborted nine days after infection were negative for *Toxoplasma* infection by both PCR and MI. Placental cotyledons from three precolostral antibody-positive lambs were positive by PCR but negative by MI and cotyledon from one other precolostral antibody positive lamb was MI positive, but PCR negative. All positive PCR results were confirmed by MI (except in the case of the foetus unsuitable for MI). Placental cotyledons from sentinel ewes

were negative by both MI and PCR. From the foetus, the tissues of which were unsuitable for MI, the placental cotyledon gave a positive PCR result. Typical results of a PCR gel are shown in Figure 2.

Figure 1. B1 PCR sensitivity using digestions of mock-infected placental cotyledon samples. A serial dilution of 10^6-10^1 tachyzoites was added to 500mg placental cotyledon samples prior to tissue digestion (lanes 1-6), cotyledon from a sentinel ewe (lane 7), 10ng *T. gondii* DNA (lane 8), distilled water negative control (lane 9), molecular weight marker (lane 10). Amplification products from PCRs, using 5ml of each tissue digest, were analysed by electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualised by ultra-violet light trans-illumination. The 94 base pair *T. gondii* specific amplicon is indicated.

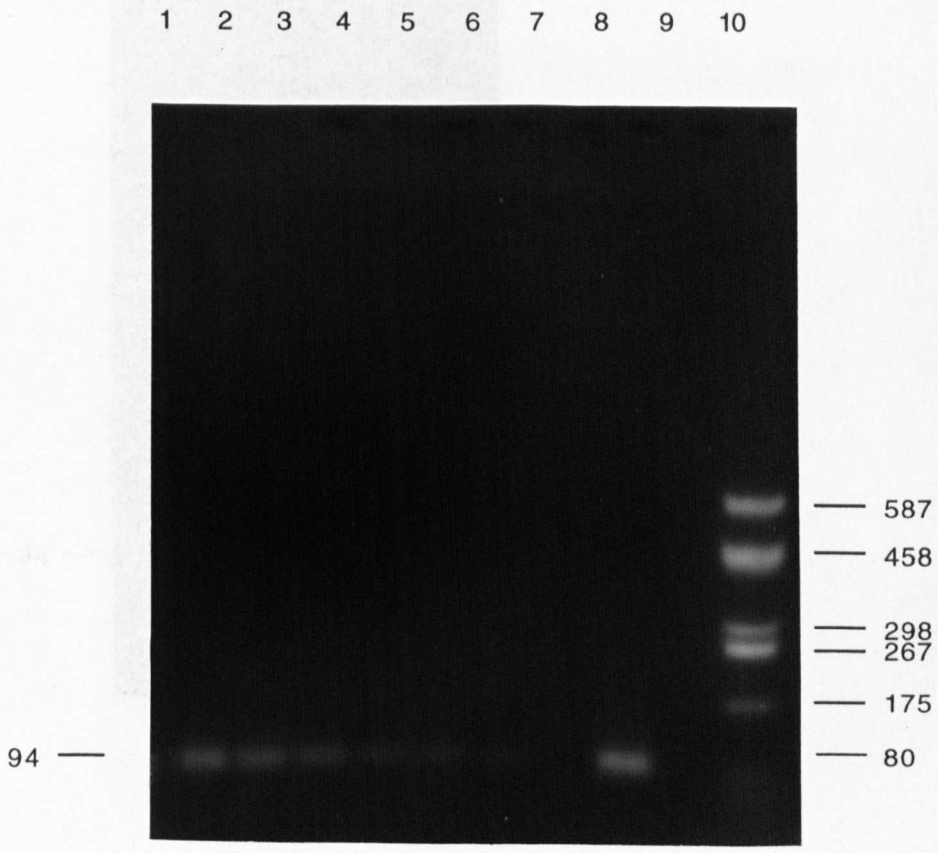

Figure 2. PCR amplification of the *Toxoplasma* B1 gene from tissue digests of a foetus from an experimentally infected ewe. 30ml aliquots of PCRs were electrophoresed on ethidium stained 2% agarose gels: placental cotyledon (lane 1), foetal brain (lane 2), foetal lung (lane 3) and foetal liver (lane 4). Non-infected cotyledon was used as a negative control (lane 5) and molecular weight markers are shown in lane 6. The *T. gondii* specific amplification product (94bp) is indicated.

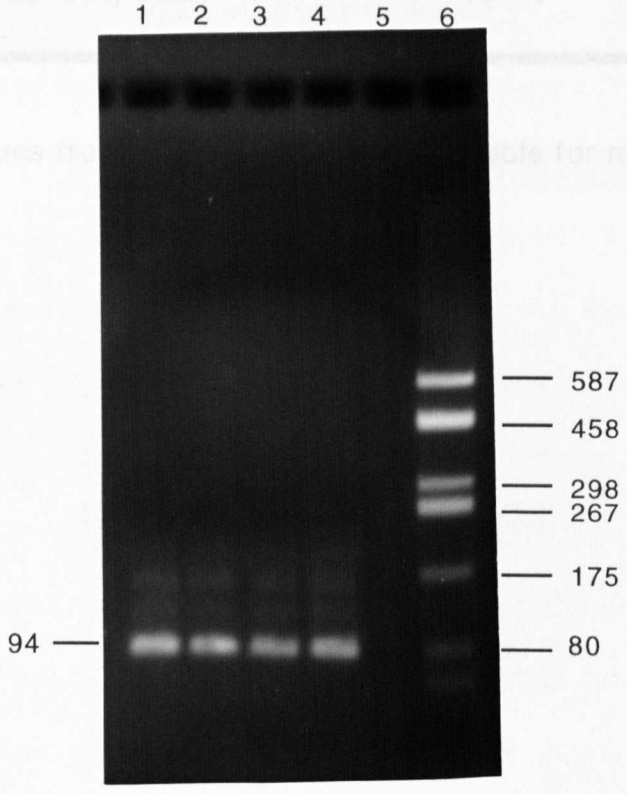

Table 1. The detection of *Toxoplasma gondii* by mouse inoculation (MI) and polymerase chain reaction (PCR) amplification in foetal tissues after experimental infection of ewes.

Tissue	MI[a] No. pos/No. tested	PCR No. pos/No.tested
Brain	3/6	3/7
Lung	3/6	2/7
Liver	4/5	2/6
Placental cotyledon	13/18	16/19

[a] Tissues from one foetus were unsuitable for mouse inoculation.

There are a number of reports of the use of PCR to detect *T. gondii* in cases of human toxoplasmosis (Burg and others 1989; Casenave and others 1991; Filice and others 1993; and many others) and in mock -infected samples from several species of domesticated animals (MacPherson and Gajadhar 1993, Stiles and others 1996). B1 gene PCR was successfully used to detect *T. gondii* in peripheral blood, efferent lymph and lymph node samples taken from experimentally infected ewes (Wastling and others 1993), and there are reports of the use of a P30 (surface antigen one, "SAG1") gene based PCR test to detect *T. gondii* in abortion tissues from presumed field cases of maternal toxoplasmosis (Wheeler and others 1990). The studies reported here, using tissues infected under controlled conditions, demonstrate the value of the PCR test for diagnosing toxoplasmosis in ovine abortions and show it to be as sensitive as MI, the accepted definitive diagnostic method. Most significantly, from a practical point of view, a protocol has been developed and validated involving simple methods of tissue digestion and without the need for DNA extraction. *T. gondii* was readily detectable in placental cotyledons and, since this tissue is the most likely to be available after abortion, the PCR test is highly suited to the examination of abortions for *T. gondii* infection. In no case was *T. gondii* DNA detected in samples of brain, lung or liver in the absence of a positive PCR result for placental cotyledon. However, in clinical cases when placental cotyledons may not be available, our results suggest that

brain, lung and liver may be used for PCR. The sensitivity of this PCR test, which detected 10 parasites in mock-infected placental cotyledon, is similar to detection sensitivities reported by others using B1 gene based PCR (Burg and others 1989; Stiles and others 1996) and results using a small sub-unit ribosomal RNA gene based PCR (MacPherson and Gajadhar 1993). The B1 PCR was more sensitive for detection of *T. gondii* than SAG1 PCR (Savva and others 1990) in all tissues and mock-infected tissues (data not published), a finding also observed by Wastling and others (1993). The greater sensitivity of B1 PCR over SAG1 PCR is presumably because the B1 sequence is a multicopy gene (approximately 35 copies in the genome, Burg and others 1989) in contrast to SAG1, which is a single copy gene.

For most tissues, results of PCR and MI were in agreement. However, in one case, cotyledons from a seropositive (pre-colostral serum) live lamb were positive for *Toxoplasma* infection by MI, but repeatedly negative by PCR and in three cases, PCR was positive when MI was negative. These discrepancies may be due to a very sparse and focal distribution of parasites in some cases, such that some tissue samples fail to contain parasites. In addition, MI fails if parasites are present but are non-viable, whereas PCR will detect parasite DNA which is relatively resistant to degradation. This is particularly so if tissues are autolysed, or contaminated, hence unsuitable for MI. For these latter reasons, PCR is potentially more useful and sensitive for detecting *Toxoplasma* infection.

The specificity of this PCR test for *T. gondii* infection is particularly important in the light of recent reports of foetal infection following experimental *Neospora caninum* infections of pregnant ewes (M^{c}Allister and others 1996; Buxton and others 1996). Although one natural infection of a lamb has been reported (Dubey and others 1990), there is no evidence to date of naturally occurring *Neospora*-induced abortion in ewes. Nonetheless, because of the similarities in the gross pathology and histology of *N. caninum* and *T. gondii* infections, a molecular test which can differentiate between them will be valuable.

Previous studies (Buxton and others 1988, 1991; M^{c}Colgan and others 1988; Owen and others (submitted, b)) have recorded a number of early or acute phase abortions following a single dose of 2000 *T. gondii* oocysts at 80-90 days of gestation, in which foetal death occurs during or soon after oocyst inoculation and probably as a result of the pyrexia associated with infection (M^{c}Colgan and others 1988). These acute phase abortions are not considered typical of natural cases of ovine toxplasmosis, (Buxton and others 1993), but the extent to which such abortions occur in natural infections may be under-estimated (Trees and others 1989). To minimise the occurrence of acute phase abortions in this study, *T. gondii* oocysts were given in divided doses over five days. This resulted in only one acute phase abortion, the tissues of which were negative for *T. gondii* infection by MI and by PCR. We have consistently been unable to detect *T. gondii* in tissues from acute phase abortions by MI or PCR and consequently they

remain a diagnostic challenge to the clinician (Owen and others (submitted, a)).

In conclusion, we have demonstrated that under controlled experimental conditions, PCR reliably detects *T. gondii* infection, with a sensitivity equal to that of MI, confirming its potential use as a diagnostic test for ovine abortion. The preferred method is to examine placental cotyledon, using a nested PCR with primers which amplify the B1 gene. DNA extraction with phenol/chloroform is not necessary and the PCR will detect parasites in poorly stored tissue or contaminated samples which are unsuitable for MI. The use of this diagnostic PCR would be further simplified by use of a one-tube nested format (M^{c}millan and others 1996) and the combination of the PCR with colorigenic ELISA to enable automated assay of PCR products (Shirley and others 1990; Muller and others 1996).

ACKNOWLEDGEMENTS

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MANUSCRIPT II

ACUTE PHASE OVINE *TOXOPLASMA* ABORTIONS - A DIAGNOSTIC CHALLENGE

M. R. OWEN, M. J. CLARKSON* and A. J. TREES

Veterinary Parasitology, Liverpool School of Tropical Medicine/Faculty of Veterinary Science, University of Liverpool, Pembroke Place, Liverpool. L3 5QA.

*Veterinary Clinical Science and Animal Husbandry, Veterinary Teaching Hospital, Leahurst, University of Liverpool, Chester High Road, Neston, S. Wirral. L64 7TE.

ABSTRACT

Eleven abortions occurred within thirteen days of the experimental infection of fifteen oestrous synchronised-ewes with 2000 sporulated oocysts of *Toxoplasma gondii* at 80 to 90 days of gestation. Infection induced a pyrexia and specific antibody in all ewes. One ewe resorbed its foetus, 11 ewes aborted and 3 delivered, at full term, live congenitally infected lambs (antibody positive pre-colostral serum). Tissues from aborted foetuses and placentae from live lambs were examined for *Toxoplasma* infection by polymerase chain reaction (PCR) amplification of the B1 gene and by mouse inoculation (MI). In contrast to tissues from the live lambs, all of which were shown to be infected by PCR and MI, there was no evidence of infection by PCR or MI in any tissues from acute phase abortions, confirming that these fatalities occurred before invasion of the placenta or the foetus by *T. gondii*. Such *Toxoplasma*-induced, acute-phase abortions are likely to be impossible to diagnose from foetal tissues. Their frequency following field infection is unknown. These results have implications not only for the diagnosis of naturally occurring ovine abortion but also for the understanding of the pathogenesis of *Toxoplasma*-induced abortion.

Toxoplasmosis is a significant cause of ovine abortion, neonatal mortality and reproductive losses in Britain (Blewett and Watson 1984; Blewett and Trees 1987), and many other countries (Dubey and Towle 1986). Typically, reproductive losses only occur when a ewe contracts a primary infection during pregnancy. Infection during early pregnancy may lead to foetal death and resorption, hence the ewe appears barren, whilst infection between approximately 50 to 120 days gestation classically leads to abortion late in gestation, or the birth of stillborn or weakly lambs, which may be accompanied by a mummified foetus (Beverley 1976; Buxton 1989). Placental cotyledons from these infected lambs and aborted foetuses may show characteristic small white necrotic foci whilst intercotyledonary membranes are normal. In such cases, characteristic gross pathology and specific antibody to *T. gondii* in foetal fluids, detectable three weeks after infection of the ewe, are diagnostic of toxoplasmosis. However, experimental infections of pregnant ewes with *T. gondii* oocysts indicate that a proportion of ewes abort as soon as seven to twelve days post infection (Dubey and others 1987; Trees and others 1989), presumably due to the pyrexia of infection (M^{c}Colgan and others 1988). The placentae from such abortions do not show the characteristic lesions of focal necrosis typical of toxoplasmosis (M^{c}Colgan and others 1988). Foetal fluids from such abortions will be negative for *Toxoplasma* antibodies (Dubey and others 1987;Trees and others 1989).

The extent to which acute phase abortions occur in natural cases of ovine toxoplasmosis is unknown and may well be under-estimated (Trees and others 1989), owing to the difficulty in their diagnosis. Using a nested polymerase chain reaction (PCR) technique for *T. gondii* detection in ovine abortion tissues, we investigated tissues from acute phase abortions, following experimental infections, for evidence of *T. gondii* infection. This PCR technique has been shown to be equal in sensitivity to mouse inoculation (MI) (Owen and others (submitted, b)).

MATERIALS AND METHODS

Experimental infections

Eighteen Welsh half-bred ewes, without detectable *T. gondii* specific antibody, were oestrous-synchronised, mated and confirmed pregnant at 60-70 days gestation by ultrasound examination. Between days 80-90 days of gestation, fifteen ewes were orally infected with 2000 sporulated *Toxoplasma* oocysts of the M1 strain (Buxton and others 1979). The three remaing uninfected ewes were kept as sentinels with the infected groups to detect adventitious *Toxoplasma* infection. Rectal temperatures were measured from two days pre-infection to 14 days post-infection (dpi). Post infection pyrexias and an eightfold rise in *T. gondii* antibody titre at 21 dpi confirmed infections (Blewett and others 1983). Tissues from abortions and foetal membranes from live lambs were collected and tested for *Toxoplasma* infection by MI and PCR. Thoracic fluid from aborted foetuses,

pre-colostral sera from live lambs and ewe sera, taken pre-infection and 7,14 and 21 dpi were tested for *Toxoplasma* specific antibody.

