reflects the predominance of this breed of cattle in UK. Infection and abortions have been diagnosed in beef herds but more research is needed to determine the significance of neosporosis in beef breeds in Britain.

3.4.3. Neosporosis in other species

The only recorded case of natural ovine infection occurred in Britain. A lamb presenting with neonatal paresis and encephalomyelitis was retrospectively shown to be infected by immunocytochemical examination of brain tissue [33,100,101]. Because of the large sheep population in the UK (40 million, VIDA Report, 1998), the importance of abortion and the possibility of misdiagnosis of *T. gondii* as a cause, a specific search for further ovine *N. caninum* infections has been made. In a prospective study, Otter and colleagues [26] examined fixed tissues from 281 aborted lamb foetuses submitted to Veterinary Investigation Centers throughout England and Wales and foetal fluids were tested for antibodies to *N. caninum* by IFAT at a cut-off titre of 1/50. No evidence of *N. caninum* infection was found by either method. Furthermore, serological surveys, using IFAT, ELISA and inhibition ELISA, of sheep flocks, some of which were grazed with cattle on *Neospora* – endemic farms, have not found evidence of natural ovine infection (Helmick et al., unpublished observations; McGarry et al., unpublished observations). This is despite the demonstrated susceptibility of sheep to experimental parenteral infection [9]. It is clear that natural sheep infections occur very rarely and neosporosis is not a frequent cause of ovine abortion.

Of 54 fox sera examined by IFAT, one had a titre of 1 in 200 which in dogs is considered indicative of exposure to *N. caninum* infection [33]. Associated with a serious outbreak of *N. caninum* abortion, 16 foxes were shot and sampled over a 2 year period on one farm in Cornwall, but none was found antibody positive by IFAT [102]. Further work is needed to determine the role of the fox as a potential host of *N. caninum* since negative serology may not indicate lack of infection. Neosporosis has not been confirmed in any other species and serological screening has been carried out in rodents (Williams, unpublished observations) and pigs (Helmick et al., unpublished observations). We have examined the sera of more than 400 farm workers and over 100 women who had suffered recurrent abortion and found IFAT titres of $<$1/200 in two individuals, the significance of which is unclear (Trees et al., unpublished observations). In Northern Ireland, sera from 199 blood donors and 48 agricultural workers were tested by IFAT and none were found positive using a cut-off dilution of 1/160 [103].

3.4.4. Epidemiology

In dogs, retrospective and prospective serological examination of family groups has shown that the efficiency of vertical transmission is insufficient to sustain the observed prevalence of infection and implies that postnatal infection is crucial to the maintenance of the parasite [93]. This is supported by age-prevalence studies indicating a rising antibody prevalence with age in four populations, although the data also suggested a secular decline in prevalence over time (Barber et al., unpublished observations). The high antibody prevalence in hounds (66% in adult dogs), which are fed predominantly bovine carcasses is consistent with recent findings of the role of the dog in the transmission of *N. caninum* [3].

In cattle, results of age-prevalence studies [11], analysis of familial distribution of sero-positivity in endemic herds (Davison and Trees, unpublished observations) and antibody assays on dam-calf pairs [31], have all shown that vertical transmission is the predominant route of infection. By testing pre-colostral serum samples, the efficiency of vertical transmission (based on antibody) was 95% [31]. Nonetheless, modeling has suggested the importance of post-natal sources of infection on maintaining prevalence levels [47], and prospective studies on calves which were antibody-negative at birth has established an incidence of post-natal infection (based on sero-conversion) of 1.9 per 100 heifer years at risk [31]. Great care is necessary in studies which rely on changes in antibody level to indicate de novo infection because antibody levels can fluctuate below the cut-off level. Thus in the prospective study calves were recruited at birth, at which age the sensitivity of antibody detection is maximal [31]. The source of post-natal infection for cattle in the UK (and elsewhere) has not yet been determined but is presumed to be most likely the result of oral ingestion of oocysts from dogs or other canids. Another possible source of infection is bovine to bovine transmission via milk or calving fluids and membranes. Although experimentally, milk spiked with *N. caninum* tachyzoites can infect calves [66], we have failed to infect new-born calves which were foster-suckled onto naturally-infected newly calved cows and attempts to infect cows or calves with placental tissue were also unsuccessful (Davison et al., unpublished observations).