Serology

Toxoplasma antibody in sheep and live lambs was assayed using the latex agglutination test (LAT) (Eiken Chemical Company, Japan), (Trees and others 1989), from a dilution of 1/64 and in mice and aborted lambs by the modified agglutination test (MAT), (Biomerieux, Marcy L' Etoile, France), which shows superior sensitivity to the LAT for aborted lambs (Dubey and others 1987). For the MAT, sera were assayed at both at 1/40 dilutions and at 1/4000 dilutions to detect false negative results which can occur when testing serum with high antibody titre at low dilutions.

Tissue collection

Tissues were collected with single-use disposable gloves and implements in order to prevent cross-contamination. From all abortions and live-born lambs (killed at birth by lethal injection), $1cm^3$ samples of placental cotyledon, foetal brain, lung and liver were collected for PCR and MI. Samples for PCR (placental cotyledon) were stored in sterile bijoux bottles at -20°C. Samples (all tissues) for mouse inoculation were stored at 4°C, prepared and injected within 8 hours of collection.

Mouse inoculation and PCR

Detailed methods of tissue preparation for MI and for PCR were as previously described (Owen and others (submitted, b)). Tissues for MI (placental cotyledon, foetal brain, lung and liver) were homogenised with sterile phosphate buffered saline (PBS), pH 7.2, supplemented with 100 i.u./ml penicillin G and 100μg/ml dihydrostreptomycin (Gibco, Paisley, UK). Each tissue homogenate was inoculated into two *Toxoplasma* susceptible Balb/c mice. Tissues were considered infected with *T. gondii* if either mouse developed specific *Toxoplasma* antibody at dilutions of 1/40 or 1/4000 eight weeks after inoculation. Previous studies (Owen and others (submitted, b)) showed that PCR examination of placental cotyledon alone was adequate to determine foetal infection, since in no case of foetal infection was placental cotyledon PCR negative when a positive PCR result was obtained from other foetal tissue. Hence for PCR, placental cotyledon samples were finely chopped, powdered under liquid nitrogen, then incubated at 37°C for 15 minutes in erythrocyte lysis buffer (10mM Tris, 155mM NH_4Cl, pH 7.2) at a ratio of 1 part tissue to 9 volumes buffer. After 3 washes in sterile PBS, pH 7.4, 500mg of each tissue was digested in 1ml digestion buffer (50mM Tris, 1mM EDTA, 0.5% Tween 20, 200μg/ml proteinase k, pH 8.5) at 55°C for 2 hours, boiled for 8 minutes to inactivate the proteinase and coagulate proteins. Digested samples were centrifuged for 30 seconds at 11500g and 5μl of supernatent was used in the first of a nested PCR. Amplifications were conducted in 100μl reactions using primers that amplify part of the *Toxoplasma* B1 gene (Burg and others

1989; Wastling and others 1993), using a reaction mixture and thermal profile as previously described, (Owen and others submitted,b)). For positive control *T. gondii* DNA, 5μl of supernatent from the digestion of 10^4 *Toxoplasma* tachyzoites was amplified in each experiment. Following the successful amplification of a 193 base pair *T. gondii* specific product, 1μl of each reaction was used in the nested PCR, with a second primer pair to produce a 94 base pair amplicon. PCR products were visualised following electrophoresis on ethidium bromide stained 2% agarose gels, trans-illuminated with ultra-violet light. Distilled water and digestion supernatent from non-infected tissues were used as negative controls and were amplified with experimental tissues to monitor for cross-contamination. Disposable gloves and single-use plastic wear were used to prevent cross-contamination.

RESULTS

Clinical findings and antibody results

All infected ewes developed a pyrexia (greater than 41°C) five dpi which lasted an average of four days (Figure 1). Pyrexic ewes had increased respiratory rates but showed no other signs of illness and continued to eat normally. There was no pyrexia in the sentinel ewes. In the infected group, 11 abortions occurred within 13 days of infection (eight at 10dpi, two at 12dpi and one at 13dpi) (Figure 1 and Table 1). At full term, three ewes produced live lambs but small white foci, characteristic for

toxoplasmosis (Beverley and others 1971), were visible on the cotyledons of their associated placentae. One ewe resorbed its foetus. All sentinel ewes delivered live lambs at full term, with no visible lesions on their placental cotyledons. *Toxoplasma* specific antibody was first detected at a serum dilution of 1/64 fourteen dpi in eight of the infected ewes and by 21dpi, all fifteen infected ewes developed *T. gondii* specific antibody, with titres ranging from 1/64-1/1024. *T. gondii* antibody was not detected in foetal fluid samples obtained from the eleven abortions. All three live lambs had a precolostral *T. gondii* antibody titre of 1/4096, confirming infection. Sentinel ewes and their lambs (which numbered three) did not develop *T. gondii* antibodies.

PCR and Mouse inoculation

All tissues from live lambs delivered to sentinel ewes (tested by PCR and MI) and placental cotyledon samples from cases of early abortion (tested by PCR) were negative for *T. gondii* infection. *T. gondii* was detected in the cotyledons from all three live-born lambs by PCR and MI. (Table 2) but none of the mice inoculated with placental cotyledon, foetal brain, lung or liver from early abortions developed *Toxoplasma* antibodies by eight weeks post injection, indicating that these foetal tissues were not infected with viable *T. gondii* organisms.

Table 1. Outcome of pregnancies in fifteen ewes infected with 2000 *Toxoplasma gondii* oocysts *per os* at 80-90 days gestation.

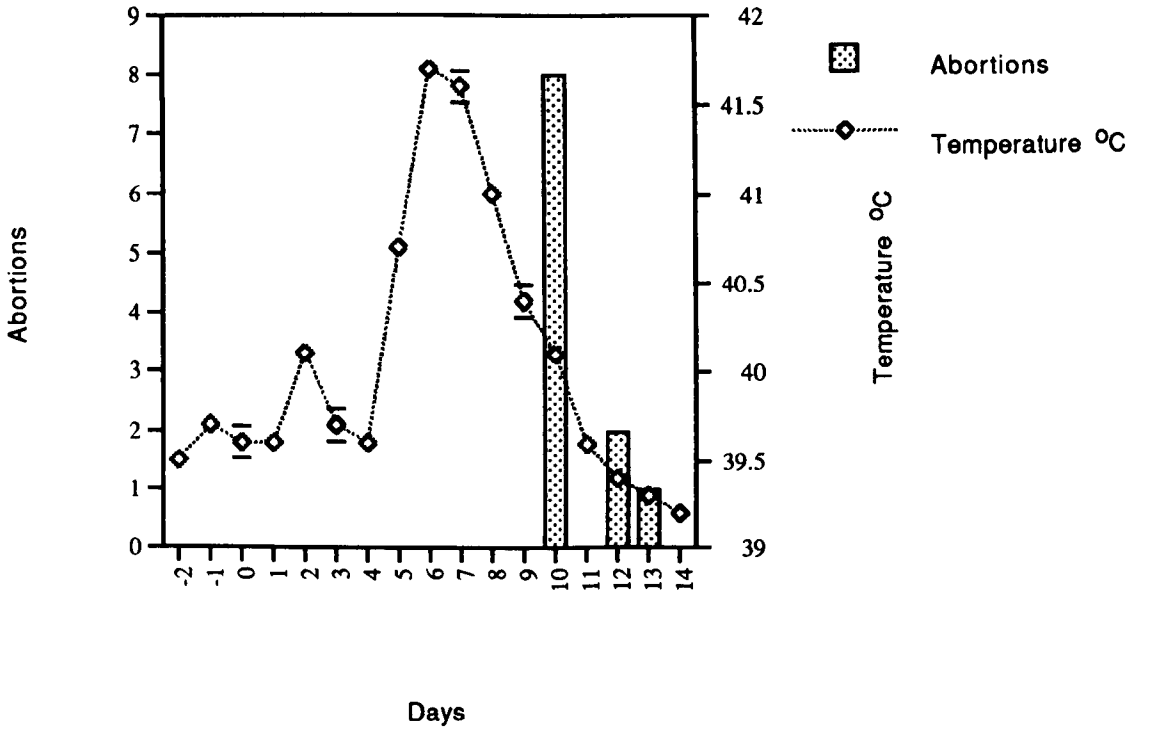
Outcome of Pregnancy	Ewes	No. foetuses/ live lambs	No. foetuses/lambs with detectable *T. gondii* antibody[a]
Resorption	1	0	-
Abortions	11	11	0
Live lambs	3	3	3

[a] Aborted foetuses were tested using the modified agglutination test, live lambs were tested by the latex agglutination test.

Table 2. *Toxoplasma gondii* detection in tissues by polymerase chain reaction (PCR) or mouse inoculation (MI) in tissues from foetuses aborted within 13 days of infection and lambs born at full term to experimentally infected ewes.

	Tissue (No. positive/No. tested)				
	Placental cotyledon		Brain	Lung	Liver
	PCR	MI	MI	MI	MI
Aborted foetuses	0/11	0/11	0/11	0/11	0/11
Live lambs	3/3	3/3	3/3	1/3	2/3

Figure1. The mean (+/- sem) temperature response and abortions in fifteen ewes experimentally infected with 2000 sporulated *Toxoplasma gondii* oocysts *per os* on day one.



DISCUSSION

These results present data from eleven-acute phase abortions following experimental infection of pregnant ewes with *T. gondii* oocysts. They confirm that these abortions can occur without demonstrable *Toxoplasma* infection in the foetus or placental cotyledon, as determined by PCR and MI. Following oral infection of sheep with *T. gondii* oocysts, parasites are detectable in the blood by MI six days post-infection (Dubey and Sharma 1980), but the earliest time at which parasites have been detected in the uterus by MI is 12 days and they may not be detected until 20 days (Dubey and Sharma 1980; Dubey and others 1987). That no parasites were detected by PCR or MI in placental samples in any of eleven acute-phase abortions in this study suggests that the placenta is not normally infected until the 14th day of infection in the ewe. Classically, *Toxoplasma* abortion occurs when tachyzoites multiply in placental cotyledons causing placental insufficiency. Accompanied by replication of parasites within the developing foetus, focal lesions develop in both the placental cotyledons and in foetal organs (Buxton and Finlayson 1986). Infected foetuses develop focal leucomalacia of the brain, probably as a result of anoxic damage due to placental insufficiency, which is often so severe as to lead to foetal death and abortion. The mechanism by which acute-phase abortions occur within 14 days of infection is unknown. However, pyrexia associated with infection can induce abortion (Kline and others 1985) and in this study, because of the absence of characteristic gross pathological *Toxoplasma* lesions nor evidence of *T. gondii* infection by either PCR or MI

in any of the abortion tissues examined, we consider that these abortions occur in association with the fever of infection in the dam, (M°Colgan and others 1988). The mechanism by which such abortions occur in sheep is poorly understood. In mice, the cytokines interferon gamma, interleukin two (IL-2) and tumour necrosis factor alpha induce abortion in response to administration of lipopolysaccharide, a known pyrogen (Gendron and others 1990). The fever associated with parasitemia in ewes may induce similar events culminating in foetal death and expulsion. Whether abortion occurs as a direct result of pyrexia, whether abortion follows disturbances, such as uterine contraction, induced by the release of cytokines, in response to pyrexia or whether pyrexia and cytokine-induced abortion are independent but coincident events, is unknown. In ewes, pyrexia can occur following the ingestion of 20 oocysts (Buxton and others 1991) and acute-phase abortions can occur when pregnant ewes ingest as few as 200 oocysts (M°Colgan and others 1988). In view of the fact that during a primary *Toxoplasma* infection, a cat can excrete millions of oocysts (Dubey 1976) and that sheep can be infected at grazing, presumably as a result of the direct contamination of pasture by infected stools (Owen and others (submitted, c)), infective doses of oocysts sufficiently large to provoke acute phase abortions can credibly occur.

In conclusion, it has been shown in experimental infections that *Toxoplasma*-induced abortions can commonly occur within 14 days of oocyst infection and that *T. gondii* infection cannot be demonstrated in

foetuses from such abortions, either by PCR or MI. The only aid to the diagnosis of such cases is by maternal antibody testing approximately seven days after abortion when ewes will have developed IgM antibodies to *T. gondii* (Trees and others 1989). When presented to the clinician in the absence of maternal serum, these abortions will remain undiagnosable.

Acknowledgements

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MANUSCRIPT III

VERTICAL TRANSMISSION OF *TOXOPLASMA GONDII* IN WILD RODENTS

M. R. OWEN and A. J. TREES

Veterinary Parasitology, Division of Parasite and Vector Biology, Liverpool School of Tropical Medicine/Faculty of Veterinary Science, University of Liverpool, Pembroke Place, Liverpool. L3 5QA.

SUMMARY

Captive-bred *Mus musculus* (house-mice), *Apodemus sylvaticus* (field mice) and *Clethrionomys glareolus* (bank voles), were infected with oocysts of *Toxoplasma gondii* M1 strain *per os* and infection in them and their offspring was assessed by serology, using the modified agglutination test (MAT) and polymerase chain reaction (PCR) amplification of the *T. gondii* B1 gene from brain tissue. A dose of 50 oocysts established chronic infections in *Apodemus* and *Mus* with minimal mortality but for *Clethrionomys*, mortality was 100% when the dose exceeded 25 oocysts and it was not possible to confirm infection serologically in animals given 25 or fewer oocysts. Chronically infected female *Apodemus* ($n=10$) and *Mus* ($n=23$) were mated at least six weeks after infection (and subsequently to produce up to six litters) and their pups examined three weeks after weaning at six weeks of age. By PCR, in offspring of *Apodemus* and *Mus* respectively, vertical transmission was demonstrated in 82.7% ($n=83$) and 85.0% ($n=207$) of all pups (NS, $p>0.05$), 95% ($n=21$) and 100% ($n=30$) of all litters (NS, $p>0.05$), with a mean (+/- s.e.) proportion of each litter infected of 0.87 (0.06) and 0.86 (0.04) (NS, $p>0.05$). There was no change in any of these variables between first and subsequent litters. By serology, whilst MAT indicated 100% vertical transmission in *Apodemus*, it under-estimated rates of infection in offspring of *Mus*. A limited series of bioassays from *Mus* tissues confirmed the good correlation of PCR and the poor correlation of MAT with mouse inoculation.

These results indicate that vertical transmission in *Apodemus sylvaticus* and *Mus musculus* is extremely efficient and probably endures for the life of the breeding female. This mechanism favours parasite transmission and dispersion by providing a potential reservoir of infection in hosts predated by the cat.

Toxoplasma gondii is an obligate intracellular protozoan parasite with a world-wide distribution which is capable of infecting all warm-blooded animals. Owing to its zoonotic potential, it is of both medical and veterinary importance. Felids are the only known definitive hosts for *Toxoplasma*, in which infection leads to sexual replication in intestinal epithelial cells, resulting in the shedding of millions of oocysts (Dubey & Frenkel 1970). Infection in other hosts, including rodents leads to disseminated infection with multiplication of *T. gondii* in various body tissues, leading to the formation of tissue cysts which can remain viable throughout life (Beverley, 1976). Such chronically infected animals become a potential source of infection for cats by carnivorism. Chronically infected laboratory mice (Swiss and NIH strains) have been shown to produce congenitally infected offspring repeatedly in successive litters (Beverley, 1959; Remington *et al.* 1961). However, both the parasite and host genotype can substantially affect the biology of *T. gondii* (Remington *et al.* 1961; Roberts & Alexander 1992). Congenital transmission has not been investigated in wild rodents, other than *Rattus norvegicus* (Webster, 1994), although it is thought to be the most significant route of *Toxoplasma* transmission in wild *Apodemus sylvaticus* (Wood mouse) and *Clethrionomys glareolus* (Field vole) (Jackson, Hutchison & Siim, 1986).

The purpose of this study was to investigate the vertical transmission of *T. gondii* in captive *Mus musculus, A. sylvaticus* and *C. glareolus* and hence determine its role in the epidemiology of toxoplasmosis using the polymerase chain reaction (PCR) to specifically detect *Toxoplasma* infection in tissues after experimental infections.

MATERIALS AND METHODS

Serology

Detection of *Toxoplasma* specific antibodies was performed using the modified agglutination test (MAT) (Biomerieux, Marcy L' Etoile, France), which correlates well with the dye test (Dubey *et al.* 1995a). Sera were assayed using a screening dilution of 1/40 and also at 1/4000 to prevent the false negative results which can occur at low dilutions using sera with high antibody titres. Detection of *Toxoplasma* specific antibodies at either screening dilution was taken as evidence of infection.

T. gondii oocysts

T. gondii oocysts were obtained by the oral infection of a seronegative cat with approximately 200 *T. gondii* tissue cysts of the M1 strain (Buxton, Reid & Pow, 1979), in mouse brain homogenate. The cat was orally dosed each day with 2ml of lactulose to prevent the constipation frequently encountered when cats are infected with *T. gondii* and faeces were collected from days one to fourteen post-infection Oocysts were recovered

by flotation in saturated sodium chloride solution, and allowed to sporulate in 2% H_2SO_4 for 4 days at room temperature, then stored in 2% H_2SO_4 at 4°C.

Establishment of chronic infections

Prior testing of sera from breeding colonies of *Apodemus sylvaticus* and *C. glareolus* (supplied by the Royal Liverpool Hospital Animal Unit, UK) and from *M. musculus* (gift from A. Brigham, Rentokil Ltd, East Grinstead, UK), did not detect *Toxoplasma* antibody confirming the absence of toxoplasmosis in the breeding colonies of the three species investigated. Preliminary dose titration experiments were conducted to determine an appropriate infective dose of oocysts which was sublethal but established chronic infection. For each species, groups of 10 females of three to six weeks of age were infected with 25, 50, 100, or 200 sporulated *T. gondii* oocysts by stomach tube. For *C. glareolus*, two further groups of females were dosed with 5 and 10 oocysts. Three weeks post infection, surviving animals were tested for *T. gondii* antibody. Animals without detectable antibody were re-infected, and only recruited into experimental groups upon detection of specific antibody.

Investigation of vertical transmission of T. gondii

Six weeks post infection, seropositive females were paired with males for mating except for *Mus*, in which three females were housed with one male until confirmed pregnant, then females were housed separately to rear their

young. All offspring were weaned at three weeks of age, and killed by exsanguination under isofluothane anaesthesia at six weeks of age. Offspring sera were tested for *T. gondii* antibodies and their whole brains were examined for *T. gondii* by nested PCR directed towards the *T. gondii* specific B1 gene.

PCR

Brains were removed under sterile conditions with single use, sterile scalpel blades and cocktail sticks. Whole brains were homogenised, using a descending needle series (19-23 gauge), and then digested in 1ml digestion buffer (50mM Tris (Sigma Chemical Co., Poole, Dorset, UK), 1mM EDTA (Sigma), 0.5% Tween 20 (BDH Lab Supplies, Poole, Dorset, UK), 200μg/ml proteinase K, (Boehringer Mannheim, UK), pH 8.5) at 55°C for 2 hours. Digested samples were boiled for 8 minutes to inactivate the proteinase and coagulate proteins. Samples were centrifuged for 30 seconds at 11500g and 5μl of supernatent fluid was used in the first of a nested PCR. PCRs were conducted in 100μl of reaction mixture consisting of 20mM $(NH_4)_2SO_4$, 75mM Tris HCl, pH 9.0, 0.01% Tween 20 (w/v), 2.5mM $MgCl_2$, 0.1mM d-NTPs (Advanced Biotechnologies, Leatherhead, Surrey, UK), 0.2μM each primer (Severn Biotech Ltd, Kidderminster, Worcestershire, UK), 0.3 units Red Hot Taq polymerase (Advanced Biotechnologies). PCR was directed toward the B1 gene, (Burg *et al.* 1989), using primers and thermal profile as described by Wastling *et al.* 1993, except that the denaturing, annealing and extension times used were each 1 minute. In the

first PCR, amplification was performed over 25 cycles of 93°C, 50°C and 72°C, each for one minute with primers GGAACTGCATCCGTTCATGAG and TCTTTAAAGCGTTCGTTCGTGGTC to produce a 193 base pair product. Following a successful first round of PCR, 1μl of each reaction was used in a second PCR, with a new primer pair, TGCATAGGTTGCAGTCACTG and GGCGACCAATCTGCGAATACACC, over fifteen cycles of the same thermal profile to produce a 94 base pair amplicon. PCR products were visualised after electrophoresis on ethidium bromide stained 2% agarose gels, trans-illuminated with ultra-violet light. Distilled water and non-infected brains were used as negative controls and were run with tests to monitor for cross-contamination. Disposable gloves and single-use plastic wear were used to prevent contamination of tests.

Sensitivity and Specificity of B1 PCR

T. gondii tachyzoites, grown in Vero cells, were isolated by scraping the cell monolayer with a rubber policeman and homogenizing through a descending series of needles (19-25 gauge). The cell suspension was washed three times with phosphate buffered saline (PBS), pH 7.2, then eluted through a G25 sepharose minicolumn (Pharmacia Biotech, UK). Mock infected brains, containing from 10^0-10^6 parasites, created by adding purified tachyzoites to brains from *Toxoplasma*-free mice prior to homogenisation and digestion, were examined for *T. gondii* by B1 PCR. *Neospora caninum* DNA was prepared by standard methods from *in vitro* cultured tachyzoites of *N. caninum*, Liverpool isolate (Barber *et al.* 1995).

Bioassay of brains from offspring of chronically infected Mus

Brains of twelve offspring from 6 *Mus*, considered chronically infected with *T. gondii* (detection of specific antibody, following oral infection with oocysts), were tested by bioassay in susceptible Balb/c mice. Whole brains were removed under sterile conditions and sagitally sectioned. One half of each brain was tested for *T. gondii* infection by PCR (as above) and the other half by bioassay, as follows. Using a descending series (19-23 gauge) of hypodermic needles, half brains were homogenised in sterile phosphate buffered saline (PBS), pH 7.2, supplemented with 100 i.u./ml penicillin G and 100μg/ml dihydrostreptomycin (Gibco, Paisley, UK). For all samples, each of two Balb/c mice were inoculated, by intraperitoneal injection, with 0.5mls of brain homogenate. Mice were tested for *Toxoplasma* specific antibody 28 days post injection and tissues were considered infected with *T. gondii* if either mouse developed specific antibody.

Statistical methods

Data were analysed by Student's *t* test or chi-square test with Yates' correction where necessary.

Oral infections with T. gondii oocysts

Following a single oral infection with 50 oocysts, five of nine surviving *Mus* and *Apodemus* females produced *Toxoplasma* specific antibody, confirming infection. Higher doses of oocysts increased the proportion of seroconversion in survivors but were associated with unacceptable increases in morbidity and mortality. For *Clethrionomys*, oral infection with 50-200 oocysts resulted in 100% mortality. Smaller doses of 5-25 oocysts resulted in increased survival rate but there was no serological evidence of infection at 28 days post-infection (Table 1). Accordingly, without ante-mortem evidence of infection, *Clethrionomys* was not entered into the vertical transmission experiments. However, post-mortem PCR examination of brain tissue revealed infection in 11 of 15 survivors. Results from this preliminary experiment suggested that the optimum oocyst dose for inducing chronic infection in *Mus* and *Apodemus* was 50 oocysts and this dose was used throughout the vertical transmission experiments. After the vertical transmission experiments, breeding *Mus* and *Apodemus* females were killed and their brains were examined for *T. gondii*, by PCR. *T. gondii* was detected in the brains of all breeding females (data not shown), confirming chronic infection.

B1 PCR-sensitivity and specificity

DNA from as few as 10 *T. gondii* tachyzoites in a mock-infected mouse brain digest could be detected by ultra-violet light trans-illumination of the electrophoresed PCR product on ethidium bromide stained 2% agarose gels. PCR positive results for *T. gondii* were not obtained with either non-infected mouse brain or *Neospora caninum* DNA.

Vertical transmission

Ten *Apodemus* and 23 *Mus musculus,* with chronic *T. gondii* infections, were initially bred and a reducing number were subsequently bred up to six times for *Apodemus* and twice for *Mus*, producing a total number of pups which survived to six weeks of age of 83 and 207, respectively. The mean size of litters at weaning was 3.77 (+/- 0.48 s.e.) for *Apodemus* and 6.9 (+/- 0.46 s.e.) for *Mus*. Cannibalism of neonates reduced the total number of litters reared and the mean litter size at weaning for *Apodemus*. The frequencies of vertical transmission of *T. gondii* in *Mus* and *Apodemus* as determined by serology and by PCR for first litters, second litters, and for *Apodemus,* for subsequent litters, are given in Tables 2 and 3. Typical PCR results are shown in Figure 1.

Detection of vertical transmission of T. gondii in Mus. by bioassay

PCR was as sensitive as MI in detecting infection in the offspring of infected dams, but the detection of antibody by MAT did not reflect infection status (Table 4).

Table 1. Outcome of oral infection of wild rodent species[a] with *Toxoplasma gondii* oocysts.

No. of oocysts	*Mus musculus*		*Apodemus sylvaticus*		*Clethrionomys glareolus*	
	No. died[b]	No. antibody positive[c]	No. died	No. antibody positive	No. died	No. antibody positive
5	ND[d]		ND		5	0
10	ND		ND		5	0
25	0	2	0	3	5	0
50	1	5	1	5	10	0
100	3	5	3	5	10	0
200	6	4	4	5	10	0

[a] 10 animals per group

[b] by 21 days post-infection

[c] at 21 days post infection in survivors

[d] ND = not done

Table 2. Vertical transmission of *Toxoplasma gondii* in 10 chronically infected *Apodemus sylvaticus*.

	1st litters			2nd litters			3rd and further litters		
Variable	No.	MAT	PCR	No.	MAT	PCR	No.	MAT	PCR
Proportion of litters	10	1.0	0.9	6	1.0	0.83	6	1.0	1.0
Mean (s.e.) proportion of each litter	10	1.0	0.75 (+/-0.24)	6	1.0	0.77 (+/-0.17)	6	1.0	0.81 (+/- 0.03)
Proportion of all pups	26	1.0	0.73	27	1.0	0.85	30	1.0	0.80

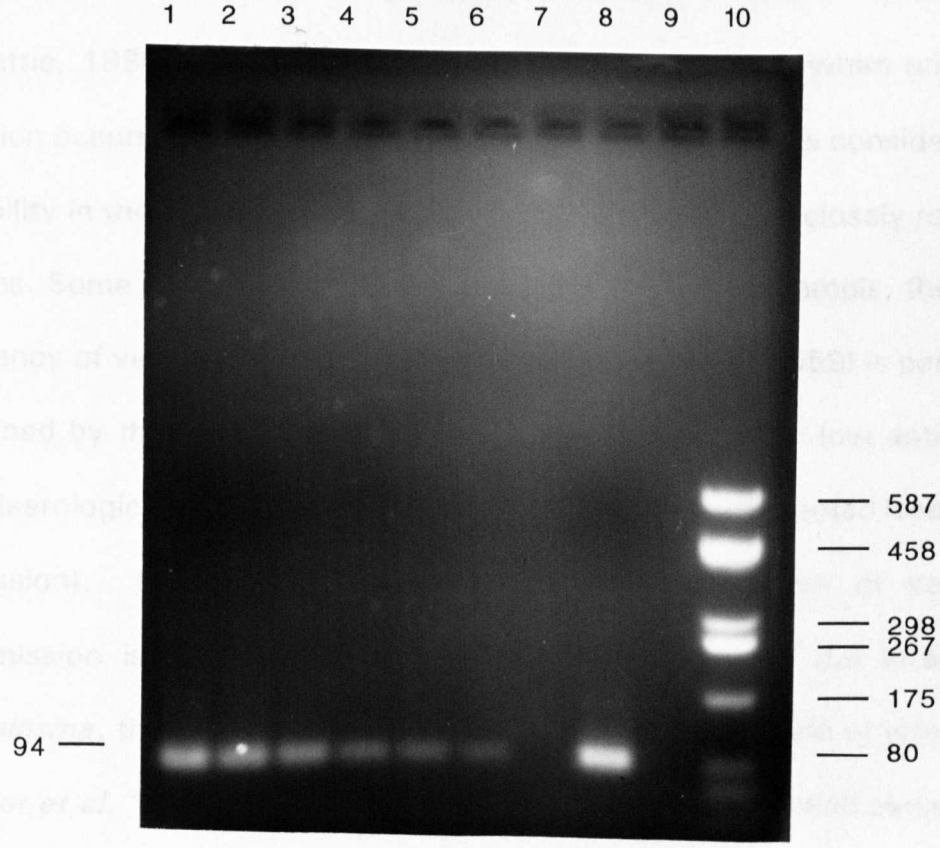
Table 3. Vertical transmission of *Toxoplasma gondii* in 23 chronically infected *Mus musculus.*

Variable	1st litters			2nd litters		
	No.	MAT	PCR	No.	MAT	PCR
Proportion of litters	23	0.91	1.0	7	0.86	1.0
Mean (s.e.) proportion of each litter	23	0.63 (+/- 0.07)	0.89 (+/- 0.04)	7	0.48 (+/- 0.14)	0.78 (+/- 0.30)
Proportion of all pups	165	0.65	0.90	42	0.62	0.88

Table 4. Detection of *Toxoplasma gondii* infection in *Mus* offspring - comparison of the modified agglutination test (MAT) and polymerase chain reaction (PCR) amplification of the B1 gene with mouse inoculation (MI).

MAT Result	No. offspring	Detection of *T. gondii*	
		MI	PCR
Positive	5	4/5	5/5
Negative	7	5/7	5/7

Figure 1. Detection of *Toxoplasma gondii* infection in offspring from chronically infected *Mus musculus*. Amplification products from PCRs on brain digests, from chronically infected dam (lane 1), offspring (lanes 2-6), uninfected mouse (lane 7), 1ng *T. gondii* DNA (lane 8) and from distilled water (lane 9) were analysed by electrophoresis on ethidium bromide stained 2% agarose and photographed under ultra-violet light. The diagnostic amplification product from *T. gondii* specific PCR (94bp) is indicated. Lane 10 contains molecular weight markers.