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3.5. Neospora caninum *in Sweden*

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Research on cyst-forming coccidia with emphasis on *Toxoplasma gondii*and later *Neospora caninum*has been performed since the early 1980's at the Swedish University

of Agricultural Sciences and the National Veterinary Institute in Uppsala. Our studies on *N. caninum* have had emphasis on the development of serological and molecular tools for diagnosis and their use to investigate modes of transmission and prevalence of the parasite. The major achievements will be briefly reviewed in this communication.

3.5.1. The description and identity of N. caninum

Immunohistochemical labelling techniques employing specific antibodies are important tools with which to visualize and identify protozoan parasites in histological sections. *T. gondii* and *N. caninum* organisms are morphologically very similar but they have fundamental antigenic differences, and therefore the use of immunohistochemistry is important for their differentiation at post-mortem investigations. Indeed, the fact that *Toxoplasma*-like organisms in the tissues of dogs were not labelled when *T. gondii* antibodies were applied with an immunoperoxidase method [104] was a significant observation in the characterization of *N. caninum* in American dogs by Dubey et al. [105], as in the early reports of Swedish canine cases of neosporosis [106–108].

Soon after the description of *N. caninum,* it was isolated in the USA from dogs and some years later from cattle. With the availability of culture-grown organisms it was possible to perform detailed antigenic analyses as well as genetic studies of the parasite. In our studies on the identity of *N. caninum* we used a strategy based on DNA sequence analysis of ssrRNA genes. The results after comparing the *N. caninum* ssrRNA gene sequence with those of other parasitic protozoa classified in the phylum Apicomplexa, confirmed the placing of *N. caninum* in the family of Sarcocystidae. We also showed that *N. caninum*and *T. gondii* are phylogenetically very closely related, and the question arose whether or not they should be placed in separate genera [109]. This issue is still under debate as more data from other genera in the family of Sarcocystidae are added to the phylogenetic analysis.

The first European canine isolate of *N. caninum*(NC-Liv) was obtained in England by Barber et al., [25] and a bovine isolate (Nc-SweB1) in Sweden by Stenlund et al., [110]. The Swedish bovine Nc-SweB1 isolate appears to have some interesting features compared with other known *N. caninum*isolates. It grows considerably slower in cell culture, and Atkinson et al. [111] reported that it was less pathogenic in mice than was the NC-Liv isolate. By RAPD-PCR they also found distinct genetic differences between the two isolates. Further investigations into the possibility of variations in pathogenicity between strains of *N. caninum* isolated from different host species and geographic areas would be of considerable interest. In preliminary studies we have shown that the Nc-SweB1 isolate is accessible to genetic manipulation through transfection experiments (J. Mattsson, unpublished data). These are important tools with which to genetically manipulate the parasite in order to explore the function of various *N. caninum*genes.

For several years after the first isolation of *Neospora* from bovines in the USA, it was not clear whether this parasite in cattle was identical to the previously described *N. caninum* [112]. However, after further molecular and antigenic studies including the European bovine and canine isolates, we suggested that canine and bovine *Neospora* were the same species, i.e. *N. caninum* [113].

3.5.2. Molecular diagnosis

As alternatives to immunohistochemistry, the PCR and DNA hybridization assays have emerged to be important methods for the detection of protozoa and other microbes in tissues and body fluids of infected individuals. After sequence analysis of the internal transcribed spacer 1 (ITS1) from *N. caninum*and *T. gondii,* we were able to develop a PCR for the demonstration of *N. caninum.*The PCR system made possible the specific detection of *N. caninum* organisms as no amplification was observed with any of the other cyst-forming coccidia tested, including *T. gondii.*This PCR could be used to demonstrate the presence of *N. caninum* DNA in brain and lung tissue samples from experimentally infected mice [114]. It was later also used to detect *N. caninum* in sheep [9] as well as in cattle [66].