This study, using detection of parasite DNA in brain as evidence of infection, demonstrates the efficient nature of the vertical transmission of *T. gondii* from chronically infected *Apodemus* and *Mus* which probably persists for the life of the breeding female. This consistently high rate of transmission is substantially higher than the lower rates of transmission observed in previous studies using Swiss and NIH strains of laboratory mice (Beverley, 1959; Remington, Jacobs & Melton, 1961), and hamsters (De Roever-Bonnet, 1969) and is in contrast to the complete lack of transmission observed from chronically infected Balb/c and Balb/K strain mice (Roberts & Alexander, 1992), sheep (Beverley, 1976) and man (Dubey & Beattie, 1988) in all of which transmission occurs only when primary infection occurs during pregnancy. It is apparent that there is considerable variability in the occurrence of vertical transmission even in closely related species. Some of this is related to the criteria used - for example, the low frequency of vertical transmission observed by Beverley (1959) is partially explained by the erroneous assumption that offspring with low antibody titres (serological test not specified) were considered non-infected (see later discussion). Nonetheless, it is apparent that the nature of vertical transmission is also determined by the host genotype, the strain of *Toxoplasma*, the life-cycle stage used to infect and the route of infection (Zenner *et al.* 1993). In this study, oocyst infection was used since it is reasonable to assume that this may occur naturally and quite frequently as

rodents feed within an oocyst contaminated environment. In rats, it has been shown that of different stages and routes, oral infection results in the highest frequency of vertical transmission (Dubey & Shen, 1991). The usefulness of PCR to investigate vertical transmission and the limitations of serology, discussed below, indicate that the accepted dogma that, for example, chronically infected sheep do not infect their offspring may merit re-appraisal and re-investigation.

Vertical transmission can occur both *in utero* and by the transmammary route (Eichenwold, 1948), and whilst the former route is likely to be of greater significance, both routes of transmission were possible and in these experiments, we have made no attempt to distinguish between them. The high frequency of transmission observed in wild rodents in this experiment could maintain a reservoir of infection of *T. gondii* in the environment (Beverley, 1976), capable of infecting cats and hence the dissemination of toxoplasmosis to animals and man. However, the antibody prevalence reported in wild rodents ((Hay *et al.* 1983; Jackson, Hutchison & Siim, 1986; Smith & Frenkel 1995; Dubey *et al.* 1995b) is much lower than might be expected from the results presented. *T. gondii*- infected rodents are more susceptible to predation (Hay, *et al.* 1983) and in experimental studies, have smaller litters (Beverley 1959), hence in nature, the prevalence of infection will be considerably lower than that which can be achieved by experimental infections in laboratory breeding colonies.

For laboratory mice, the duration of maternally derived antibody is sixteen days (Weir, 1978), hence *Toxoplasma* specific antibody detected in *Mus* and *Apodemus* offspring 21 days after weaning probablyindicates infection. For *Apodemus*, the MAT is a sensitive and reliable indicator of infection in offspring following vertical transmission, with all offspring from chronically infected dams, including some which tested negative by PCR, having detectable *Toxoplasma* specific antibody at a serum dilution of 1/40 and most having a titre of 1/4000 at six weeks of age (data not shown). Whilst there are no published details on the sensitivity and specificity of the MAT in mice, we have tested hundreds of mice of several laboratory strains during the passage of *T. gondii* and following oral infection with oocysts and we have found the MAT to be a reliable indicator of infection in these laboratory strains. Furthermore, Dubey Thulliez & Powell (1995a) consider the MAT a sensitive test for *Toxoplasma* infection in Swiss-Webster mice at a serum dilution of 1/50. However, in our hands, the MAT was not a reliable test for detection of vertically acquired infection in *Mus*, when compared to parasite detection by PCR. Dubey *et al.* (1995a), have also found false negative results for *Toxoplasma* infection, by MAT, in wild *Mus* and *Peromyscus* species, even using a lower screening dilution of 1/25. Suzuki & Kobayashi (1990) suggest that the antibody responsiveness of young mice born to chronically infected dams may be compromised, with some individuals exhibiting tolerance to *Toxoplasma* infection. The limitations of the MAT in detecting antibodies in experimentally infected *Mus*, in which infections were confirmed by PCR, suggests that whilst the

MAT is useful in laboratory strains of mice, it is not a reliable test for diagnosis nor epidemiological study of toxoplasmosis in wild mice. Whilst the MAT was a poor predictor of vertical transmission for *Mus*, PCR results showed good agreement with bioassay in the small group of *Mus* offspring tested and this same PCR protocol is as sensitive as mouse inoculation in infected ovine tissues (Owen *et al*, (submitted,b)). It is apparent that serological examination alone should not be relied upon as definitive evidence of vertical transmission of *Toxoplasma* infection in these rodents and PCR offers a more reliable alternative. It is not known if similar disparities between serological and parasitological detection exist for other rodent species. However, our results suggest that caution needs to be applied before the determination of vertical transmission of *T. gondii* in rodents is established by serological investigation alone.

ACKNOWLEDGEMENTS

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MANUSCRIPT IV

OVINE TOXOPLASMOSIS: THE INCIDENCE OF INFECTION IN GRAZING SHEEP INDICATES PASTURE CONTAMINATION WITH *TOXOPLASMA GONDII* OOCYSTS

M. R. OWEN, M. J. CLARKSON* and A. J. TREES

Veterinary Parasitology, Liverpool School of Tropical Medicine/Faculty of Veterinary Science, University of Liverpool, Pembroke Place, Liverpool. L3 5QA.

*Veterinary Clinical Science and Animal Husbandry, Veterinary Teaching Hospital, Leahurst, University of Liverpool, Chester High Road, Neston, S. Wirral. L64 7TE.

ABSTRACT

The incidence of *Toxoplasma gondii* infection in sheep on three farms, over a period of 9-18 months, was determined by the regular prospective determination of antibody prevalence, using the latex agglutination test (LAT), in cohorts of initially sero-negative animals. *T. gondii* antibody prevalence increased throughout the periods studied for all farms, although the incidence of infection varied both between farms and for different calendar periods on any one farm. On all farms, the *de novo* detection of *T. gondii* antibody in previously antibody-negative sheep, whilst grazing pasture without supplementary feed sources indicates acquisition of infection by ingestion of oocysts contaminating pasture and deposited there directly by cats. These observations demonstrate the degree of environmental contamination with *Toxoplasma* oocysts due to cats, show the impossibility of physically preventing ovine infections by management means and help explain the unpredictable nature of infection.

Toxoplasma gondii is a significant cause of abortion and disease in sheep both in the UK (Blewett and Watson 1984) and world-wide (Dubey and Towle) 1986. Ministry of Agriculture VIDA figures for 1995 indicate that *Toxoplasma* accounts for approximately one quarter of diagnosed ovine abortion incidents and seroprevalence studies indicate that in some flocks, over 90% of sheep are exposed to infection (Watson and Beverley 1971). Modelling of serological data (Blewett and Watson 1984) and clinical case studies suggest that sheep are predominantly infected by exposure to and ingestion of oocysts either disseminated in stored feed (Plant and others 1974; Dubey and others 1990), or from pasture accidentally contaminated by distribution of manure from winter-housing, containing infected cat faeces (Faull and others 1986). *Toxoplasma* induced abortions generally follow infections which occur during mid gestation (Dubey and others 1986). In the UK, where most sheep are mated in the autumn, mid-gestation for many flocks falls before the end of December. Until this time, sheep are kept at grass, generally with no supplementary source of nutrition, suggesting that many *Toxoplasma* abortions result from the ingestion of oocysts at pasture.

The purpose of this study was to investigate the incidence of ovine toxoplasmosis, by serial antibody assays of cohorts of susceptible sheep and hence to establish whether grazing sheep, not receiving stored feed

supplements, are infected by *Toxoplasma* oocysts from direct contamination of pasture with infected cat faeces.

MATERIALS AND METHODS

Selection of farms and collection of serum

Farms, which had cases of *Toxoplasma*-induced ovine abortion confirmed (antibody-positive foetal fluids and/or detection of *T. gondii* antigen by direct antibody fluorescence test (FAT)) in the previous lambing season, were investigated for evidence of continued acquisition of infections. On each farm, 50-100 tagged ewe-lambs were blood sampled by jugular venepuncture and tested for *Toxoplasma* specific antibody. Four to six weeks later, repeat samples were taken and retested for *T. gondii* antibody. Farms on which there was an increase in seroprevalence to *T. gondii* were recruited. For three farms selected for study, repeat serum sampling and testing was performed approximately every two months, as farm management allowed. Cohorts of between 70 and 100 animals were sampled throughout the study period. For farm B sheep, some new animals were recruited mid-study to increase the number of sentinels. Detailed records were kept of pastures grazed and the use of supplementary stored feed throughout the study period. On farm A, two cohorts of sheep were studied: group 1 from December 93 to July 94; group B from August 94 to August 95. A single cohort of sheep was studied on each of farms B and C. On all farms, sheep were housed by night from January until lambing,

with most ewes lambing in March at which time they were turned out to pasture. During winter-housing, weather permitting, by day, sheep grazed on local pasture in addition to receiving supplementary stored feed. Ewes continued to receive stored feed for 3-4 weeks after turn-out on farms B and C. On farm A, supplementary feed consisted of silage only and was only used during the housing period. Sheep on farm B were fed hay and a home prepared coarse mix, whilst sheep on farm C were fed hay and a commercially prepared concentrate, which was stored on the farm in sealed bags. Lambs were fed commercially prepared, bagged creep feed on all farms. Deep litter bedding from last seasons winter-housing was not spread onto pastures grazed by sheep on any farms. On farm A, no ewe received any food other than grass from mid-April until the end of the periods studied. On farm B ewes received grass only from April until the end of January, when they were housed. Ewes ate only grass from April to the end of the study period on farm C.

Serology

Whole blood was obtained by jugular venepuncture in Vacutainers (Becton Dickinson,UK), allowed to clot at room temperature for at least one hour, and serum was separated following centrifugation at 3000g for 10 minutes. Sera were examined for *Toxoplasma* specific antibody using the latex agglutination test (LAT) (Eiken Chemical Company, Japan), (Trees and others 1989), in round-bottomed 96-well plates (Serowell, Bibby Sterilin Ltd, UK) using a screening dilution of 1/64. All serum titrations were

conducted using a single batch of test kits to eliminate artifactual variation in results, following storage of sera at -20°C. Infection was considered to have occurred in previously antibody-negative, sheep (titre of less than 1/64) when a minimum of an eight-fold rise in antibody titre was detected in at least one subsequent serological test (Blewett and others 1983).

RESULTS

On all farms, *Toxoplasma* infections in sheep occurred throughout the periods of investigation (Tables 1-4 and Figures 1-4). Farms A (1993-94 and 1994-95) and B both show substantial increases in *Toxoplasma* antibody prevalence during spring and summer whilst sheep were at pasture and fed only grass. On farm C (Table 4 and Figure 4), *Toxoplasma* antibody prevalence was 60% at the first sampling prior to housing. Further *Toxoplasma* infections occurred during winter housing and in the period whilst sheep were grazing.

Table 1. Farm A 1993-1994 (Group 1). Seroprevalence and incidence of infection with *Toxoplasma gondii* in 73 ewe-lambs, detected using the latex agglutination test (LAT).

Date	Cumulative prevalence (%)	Interval seroconversions	
		No.	%
12/93	35.6	-	-
1/94	37.0	1	2.1
3/94	42.5	4	8.7
5/94	63.0	16	38.1
7/94	68.5	4	17.4

Table 2. Farm A 1994-1995 (Group 2). Seroprevalence and incidence of infection with *Toxoplasma gondii* in 70 ewe-lambs, detected using the latex agglutination test (LAT).

Date	Cumulative prevalence (%)	Interval seroconversions	
		No.	%
8/94	2.86	-	-
10/94	4.30	1	1.47
12/94	5.70	1	1.49
1/95	11.4	4	6.06
3/95	21.4	7	11.3
6/95	27.1	4	7.27
8/95	43.1	6	15.4

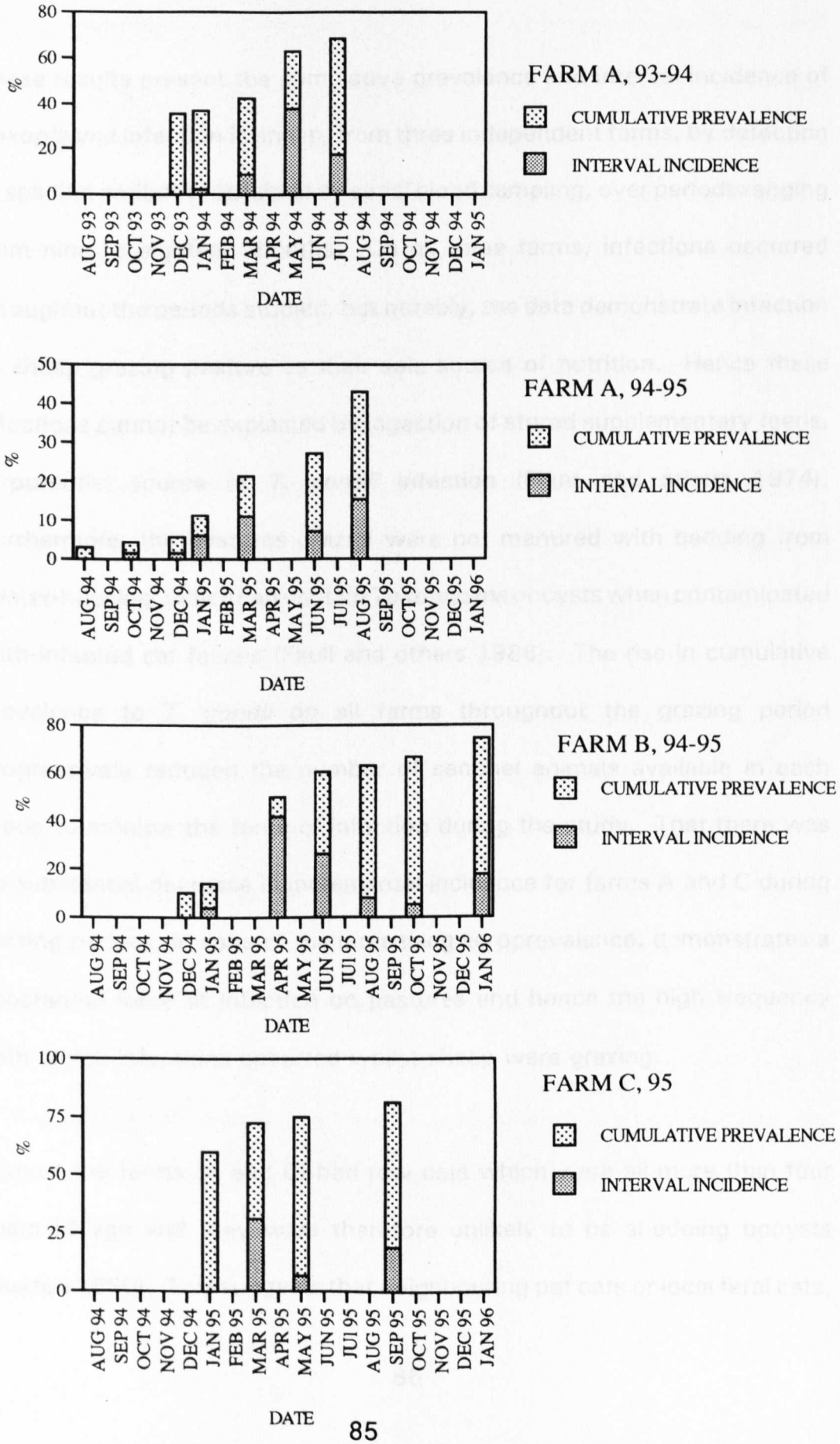
Table 3. Farm B. Seroprevalence and incidence of infection with *Toxoplasma gondii* in 158 ewe-lambs, detected using the latex agglutination test (LAT).

Date	Cumulative prevalence (%)	Interval seroconversions	
		No.	%
12/94	10	-	-
1/95	14	4	4
4/95	50	28	41.8
6/95	60.2	11	26.2
8/95	63.0	5	7.94
10/95	66.7	3	5.26
1/96	74.5	7	17.5

Table 4. Farm C. Seroprevalence and incidence of infection with *Toxoplasma gondii* in 80 ewe-lambs, detected using the latex agglutination test (LAT).

Date	Cumulative prevalence (%)	Interval seroconversions	
		No.	%
1/95	60	-	-
3/95	72.5	10	31.3
5/95	75	2	6.7
9/95	81.5	6	19.4

Figure 1. The cumulative prevalence and interval incidence of ovine toxoplasmosis for four cohorts of ewe-lambs (n) on three farms (A, B and C). Farm A, 93-94 n=73,94-95 n=72; farm B, n=158; farm C n=80.



These results present the cumulative prevalence and interval incidence of *Toxoplasma* infection in sheep, from three independent farms, by detection of specific antibody, obtained by serial blood sampling, over periods ranging from nine to eighteen months. On all three farms, infections occurred throughout the periods studied, but notably, the data demonstrate infection of sheep grazing pasture as their sole source of nutrition. Hence these infections cannot be explained by ingestion of stored supplementary feeds, a potential source of *T. gondii* infection (Plant and others 1974). Furthermore, the pastures grazed were not manured with bedding from winter-housing, which can contain *Toxoplasma* oocysts when contaminated with infected cat faeces (Faull and others 1986). The rise in cumulative prevalence to *T. gondii* on all farms throughout the grazing period progressively reduced the number of sentinel animals available in each group to monitor the force of infection during the study. That there was no substantial decrease in incremental incidence for farms A and C during grazing periods, in spite of the increasing seroprevalence, demonstrates a substantial force of infection on pastures and hence the high frequency with which infections occurred whilst sheep were grazing.

Two of the farms (A and C) had few cats which were all more than four years of age and they were therefore unlikely to be shedding oocysts (Buxton 1990). This suggests that neighbouring pet cats or local feral cats,

the latter of which can have territories of 60-80 hectares (Macdonald 1980), might have been responsible for pasture contamination. Millions of infective oocysts are contained within each stool produced by a cat during a primary *Toxoplasma* infection (Dubey 1976), and ewes can become infected by the ingestion of as few as 20 oocysts (Buxton and others 1991). Whilst experimental evidence suggests that oocysts can be transported from stools and spread around the environment, for example, by birds, or flies (Wallace 1971), this may be unnecessary for infection to occur in many sheep in an oocyst contaminated pasture. Whilst sheep prefer to ingest certain pasture plants, many factors influence the regions of pasture grazed (Veterinary Laboratories Agency 1995). When grass is limited in supply, sheep nibble grass very close to the ground (Waldeland 1977) and even consume herbage contaminated with faeces, when necessary (Fraser and Broom 1990). Even on good pasture land, sheep travel at least one kilometre per day and possibly as a result of allelomimetic behaviour, they often form specific paths between patches of preferred herbage and water sources (Fraser and Broom 1990). In a flock, sheep often form subgroups that follow a routine of movement around the pasture. Limited grass availability encourages any one individual to sample all of a pasture over a period of time, including any small areas of herbage contaminated with infected cat faeces, consequently it is not necessary for oocysts to be widely dispersed for many sheep to become infected.

The finding of *Toxoplasma*-infected wild rodents on Farm A (*Apodemus sylvaticus* and *Clethrionomys glareolus*) as part of a separate study (Owen and others (submitted, b)), demonstrates the existence of the environmental reservoir of infection for *Toxoplasma*-susceptible cats. The longevity of oocysts in the environment (Waldeland 1977) which can remain viable for up to 18 months (Frenkel and others 1975), potentially enables oocysts from the infection of a single cat to cause ovine toxoplasmosis and hence abortions, in two successive lambing seasons. In some species of wild rodents, *Toxoplasma* infection can be transmitted vertically (Owen and Trees (submitted, a), hence it is likely that there will constantly be a proportion of the wild rodent population infected with *T. gondii.* Since cats only shed millions of oocysts during a primary infection, after which they develop immunity and tend not to re-shed oocysts (Dubey and Beattie 1988), the frequency of infection in *Toxoplasma*-susceptible cats and subsequent oocyst dissemination is a function of the local cat population and will tend to be sporadic in areas with small cat populations. Accordingly, ovine toxoplasmosis tends to be a sporadic disease, occurring when ewes are exposed to oocysts for the first time during pregnancy. Sporadic and unpredictable exposure of sheep to oocysts, even on farms with recurrent cases of ovine toxoplasmosis, demonstrates that it is not possible to naturally infect, hence immunize, ewes prior to mating, to avoid *Toxoplasma* abortions. Furthermore, the contamination of pasture with oocysts has implications for *Toxoplasma* control programmes utilising oral anti-coccidial drugs (Blewett and Trees 1987, Buxton and others 1988),

since for effective prevention of toxoplasmosis, ewes would need medication throughout pregnancy. The presumed oocyst contamination of pasture by non-resident cats suggests that attempts to reduce the exposure of sheep to oocysts by reducing farm cat populations (Buxton 1990) and preventing cat access to sheep housing and feed (Dubey and others 1990), will not necessarily reduce the number of *Toxoplasma* infections or abortions in sheep. Currently, the most reliable prevention of ovine *Toxoplasma* abortion is by vaccination with the live, incomplete, attenuated S48 strain before ewes are mated (Buxton and others 1991).

Toxoplasma oocysts are only produced by cats and for natural abortions to occur, sheep must be infected with oocysts. However, there are a number of ways in which sheep can be exposed to oocysts - through contaminated conserved feed such as hay or grain, through contaminated bedding subsequently spread on pasture and as these data now clearly show, directly from contaminated pasture. This latter illustrates the degree of environmental contamination with *Toxoplasma* due to cats, emphasizes the impossibility of physically preventing infection by management means and helps explain the unpredictable nature of exposure to infection.

ACKNOWLEDGEMENTS

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MANUSCRIPT V

LINEAGE TYPES OF *TOXOPLASMA GONDII* INFECTING SHEEP IN THE UK DETERMINED BY DIRECT PCR-RFLP FROM TISSUES

M. R. OWEN[a], D. K. HOWE[b], L. D. SIBLEY[b], M. J. CLARKSON[c] and A. J. TREES[a]

[a] Veterinary Parasitology, Liverpool School of Tropical Medicine/Faculty of Veterinary Science, University of Liverpool, Pembroke Place, Liverpool. L3 5QA.

[b] Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri, USA.

[c] Veterinary Clinical Science and Animal Husbandry, Veterinary Teaching Hospital, Leahurst, University of Liverpool, Chester High Road, Neston, S. Wirral. L64 7TE.

ABSTRACT

Genotypes of *Toxoplasma gondii* were examined in naturally infected tissues from 13 ovine abortions from 10 separate farms in North England and Wales, from 2 wild rodents captured from one farm and from two infected, clinically healthy lambs from a local abattoir by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) studies of the polymorphic surface antigen II (SAG2) gene. Tissues were processed for PCR using a simple protocol which does not require parasite isolation or DNA extraction. All tissues examined were infected with *T. gondii* of the type II lineage. These results demonstrate the feasibility of direct analysis of *T. gondii* lineages from either clinical samples or non-clinically infected tissues.

The populations of several protozoan parasites appear to be made up of a limited number of genetically similar lineages (Tibayrenc *et al.* 1990), each propagated by a predominance of asexual replication. Consequently, for a number of protozoa, certain genotypes exist which can often be associated with particular hosts and diseases (Tibayrenc, 1993). The protozoan, *T. gondii* is an obligate intracellular parasite with a global distribution which infects a wide range of warm-blooded hosts including man (Buxton, 1990). Whilst sexual replication, which leads to oocyst production, occurs exclusively in the intestinal epithelial cells of felids (Dubey and Frenkel, 1972), the parasite also replicates asexually in intermediate hosts. Transmission of *T. gondii* occurs when susceptible hosts ingest oocysts shed in cat faeces or by ingestion of *T. gondii* tissue cysts in chronically infected tissues (Beverley, 1976), and in some cases by vertical transmission (Beverley, 1959, De Roever-Bonnet, 1969). Despite the abundance of recognised strains of *Toxoplasma*, which differ in certain biological properties, they are all similar antigenically and morphologically and hence a single species is recognised (Sibley *et al.* 1992). Recently, within this species, three genetically distinct clonal lineages (I, II and III), have been described (Howe and Sibley, 1995), with one lineage apparently comprising all of the isolates which are virulent in mice (Sibley and Boothroyd, 1992).

In humans, the seroprevalence of *T. gondii* infection is high, and may rise to 90% in some geographical areas (Dubey and Beattie, 1988, Chapter 2, p41), but for most individuals, infection is mild or asymptomatic. However, cases of clinical human toxoplasmosis appear to be associated with certain lineages of *T. gondii* (Howe and Sibley, 1995).

Sheep are particularly susceptible to *T. gondii* infection and ovine toxoplasmosis is common world-wide, with prevalences as high as 100% reported for some flocks (Dubey and Kirkbride, 1984). Most infections follow the ingestion of oocysts (Blewett and Watson, 1984), with as few as 20 oocysts constituting an infective dose (Buxton *et al.* 1991), sufficient to induce a chronic infection and the formation of tissue cysts. Since most infected sheep do not develop clinical signs they are routinely killed for human consumption, hence many human infections will be acquired through ingestion of under-cooked infected lamb meat. Furthermore, in addition to its zoonotic significance, ovine toxoplasmosis causes significant disease in sheep, including abortion and congenital infection of lambs (Buxton, 1990).

Whilst an association between *T. gondii* types with clinical disease in human toxoplasmosis has been made, the identity of *T. gondii* lineages infecting sheep and any relationship between parasite genotype and ovine disease have yet to be determined. The purpose of this study was to apply polymerase chain reaction amplification of the polymorphic *T. gondii*

surface antigen II (SAG2) gene, with restriction fragment length polymorphism (PCR-RFLP) studies, using a simple protocol of tissue preparation for PCR, to investigate the strain types of *T. gondii* in infected animal tissues.

MATERIALS AND METHODS

Collection of T. gondii infected tissues

T. gondii infected abortion tissues (placental cotyledon, foetal brain, liver and lung), were collected from farms with a previous history of *Toxoplasma* abortions and from aborted foetuses submitted to the Veterinary Investigation (VI) Service (Preston and Shrewsbury, UK). *T. gondii* infection in aborted foetuses was confirmed by *T. gondii* B1 gene PCR using crude tissue digests (see below) and in some cases by direct fluorescence antibody test and/or by antibody detection in foetal fluid. Wild rodents (*Apodemus sylvaticus* and *Clethrionomys glareolus)* were trapped, using baited Longworth traps, on a sheep farm in North Wales on which *T. gondii* ovine abortions occur and tested for *T. gondii* infection by detection of *T. gondii* specific antibody. Infections were confirmed by B1 gene PCR performed on crude brain digests. Hearts from slaughtered lambs were collected from a local abattoir. Potentially *Toxoplasma*-infected hearts were identified by the detection of specific antibody in serum from heart cavities. *T. gondii* from each infected heart was isolated into two susceptible Balb/c mice as previously described (Dubey *et al.* 1995). Successful isolation of

T. gondii into recipient mice was confirmed by the detection *of Toxoplasma* antibody eight weeks post-inoculation. Whole brains from mice with detectable antibody were used for PCR.

Serology

Foetal fluid from all aborted lambs, rodent sera and sera from abattoir lamb hearts were tested for *Toxoplasma* specific antibody with the modified agglutination assay (MAT) (Biomerieux, Marcy L' Etoile, France) using screening dilutions of 1/40 and a higher dilution of 1/4000 to avoid false negative results obtained sera with high levels of antibody are tested at 1/40 dilutions. Foetal fluids from VI centres were tested for *T. gondii* antibodies by the latex agglutination test (LAT) (Eiken Chemical Company, Japan) at a screening dilution of 1/64.

*Preparation of control T. gondii DNA*s

RH strain tachyzoites were maintained by serial passage every three days in Balb/c mice. *T. gondii* strains T561 and C56 (gift from Dr Peter Winstanley, University of Liverpool) were grown in Vero cell culture using standard techniques. Tachyzoites from peritoneal washes or supernates were washed three times in sterile phosphate buffered saline (PBS) , pH 7.2, prior to DNA preparation by routine methods.

PCR

Tissues (placental cotyledon, foetal brain, lung and liver) were prepared for PCR and B1 gene PCR was performed as described previously (Owen et al. (submitted, b)). When available, placental cotyledon was used for PCR since previous studies (Owen et al. (submitted, b)), have shown this to be the most suitable tissue for *T. gondii* detection in ovine abortion. Samples from which a B1 gene amplification product was obtained, confirming the presence of *T. gondii* DNA, were used for SAG2 PCR-RFLP analysis. For SAG2 PCR, 2.5μl of each tissue digest was used in a 50μl reaction mix (20mM $(NH_4)_2SO_4$, 75mM Tris HCl, pH 9.0, 0.01% Tween 20 (w/v), 0.5μM each primer, 1.5mM $MgCl_2$, 0.1mM d-NTPs, 0.1 unit Red Hot Taq polymerase (Advanced Biotechnologies, Leatherhead, UK). For each sample, two separate nested PCRs were performed, to produce two potentially polymorphic amplification products, both approximately 330 base pairs, associated with the SAG2 gene. Primers SAG2.F4 (sense strand) and SAG2.R4 (anti-sense) were used together in the first PCR to produce an amplicon which spans upstream non-coding DNA and a coding portion of the SAG2 gene. Down-stream from the SAG2 exon, primers SAG2.F3 (sense) and SAG2.R3 (anti-sense) were used in the first round of PCR to produce an amplicon from this intron sequence. In the nested PCRs, the primers used (sense with anti-sense) were SAG2.F with SAG2.R2 and SAG2.F2 with SAG.R, for the exon and intron sequences respectively. Following amplification, 15μl aliquots from the first round of PCR were analysed by electrophoresis on 2% agarose gels, stained with

ethidium bromide and trans-illuminated with ultra-violet light. For each sample, according to the concentration of PCR product, 0.1-1μl of reaction mixture, containing the amplicon, was used with a second primer pair in a nested PCR. The primers used are listed in Table 1 and their annealing positions relative the SAG2 gene are shown in Figure 1. All reactions were performed on a thermal cycler (Hybaid), using 40 cycles with denaturation (95°C 1 minute), annealing (65°C 1 minute) and extension (72°C 1 minute). For positive controls, approximately 1ng of DNA from *T. gondii* strains RH, C56 and T561 was amplified. Distilled water was used for negative controls during each amplification. General precautions taken to prevent contamination included the use of disposable gloves and single-use plastic ware, and the use of separate rooms for the two PCR stages.