3.5.3. Serological diagnosis

The first serological method applied for diagnosis of *N. caninum* infection in the live animal was the IFAT employing intact tachyzoites as antigen [115]. The IFAT is still generally regarded as a reference assay. However, for large scale testing, e.g. in epidemiological studies, the ELISA has obvious advantages. The first ELISA described for the demonstration of *N. caninum* antibodies utilized extracted tachyzoite proteins incorporated into iscoms (immunostimulating complexes) as coating antigen [116,117]. Compared with an ELISA using a crude solubilized tachyzoite antigen, our iscom ELISA improved the sensitivity and specificity to 98 and 96%, respectively, against the IFAT as indicator of true status.

The iscom preparation was analyzed by gel electrophoresis and Western blot analyses and was shown to consist of a restricted number of proteins compared with whole parasite homogenates [117]. When mAbs were raised against the *Neospora* iscoms and applied in further characterization studies, they recognized antigens with Mr of 18, 30, 32, 41 and 61 kDa [118]. The 61 kDa antigen was located intracellularly and the others on the parasite surface as well as on intracellular membranes. In *T. gondii* iscoms prepared identically as the *N. caninum* iscoms, two dominant components are the tachyzoite surface antigens TgSAG1 or Tg-p30, and TgSAG2 or Tg-p22 [119]. The antigens included in the *N. caninum* iscoms have not yet been classified according to the nomenclature suggested by Howe and Sibley [120]. However, it appears that either the 30 or the 32 kDa antigen is identical with NcSAG1, and that the 41 kDa protein might be NcSRS2.

The original iscom ELISA was developed for use with canine sera, but we have later adapted it for demonstration

of antibodies also in bovine blood and milk [24]. The possibility of detection of antibodies in milk of infected cows offers advantages in the sampling, as milk samples can easily be collected by the farmers for submission to a laboratory for testing. In addition, the presence of antibodies in bulk milk can be analyzed for the identification of infected herds. Antibodies are detectable in the bulk milk if 10–15% of the lactating cows are seropositive and thus secrete antibodies with their milk.

At investigations of individual cases as well as of outbreaks of neosporosis in groups of animals it is often desirable to get an idea of the duration of the infection. The level of *N. caninum* IgG antibodies, or demonstration of rising antibody titres in paired samples, cannot be used for this purpose as these antibodies can persist at high levels for a long time and can fluctuate during pregnancy, at least in cattle. The degree of functional affinity or avidity of specific IgG antibodies can be used as a measurement of how recent a particular infection is. We measured the avidity of IgG antibodies directed to *N. caninum* by a modification of the iscom ELISA [51]. In this assay low affinity antibodies were eluted by including an incubation with urea after the primary serum incubation. The antibody titres measured with and without urea incubation were used to calculate an avidity index. Analysis of sequential sera from experimentally infected calves revealed that this index increased during the course of infection. Naturally infected cattle infected for more than 6 months had avidities above 50%. Results of preliminary studies suggest that determination of IgG avidity is useful also for investigation of neosporosis in dogs.

3.5.4. Prevalence and epidemiology

Generally, clinical neosporosis in dogs appears to occur infrequently in Sweden, possibly due to a low infection pressure. This was suggested from the results of a serological study on 398 dogs, where only two (0.5%) had demonstrable antibodies to *N. caninum* [121]. In a country-wide survey of dairy cows, 16 (2%) out of 780 individuals were seropositive [39].