Restriction enzyme analysis

Five microlitre aliquots of reaction mixtures containing SAG2 amplicons were digested overnight in 50μl reactions, containing one unit of restriction enzyme (either Sau 3A or Hha I (Boehringer Mannheim, UK), as appropriate), in a water bath at 37°C. Digests were analysed by electrophoresis on 2% agarose gels stained with ethidium bromide and photographed under ultra-violet light.

T. gondii infection was confirmed by B1 gene PCR from 13 placental samples, from mouse brains infected with *T. gondii* isolated from two lamb hearts and from brains from two wild rodents (one *Apodemus sylvaticus* and one *Clethrionomys glareolus*) (results not shown). From all these sources, both nested PCRs produced SAG2 amplification products which flanked the two polymorphic restriction sites (Typical results showing amplicons for the polymorphic sequence flanking the Hha I site are shown in Figure 2). All amplicons from *Toxoplasma* infected tissues gave restriction digest patterns consistent with *T. gondii* type II infection and amplicons obtained from the control DNAs from *T. gondii* strains RH, T561 and C56 gave restriction digest patterns consistent with types I, II and III respectively,as expected. (Typical results are shown in Figures 3 and 4).

Table 1. Primers used for amplification of polymorphic sequences associated with the *T. gondii* SAG2 gene.

Primer	Sequence (5′-3′)	Strand
SAG2.F4	GCT ACC TCG AAC AGG AAC AC	Sense
SAG2.R4	GCA TCA ACA GTC TTC GTT GC	Antisense
SAG2.F	GAA ATG TTT CAG GTT GCT GC	Sense
SAG2.R2	GCA AGA GCG AAC TTG AAC AC	Antisense
SAG2.F3	TCT GTT CTC CGA AGT GAC TCC	Sense
SAG2.R3	TCA AAG CGT GCA TTA TCG C	Antisense
SAG2.F2	ATT CTC ATG CCT CCG CTT C	Sense
SAG2.R	AAC GTT TCA CGA AGG CAC AC	Antisense

Figure 1. The *T. gondii* surface antigen II (SAG2) gene and adjacent non-coding DNA sequences. The hatched box represents exon and the introns are represented by the thin line. Primers for nested PCRs to produce the polymorphic sequences flanking the restriction sites Sau 3A and Hha I are indicated by arrows. For type I *T. gondii,* neither restriction site is present; type II is cut only by Hha I and type III by only Sau 3A.

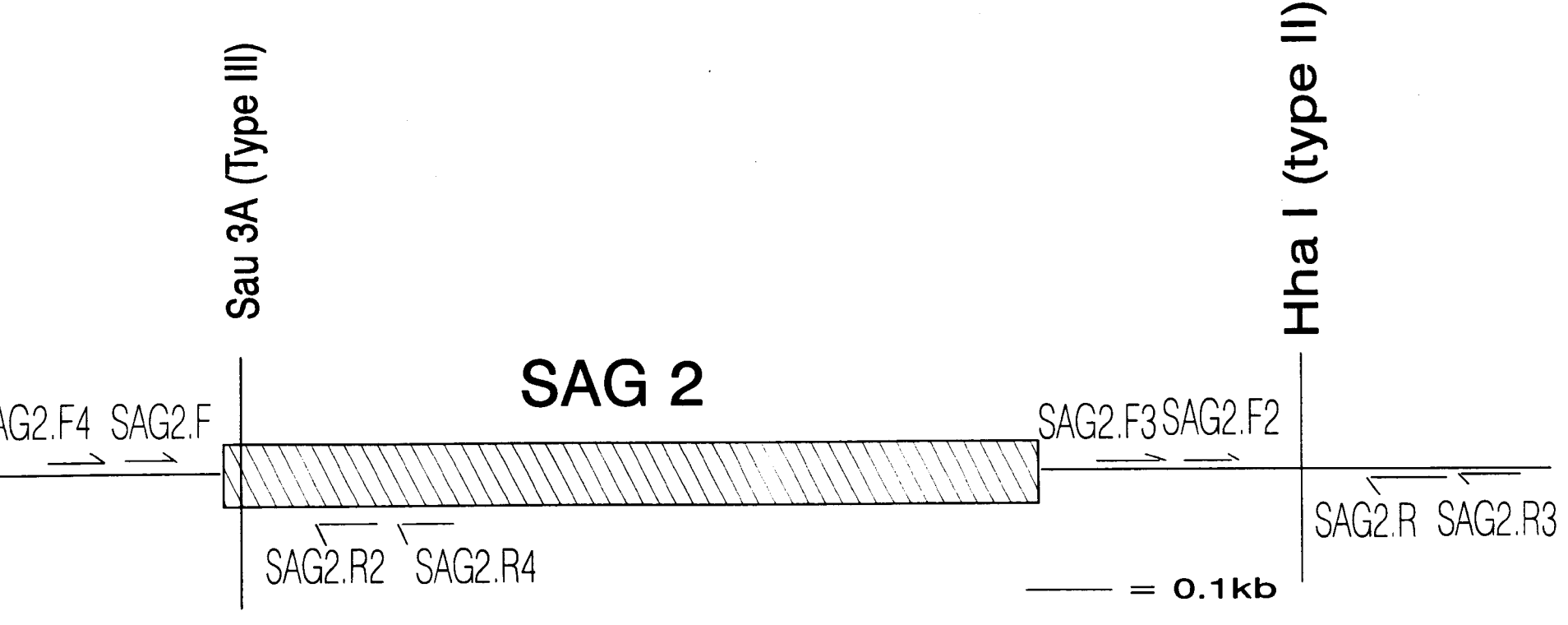


Figure 2. SAG2 (Hha I) site PCR amplification products were analysed by electrophoresis on 2% agarose gels, stained with ethidium bromide, trans-illuminated with ultra-violet light; *T. gondii* abortion tissues (lanes 3-11), abattoir lambs (lanes 12 and 13), wild rodent (lanes 14 and 15), 1ng *T. gondii* DNAs, strains RH, T561 and C56 (lanes 16-18). Lane 20 is a negative control, and lane 2 and 19 show molecular weight markers. The 220 base pair SAG2 PCR product is indicated.

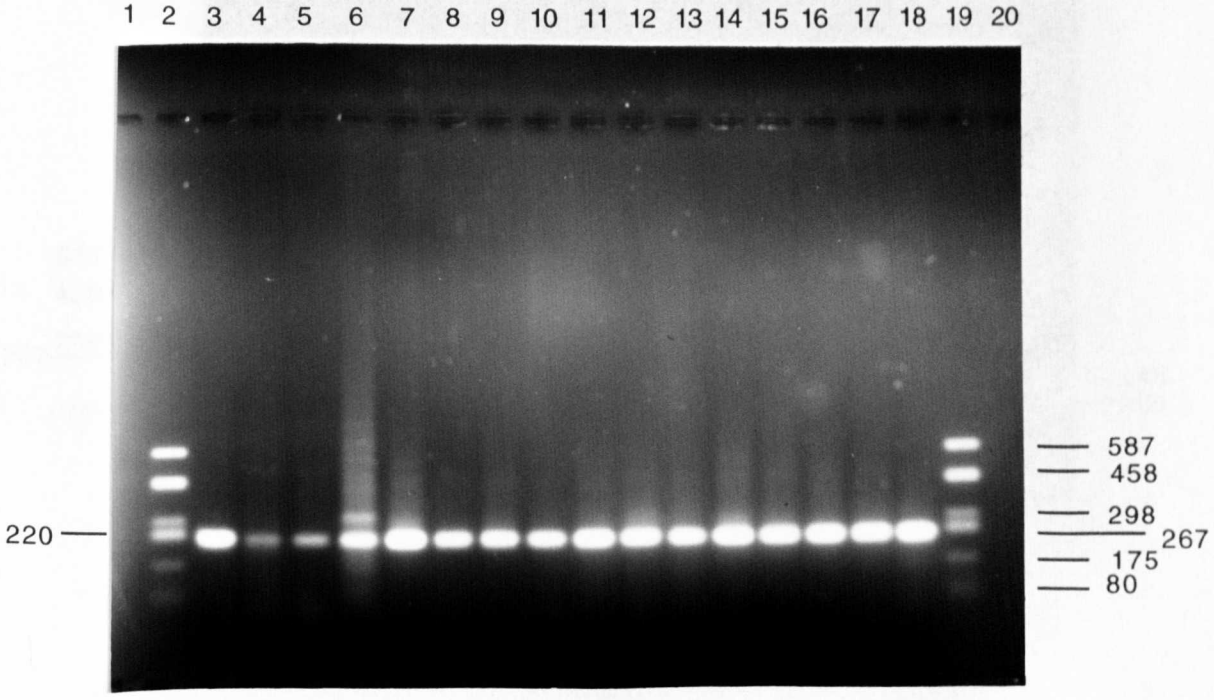


Figure 3. SAG2 (Sau 3A site) PCR amplification products (245 base pairs) from *T. gondii*-infected placental cotyledons were analysed by electrophoresis on 2% agarose gels, stained with ethidium bromide, trans-illuminated with ultra-violet light, after restriction digestion by Sau 3A. Samples were as follows: molecular weight markers (lane 1), non-digested amplicon (lane 2), C56 *T. gondii* (type III) (lane 3), placental cotyledons (lanes 4-9). The visible band of the digested amplicon indicated is approximately 200 base pairs.

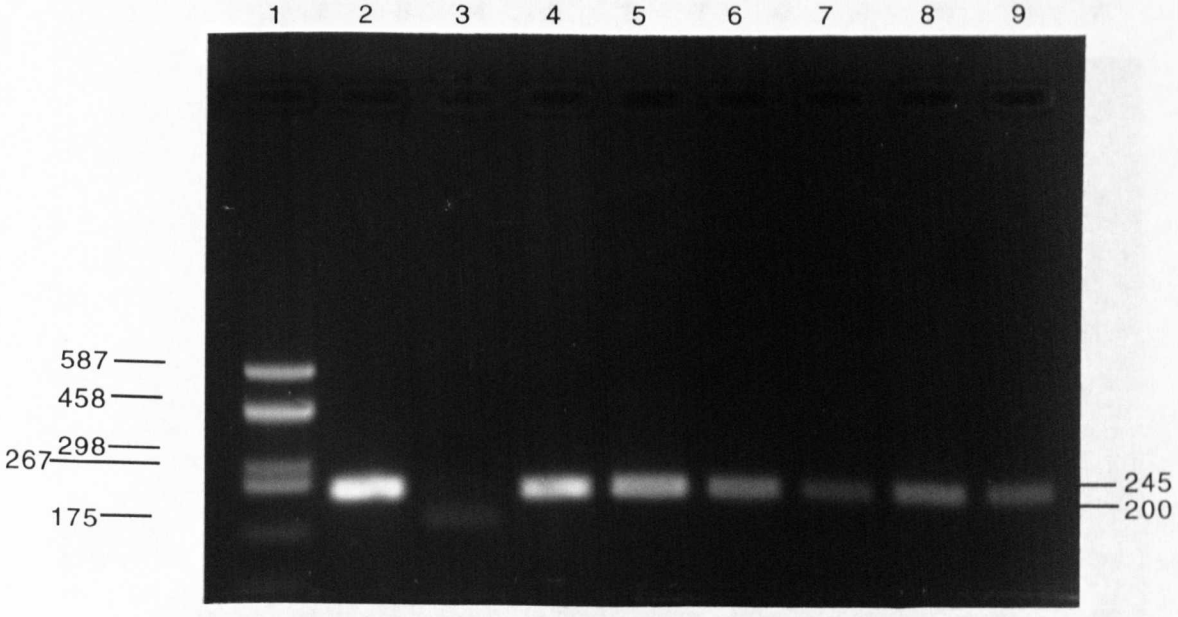
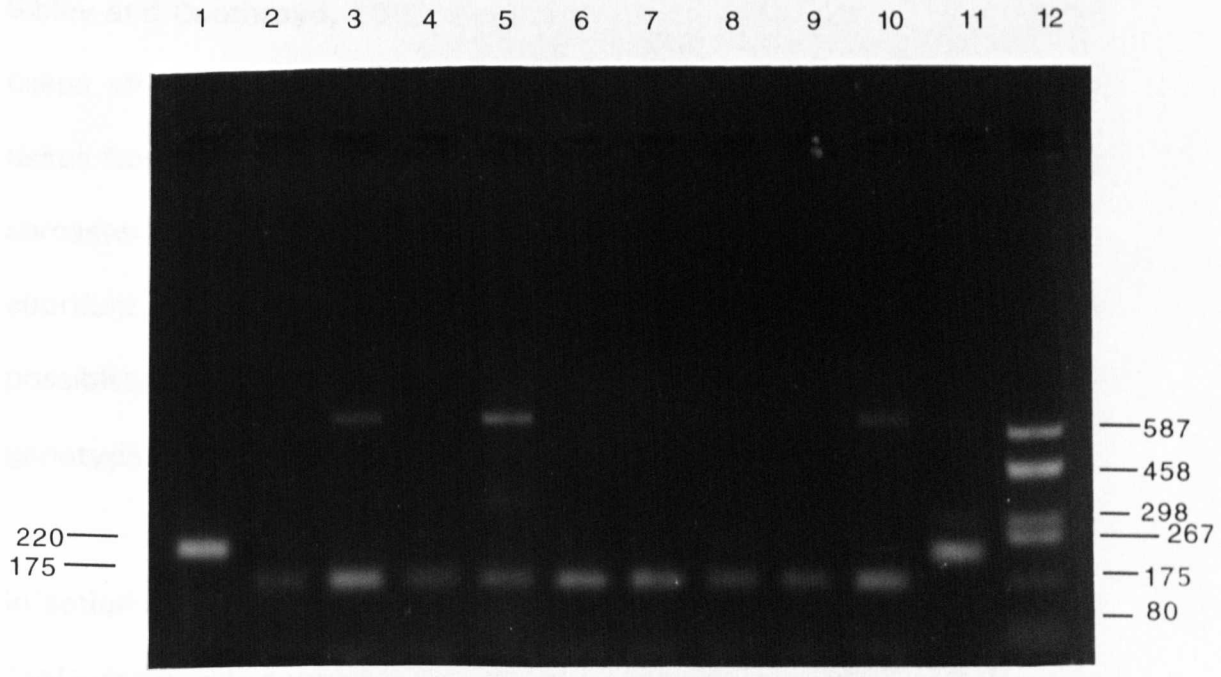


Figure 4. SAG2 (Hha I site) PCR amplification products (220 base pairs) from *T. gondii*-infected placental cotyledons were analysed by electrophoresis on 2% agarose gels, stained with ethidium bromide, trans-illuminated with ultra-violet light, after restriction digestion by Hha I. Samples were as follows: non-digested control amplicon from RH (type I) *T. gondii* DNA (lane 1), digested amplicon from T561 (type II) *T. gondii* DNA, placental cotyledons (lanes 3-10), undigested (following incubation in restriction enzyme) amplicon from C56 *T. gondii* (type III) DNA (lane 11), molecular weight markers (lane 12). The visible band of the digested amplicon is approximately 175 base pairs.



These results from a limited data set clearly demonstrate that molecular epidemiological studies on *T. gondii* can be conducted directly from infected tissue samples without the need for isolation of parasites from infected tissue into susceptible mice, nor into tissue culture. Furthermore, we have shown that without performing time-consuming DNA extraction, but using a simple tissue digestion protocol, PCR amplification products are readily obtained, which are suitable for restriction digest analysis (Owen et al. (submitted, b)) to determine the lineage type (Howe and Sibley, 1995). In contrast to studies performed in humans, in which most *T. gondii* strains have been derived from clinical cases of toxoplasmosis (Dardé et al. 1992; Sibley and Boothroyd, 1992; Howe and Sibley, 1995), analysis of animal tissue affords the opportunity to investigate strain types of *T. gondii* in tissue from both clinically healthy, infected individuals by tissue sampling carcases from abattoirs and from clinical cases of toxoplasmosis e.g. ovine abortions, at post-mortem examination. Thus, animal infections offer the possibility to more fully investigate any correlation between *T. gondii* genotype and clinical disease.

Infections in omnivorous humans can result from the ingestion of tissue cysts, or oocysts contaminating vegetables and soil but almost all infections in sheep (except congenital) occur following the ingestion of oocysts (Blewett and Watson, 1983), when sheep graze pasture or eat conserved

feed contaminated with infected cat faeces (Owen et al. (submitted, a)). During the sexual replication of *T. gondii* in the cat intestinal cells, gametogenesis and fertilisation occur for a limited period of approximately three to fifteen days (Dubey and Frenkel, 1972). In this period, should a cat become infected with two or more strains of *Toxoplasma*, the opportunity for recombination between strains exists. Whilst this is likely to be a rare event, it would lead to the expectation of greater genetic variability in *T. gondii* strains infecting sheep than humans. However, this was not supported by the observations here. The demonstration of the type II genotype in all the infected tissues examined in this study, and the very low proportion of recombinant strains observed in 106 *T. gondii* isolates in a previous study (Howe and Sibley, 1995) provides further evidence that like other protozoal parasites, *Toxoplasma* has essentially a clonal population structure (Tibayrenc, 1993), which is maintained by the rarity of recombination between lineages even after gametogomy in the definitive host.

The ovine *Toxoplasma* abortion samples investigated were obtained from a relatively small geographical area, but from ten completely independent farms in North Wales and North West England. Infection with type II *T. gondii* in all 13 abortion samples in this small data set contrasts the findings of a previous study, (Howe and Sibley, 1995), in which type II *Toxoplasma* was found only in approximately half of the isolates derived from animal infections, although the isolates examined were mainly from chronic,

subclinical infections and not cases of disease. Interestingly, the type II genotype is also the most prevalent in clinical cases of human toxoplasmosis (Dardé et al., 1992), causing approximately two-thirds of cases (Howe and Sibley, 1995).

The determination of any association between *T. gondii* genotype and its correlation with disease in animals will require further studies with larger numbers of tissue samples from *Toxoplasma* infections associated with disease and from asymptomatic cases. Such samples are readily available through post mortem material and from abattoirs. Since the majority of human infections are derived from animal infections (Howe and Sibley, 1995), the investigation of *Toxoplasma* genotype in infected meat and in cases of animal disease will provide valuable information in correlating *T. gondii* strain type with both animal and human disease.

ACKNOWLEDGEMENTS

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MANUSCRIPT VI

RIBOSOMAL INTERGENIC REGION VARIATION IN *TOXOPLASMA GONDII*: A RAPID MOLECULAR APPROACH TO THE IDENTIFICATION OF LINEAGES?

M. R. OWEN and A. J. TREES

Veterinary Parasitology, Liverpool School of Tropical Medicine/Faculty of Veterinary Science, University of Liverpool, Pembroke Place, Liverpool. L3 5QA.

ABSTRACT

Toxoplasma gondii is a single species in which three genetically distinct lineages, or types (I, II and III) are recognised. To identify genetic polymorphisms in *T. gondii* we designed primers for a nested PCR to amplify the multi-copy polymorphic non-transcribed and external transcribed spacers (NTS and ETS) in the ribosomal DNA (rDNA) unit. Analysis of published sequences of the *T. gondii* strains P, RH and SAILI demonstrated twelve polymorphisms, of which eight affected sites for restriction endonucleases. A first pair of PCR primers was designed which specifically annealed to the *T. gondii* 18S and 28S ribosomal genes and which generated the entire 2.5kb ETS/NTS sequence when genomic DNA was amplified. However, using a second pair of primers (which were theoretically *T. gondii*-specific) in a nested PCR, non-specific amplification of mammalian DNA occurred and prevented attempts to amplify *T. gondii* from infected or mock-infected tissue digests. Restriction fragment length polymorphism (RFLP) analysis of the ETS/NTS amplicons from genomic DNA of five *T. gondii* strains and two new isolates, using four endonucleases (Mae III, Rsa I, Spe I and Scr F I), differentiated RH (type I) and T561 (type II) strains from the remaining strains and the two isolates, all of which produced identicle RFLPs. This division into two demes does not exactly correlate with their lineage type. The demonstration of polymorphisms in the multi-copy NTS/ETS sequence from this small sample

of *T. gondii* DNAs suggests that NTS/ETS sequence polymorphisms may be a useful additional marker for *Toxoplasma* genotype analysis.

Toxoplasmosis, caused by the ubiquitous protozoan parasite *Toxoplasma gondii*, is a zoonotic infection affecting animals and man throughout the world. For most individuals infection is asymptomatic, but the severity of symptoms associated with disease may be influenced by the genotype of the parasite (Howe and Sibley 1995). In several species of parasitic protozoa genetically similar lineages exist for which a correlation can be made between parasite genotype and propensity to cause disease (Tibayrenc 1993). *Toxoplasma gondii* is a single species (Wong and Remington 1993), consisting of many strains which are morphologically and antigenically similar (Ware and Kasper 1987), but which differ in their virulence (Dubey and Frenkel 1973) and which have been recently ascribed to three genetically distinct lineages (Howe and Sibley 1995), of which one comprises all the mouse-virulent isolates (Sibley and Boothroyd 1992). Many other studies have examined the relatedness of *T. gondii* strains, using various techniques including isoenzyme analysis (Dardé and others 1992, Hassl and others 1994), riboprinting (Brindley and others 1993), restriction fragment length polymorphism (Cristina and others 1991, 1995, Howe and Sibley 1994, Sibley and others 1992), and random amplified polymorphic DNA - polymerase chain reaction (RAPD-PCR) analysis (Guo and Johnson 1995). All of these studies have been applied to purified parasite material obtained from propagation in tissue culture or mouse inoculation. The exquisite sensitivity of DNA detection by PCR has been

utilised to diagnose *Toxoplasma* infections in human (Burg and others 1989, Filice and others 1993, Guay and others 1993, Hitt and Filice 1992 and many others) and animal tissue samples (MacPherson and Gajadhar 1993, Owen and others (submitted,a,b; Stiles and others 1996, Wastling and others 1993). However, the opportunity to use PCR amplification of parasite DNA to investigate the molecular epidemiology of *Toxoplasma* by the direct analysis of infected tissue has yet to be exploited.

The study of a number of single-copy polymorphic genes enables the identification of *T. gondii* genotype into three lineages (Types I, II and III), (Howe and Sibley 1994, Sibley and Boothroyd 1992). Ribosomal DNA (rDNA) analysis is used extensively for phylogenetic analysis because of its highly polymorphic sequences flanked by highly conserved sequences and because in eukaryotes, there are many copies of the rDNA in the genome. There are, for example, 110 copies of the rDNA in the *T.gondii* genome (Guay and others 1992), hence enabling greater sensitivity of detection of parasites by PCR. Genetic analysis of the 18S small sub-unit rDNA gene (Luton and others 1995) and of the adjacent non-coding internal transcribed spacer (ITS1) (Homan and others 1996), have revealed very few differences between strains of *T. gondii*. However, the external transcribed spacer regions (ETS) and non-transcribed spacers (NTS) have not been investigated for strain related polymorphisms.

The purpose of this study was to investigate *T. gondii* polymorphisms in the NTS/ETS regions as possible additional markers for genotypic and phenotypic variation by PCR amplification and restriction fragment length polymorphism (PCR-RFLP) analysis.

MATERIALS AND METHODS

Toxoplasma gondii strains and DNA preparation

M1 strain (type II), *T. gondii* (Buxton and others 1979), was grown in Vero cell culture using standard techniques. *T. gondii* from brains of mice inoculated with infected lamb hearts, and *T. gondii* strains ME49 (type II), (gift from Dr J. Smith, University of Leeds); T561 (type II) and C56 (type III), (gift from Dr P. Winstanley, University of Liverpool), also in infected mouse brains were isolated into and grown in Vero cells. RH strain (type I), tachyzoites were maintained by serial passage every three days in Balb/c mice. Vero cells from tissue culture and tachyzoites from peritoneal washes and tissue culture supernates were washed three times in sterile phosphate buffered saline (PBS), pH 7.2 and DNA was extracted using routine methods.

PCR primer design

The entire rDNA sequence (Figure 1.) for three *T. gondii* strains RH, SAILI and P (see Table 1. for Genbank accession number) were aligned using the multiple alignment program PILEUP and sequence polymorphisms between the three strains were identified in the two NTS regions (NTS1 and NTS2). The small sub-unit rDNA (18S rDNA) gene sequences of seven *T. gondii* strains (Table 1.) were aligned to confirm sequence conservation at the 5' end of the 18S gene between strains. Because the 18S gene sequence for sheep was unavailable via Genbank, the *T. gondii* 18S gene was aligned with known mammalian 18S sequences; human, rat, and hamster and the *T. gondii* 28S rDNA was aligned with human and rat 28S sequences (Table 1). The 5' end of the 18S alignment and the 3' end of the 28S alignment were examined for 18-20 nucleotide sequences of maximum sequence divergence between *T. gondii* and mammalian genes. Using these 18-20mer sequences, compatible PCR primers were selected using the PCR primer selection program PRIMER (The Whitehead Institute for Biomedical Research), to specifically amplify the *Toxoplasma* NTS/ETS sequences and not mammalian NTS/ETS. To perform a nested PCR, a second set of (external) primers was selected, using the same criteria, which flanked the sequence defined by the first (internal) pair by approximately 50 and 100 nucleotides at the 5' and 3' ends, respectively.

Table 1. Source of ribosomal DNA (rDNA) sequences used in alignments

Species	Strain	Genbank Accession number
Toxoplasma gondii	P	X75453
	SAILI	X75430
	American RH	X75429
	European RH	U03070
	Canadian RH	M97703
	Australian RH	L24381
	TS4	U00458
Rat	-	K01591
Hamster	-	M33066
Man	-	M11167

In each amplification, from genomic DNA, approximately 10ng was used. From mock-infected and naturally infected tissues prepared for PCR, B1 gene PCR was performed as described previously (Owen and others (submitted, b)) using 5μl of crude digest supernate in the first PCR. The NTS/ETS sequence was amplified with external primers (RIBO 1, 5'-TCTCATCAAAGCCCTCCG and RIBO 4, 5'-AAACTTGAATGATCCGTCGC). Preliminary magnesium titration experiments using genomic *T. gondii* DNA determined that the optimum Mg^{2+} concentration for PCR was 2mM, hence amplification reactions were performed in 100μl containing approximately 10ng genomic DNA, 20mM $(NH_4)_2SO_4$, 75mM Tris HCl, pH 9.0, 0.01% Tween 20 (w/v), 2mM $MgCl_2$, 0.1mM d-NTPs, 0.2μM each primer (Severn Biotech Ltd, Kidderminster, UK), 0.3 units Red Hot Taq polymerase (Advanced Biotechnologies, Leatherhead, UK). Amplification was performed over 40 cycles of 95°C for 1 minute, 60°C for 1 minute and 72°C for 3 minutes, to produce an amplicon of approximately 2.5kb, visualised by electrophoresis of a 20μl aliquot of reaction mixture on a 1% agarose gel, stained with ethidium bromide, trans-illuminated with ultra-violet light. For the nested PCR, 1μl of reaction mixture, containing the 2.5kb amplicon was used in a second amplification to produce a 2.3kb product, using identical conditions for thermal cycling with internal primers (RIBO 2, 5'-GACGACTTTGTTGCAACCG and RIBO 3, 5'-CAGGGGAAGAGGCATGTG) and the optimum Mg^{2+} concentration for PCR was determined as 2mM.

Restriction digests

2.5kb PCR products, obtained by amplification with primers RIBO1 and RIBO4 of genomic *T. gondii* DNA, were cleaned by passage through a sepharose mini-column (Pharmacia, Uppsala, Sweden) and digested in 25μl volumes containing 5μl of PCR reaction mix, 1 unit of restriction enzyme (Boehringer Mannheim, UK), in 1 x digestion buffer. The restriction enzymes used were Mae III, Rsa I, Spe I and Scrf I. Restriction products were visualised by electrophoresis on ethidium-stained 1% agarose gels, trans-illuminated with ultra-violet light.

Toxoplasma infected tissues

T. gondii infected ovine abortion tissues (placental cotyledon and foetal brain), were collected directly from farms in Wales and North-West England and from aborted foetuses submitted to the Veterinary Investigation (VI) Service (Preston and Shrewsbury, UK). *Toxoplasma* infections detected by direct fluorescence antibody test and/or the detection of *Toxoplasma* specific antibody in foetal fluid (at VI centres) were confirmed by B1 gene PCR amplification using crude tissue digests (see below). Wild rodents were trapped on a sheep farm from which *Toxoplasma*-infected ovine abortions were obtained, using baited Longworth traps, and tested for *T. gondii* infection by detection of specific antibody and B1 gene PCR on crude brain digests. Hearts from slaughtered lambs were collected from a local abattoir, as part of another study. *Toxoplasma*-infected hearts were identified by detection of specific antibody in serum from heart cavities and

T. gondii was isolated from each infected heart into two susceptible Balb/c mice as previously described (Dubey *et al.* 1995). Isolation was confirmed by detection of *Toxoplasma* antibody in mice six weeks post-inoculation and whole brains were used for PCR-RFLP. For the preparation of mock-infected tissues, placental cotyledon, previously confirmed negative for *Toxoplasma* infection by B1 gene PCR was prepared for tissue digestion as previously described (Owen and others (submitted, b)), and divided into 200mg aliquots. A serial dilution of 10^7-10^1 *Toxoplasma* tachyzoites was added to samples prior to tissue digestion.

Serology

Foetal fluid from aborted foetuses collected directly from farms, rodent sera and sera from abattoir lamb hearts were tested for *T. gondii* antibody using the modified agglutination test (MAT), (Biomerieux, Marcy L' Etoile, France) with screening dilutions of 1/40 and 1/4000 according to manufacturers' instructions. Foetal fluids from abortions submitted to VI centres were tested for *Toxoplasma* antibody with the latex agglutination test (LAT) (Eiken Chemical Company, Japan) at a screening dilution of 1/64.

NTS/ETS Polymorphism

Twelve sequence polymorphisms were detected in the NTS/ETS regions by alignment of rDNA sequences for RH, SAILI and P strains of *T. gondii*, of which eight affected restriction sites. (Table 2.)

PCR-RFLP analysis of NTS/ETS region from genomic T. gondii DNA

PCR amplified a 2.5kb NTS/ETS sequence for approximately 10ng of genomic DNA from all five strains and the two isolates of *T. gondii*. Restriction digests of the NTS/ETS amplicons were performed using the four commercially available enzymes (Mae III, Rsa I, Spe I and Scrf I) of the nine predicted to reveal polymorphisms. RFLPs were demonstrated which differentiated RH and T561 strains from the remaining strains and isolates. (Restriction patterns for enzymes Rsa I and Spe I only shown in Figures 2 and 3). Restriction studies using a further five endonucleases (Eco R I, Hae III, Hha I, Sau 3A and Sau 96 I), revealed no polymorphisms (data not shown) between isolates or strains.

Amplification of NTS/ETS regions from T. gondii mock-infected and naturally infected tissues

Amplification with primers RIBO1 and RIBO4 did not produce a PCR product for any of the series of mock-infected tissues, though as a positive control for PCR, a B1 gene nested PCR product was obtained for all samples.

NTS/ETS PCR sensitivity was not increased by nested PCR with primers RIBO 2 and RIBO 3 since these primers were not specific for *T. gondii* and produced a 2.3kb amplicon for digests from non-infected sheep placenta and mouse brain and from Vero cell DNA. Using only primers RIBO1 and RIBO4, no NTS/ETS products were obtained by amplification of crude tissue digests from 13 placental cotyledons, two wild rodent brains nor mouse brains infected with *T. gondii* by isolation from two abattoir lamb hearts.

Figure 1. The ribosomal DNA (rDNA) unit of *Toxoplasma gondii*. The 5S, 5.8S, 18S and 28S ribosomal proteins are encoded by the respectively labelled genes, shown as boxes in the diagram. The 28S and 18S genes are separated (5' to 3') by a non-transcribed spacer (NTS 1), the 5S gene, a second non-transcribed spacer (NTS 2) and the external transcribed spacer (ETS). Between the 3' end of the 18S gene and the 5' end of the next 28S gene are respectively the first internal transcribed spacer (ITS 1), the 5.8S ribosomal protein gene and the second internal transcribed spacer (ITS 2). The annealing positions of the PCR primers for the 2.5 kb NTS/ETS amplicon (first pair RIBO 1 and RIBO 4); nested pair (RIBO 2 and RIBO 3) are indicated.

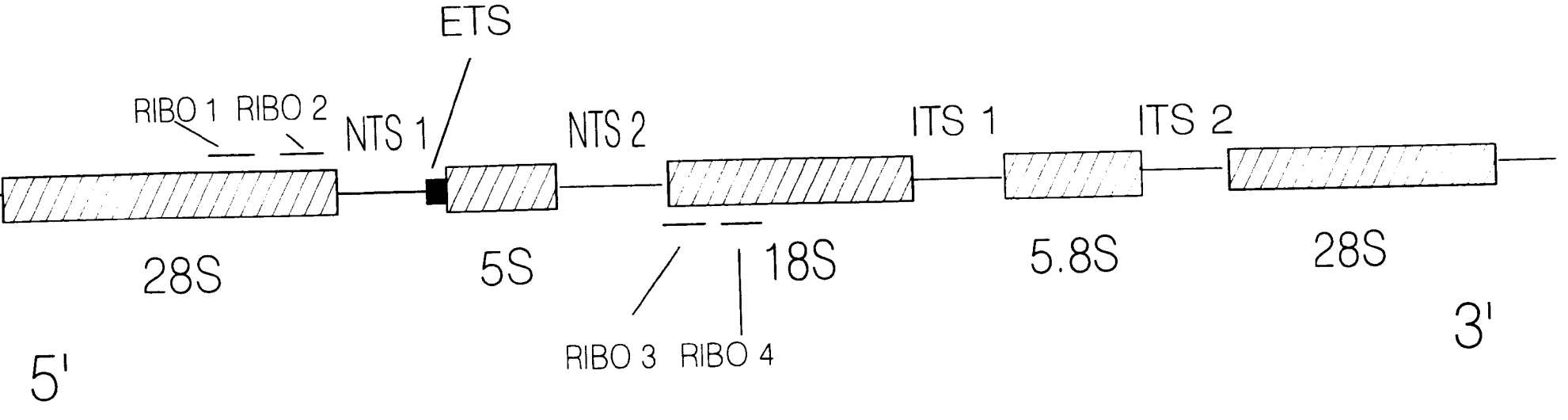

Figure 2. Restriction digests (Rsa I) of 2.5kb NTS/ETS PCR products for two isolates and five strains of *T. gondii* after electrophoresis on an ethidium bromide stained 1% agarose gel trans-illuminated with ultra-violet light. The isolate/strain identity is as follows: isolates A and B (lanes 1 and 2); strains M1 (lane 3), T561 (lane 4), C56 (lane 5), ME49 (lane 6), RH (lane 7). Lane 8 contains the undigested 2.5kb NTS/ETS amplicon and lane 9 contains molecular weight markers.

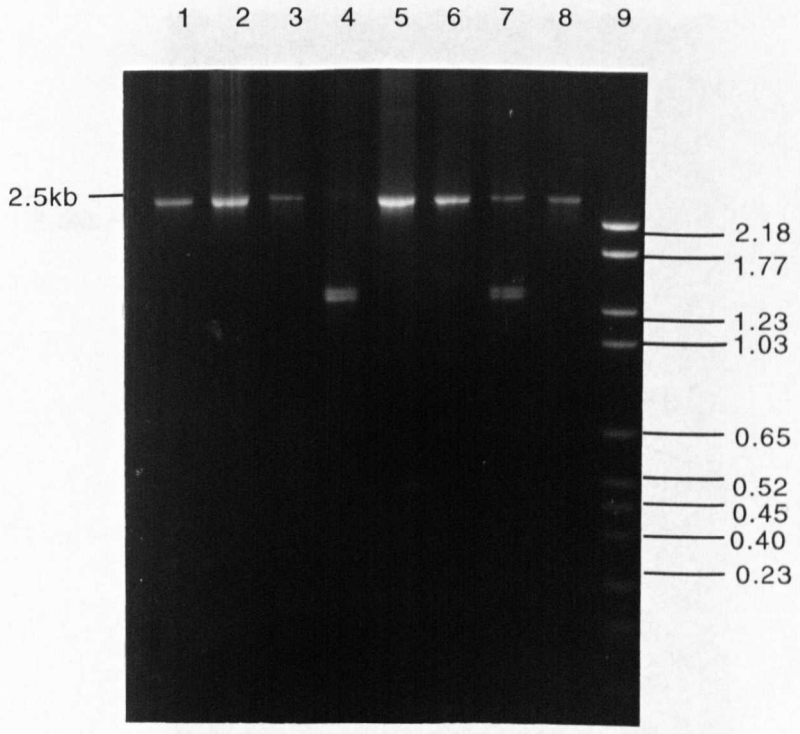

Figure 3. Restriction digests (Spe I) of 2.5kb NTS/ETS PCR products for two isolates and five strains of *T. gondii* after electrophoresis on an ethidium bromide stained 1% agarose gel trans-illuminated with ultra-violet light. The isolate/strain identity is as follows: isolates A and B (lanes 1 and 2); strains M1 (lane 3), T561 (lane 4), C56 (lane 5), ME49 (lane 6), RH (lane 7). Lane 8 contains the undigested 2.5kb NTS/ETS amplicon and lane 9 contains molecular weight markers.

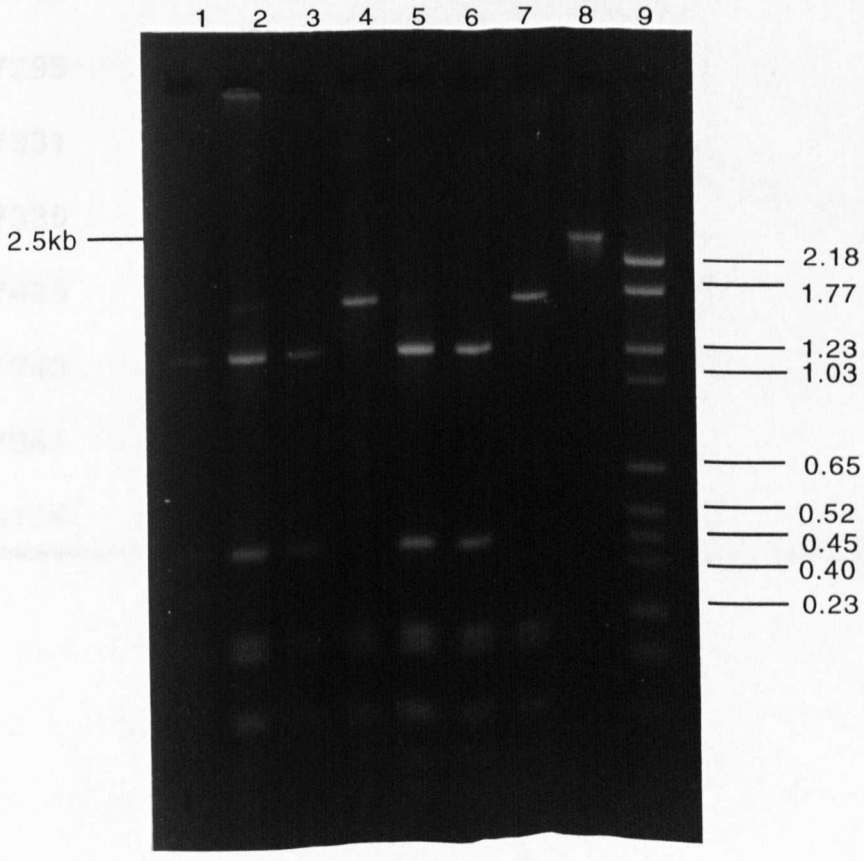

Table 2. Polymorphisms and variable restriction enzyme sites in the NTS/ETS sequence of the ribosomal DNA unit (rDNA) of *Toxoplasma gondii* for RH, SAILI and P strains. (Nucleotide numbers defined by Genbank accession X75430, SAILI strain).

Variable	Restriction site (strain)		
Nucleotide No.	(P)	(RH)	(SAILI)
6194	-	-	-
6493	Rsa I	-	Rsa I
6767	-	Hgi E II	-
6993	-	-	-
7141	Rsa I	-	-
7295	-	Spe I	-
7331	-	Mae III	-
7335	Bsr I	-	Bsr I
7425	-	-	-
7743	-	-	Scr F I
7841	-	-	-
8154	Alu I	-	Alu I

DISCUSSION

The results from this small data set of *Toxoplasma* strains and isolates demonstrate the genetic variability in the NTS/ETS regions which make them suitable targets for PCR-based molecular epidemiological studies, either by direct sequence analysis or PCR-RFLP studies. Failure to amplify the 2.5kb NTS/ETS amplicon from mock-infected and infected tissue in this experiment is likely to be due to the size of the fragment since PCR is not efficient at producing large amplicons. Long DNA strands are more susceptible to shearing forces, which can break-up long sequences, preventing *Taq* from copying them. Large DNA templates can also form complex structures which render them less amenable to duplication by *Taq* polymerase. Our attempts to amplify PCR products in excess of 1kb from the single-copy genes SAG 1 and SAG 2 in mock-infected and infected tissue have been unsuccessful, but using primers which produce amplicons of approximately 350bp, which flank polymorphic sites associated with the SAG 2 gene (Howe and Sibley, 1996, personal communication, Manuscript V), we have been able to amplify *T. gondii* DNA in infected tissues. Amplification of the NTS/ETS sequence using 10ng of pure *T. gondii* DNA is equivalent to 2×10^5 tachyzoites, hence failure to amplify the *T. gondii* NTS/ETS from mock-infected tissues to which 10^7 parasites were added probably demonstrates competition-inhibition of the PCR primers by sheep DNA.

Cross-reactivity of primers RIBO 2 and RIBO 3 with mammalian DNA was unexpected in view of the proportion of non-complementary nucleotides to mammalian sequences in the *T. gondii* primers selected. Manipulation of PCR conditions was considered unlikely to prevent non-specific amplification from non-*Toxoplasma* DNA because of the high quantity of the 2.3kb PCR amplicon obtained with non-*T. gondii* DNA and was not attempted. Consequently further molecular epidemiological studies used only *T.gondii* specific primers RIBO1 and RIBO4 with genomic *Toxoplasma* DNA.

Discrimination of RH and T561 *T. gondii* strains from the remaining isolates and strains by PCR-RFLP suggests that unlike the rDNA ITS, which is highly conserved between *Toxoplasma* strains, (Homan and others 1996), the NTS/ETS sequences are polymorphic and determination of these polymorphisms may enable differentiation between *T. gondii* genotypes. That RH and T561 produced identical RFLPs for restriction enzymes Rsa I and Spe I (and Mae III and Scr F I, data not shown) suggests that for some strains, NTS/ETS polymorphisms may not be consistent with the *T. gondii* clonal lineages recently proposed (Howe and Sibley 1995). The design of *T. gondii*-specific PCR primers, to produce small amplicons which flank the NTS/ETS polymorphisms, but which do not amplify host DNA could enable *Toxoplasma* genotyping directly from infected tissues, which has proved impossible using large PCR products of single-copy polymorphic sequences. In this respect, the fact that the target is multicopy will optimise sensitivity.

Automation of DNA sequencing may soon enable direct PCR sequence analysis to be performed, eliminating the need for restriction digestions to identify sequence polymorphisms. Currently, automated sequencing is limited to sequences not exceeding 600 nucleotides. Further investigation of *T. gondii* NTS/ETS polymorphisms would be best achieved by designing PCR primers to reproduce the NTS/ETS with over-lapping amplicons of 600 base pairs or smaller,which could be directly sequenced. Comparison of NTS/ETS sequences for many *Toxoplasma* strains would enable the identification of further markers for genotypes or lineages of *T. gondii* recently described (Howe and Sibley 1995).

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CONCLUSIONS

Toxoplasma gondii, a significant cause of clinical disease in animals and man, is detectable, directly from tissue samples by PCR. PCR based diagnosis can be applied to autolysed and/or contaminated tissues, unsuitable for mouse inoculation, hence it will be particularly useful in the detection of *Toxoplasma* infection in clinical cases of ovine abortion, which may be otherwise undiagnosable.

That *Toxoplasma* is not detectable, even by PCR, in 'acute phase' abortions which occur within 14 days of infection in ewes clearly shows parasite invasion of the placenta and/or foetus is not responsible for these abortions and raises questions as to their aetiopathogenesis.

The unequivocal demonstration of vertical transmission of *Toxoplasma* from chronically infected, wild, outbred *Mus* and *Apodemus* to their offspring provides substantial evidence to support the theory that an environmental reservoir of infection of *T. gondii* exists in rodents, capable of infecting cats, which in turn, can contaminate the environment with oocysts.

The incidence of *Toxoplasma* infection in four groups of grazing sheep, on three separate farms, with no known source of oocysts other than by ingestion from contaminated pasture demonstrates the degree and the

uncontrollable nature of environmental contamination with oocysts possible by cats.

The direct analysis of *T. gondii* lineage type from tissues by PCR-RFLP analysis from a limited number of naturally acquired infections demonstrates the feasibility of studying the molecular epidemiology of *Toxoplasma* in both clinical samples and non-clinically affected, but infected tissue.

New molecular markers for *Toxoplasma* were identified in non-coding regions of rDNA that may enable the differentiation of *T. gondii* strains into lineage types, though the polymorphisms detected appear not to correlate entirely with the *T. gondii* genotypes recently described. The new markers should, however, prove more sensitive for the genotypic identification of *Toxoplasma* in infected tissues.

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