In Sweden, antibodies to bovine virus diarrhoea virus (BVDV) is present in a large proportion of the cows and BVDV is regarded as the dominant infectious cause of bovine reproductive disturbances. Among 378 cows from 135 dairy herds with abortion problems, 7% had antibodies to *N. caninum* and 42% to BVDV and there was a statistically significant association between the presence of antibodies to *N. caninum* and BVDV [39]. It cannot be excluded that concomitant infections with these two agents may augment their detrimental effects. The possible existence of interactions between *N. caninum* and other pathogens deserves further investigation.

In cattle, the first immunohistochemically confirmed case of *Neospora*-associated abortion in Sweden was published in 1995 [122]. When we made a detailed investigation in a dairy herd in which sporadic abortions had occurred since the establishment of the farm 14 years earlier, 17 out of the 58 heifers and cows had antibodies to *N. caninum* [45]. When pedigrees were constructed for the seropositive animals it was found that they all descended from two of the original cows, no longer alive. The results suggested that these two cows were infected with *N. caninum* already when introduced to the herd. We found no indications of any route of transmission of the parasite other than the congenital in this herd. It was thus obvious that *N. caninum* can be transmitted from dam to offspring for several generations. This mode of transmission would explain why the infection can persist in a herd without any obvious external source of infection or horizontal transmission between animals.

When 18 seropositive heifers were monitored serologically during their first two pregnancies, we found that the antibody levels fluctuated in a regular manner [57]. They reached a plateau at 4–5 months of gestation and thereafter decreased from 7 months of gestation. The study showed that *N. caninum* antibody levels can remain at high levels for at least 2 years in chronically infected cattle. Furthermore, the consistent pattern of a rise in antibody titres observed during both pregnancies in all the animals indicated a reactivation of the parasite at mid-gestation. Such a reactivation of the latent *N. caninum* infection in the dam probably coincides with the transmission of parasites to the foetus, which would then usually take place at around 4–5 months of gestation. High titres were more frequently observed in the pregnancies ending in abortion than in the normal pregnancies. It can be speculated that this reflects a higher rate of parasite multiplication and more severe damage of the placental tissues leading to disturbed transmission of nutrients and oxygen to the foetus. A higher rate of multiplication may alternatively affect larger and more vital parts of the foetal CNS and other tissues, causing foetal death and abortion.

With *T. gondii,* transmission via milk has been shown to be a possible route of infection in mice and possibly also in humans. When *N. caninum* tachyzoites added to colostrum were given orally to neonatal calves, two out of four animals became infected, as shown by persistent antibody titres and presence of parasite DNA in the hippocampus region of their brains [66]. Although this study provided evidence that transmission via milk is possible in newborn calves, it is not known if this route of *N. caninum* infection occurs in the field. The parasite has not been demonstrated in milk or colostrum, neither from experimentally nor naturally infected animals.

3.5.5. Concluding remark

In Sweden, neosporosis is a sporadically occurring disease in dogs as well as cattle, and *N. caninum* has an overall low prevalence in both species. The reason for this low incidence and prevalence compared with those in many other countries is not known, but could be differences in animal husbandry, or that the parasite strains prevailing in the Swedish environment spread less easily than those in other countries. Climatic conditions may also play a role, however, we have registered cases of bovine *Neospora-*

associated abortions as far north as by the 64th latitude. In spite of the overall low prevalence of *N. caninum* infection in Sweden, as many as 60% of the cows in some dairy herds have been found infected, and in such herds abortions due to *N. caninum* infection can cause considerable problems. Generally however, the low prevalence of infection in dairy cows and dogs, together with the impression that a dominant proportion of bovine *N. caninum* infections is transmitted vertically, suggests that control of bovine neosporosis in Sweden may be achievable by gradually culling seropositive cattle.

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4. Immunology

4.1. Immunology of Neospora caninum *infection in cattle and mice*

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4.1.1. Introduction

Neospora caninum can be transmitted to intermediate hosts either from mother to foetus or through the consumption of oocysts, shed by the definitive host, the dog [15]. The outcome of *N. caninum* infection is largely determined by the effectiveness of the immune system of the host. The disease neosporosis occurs in pregnant animals and manifests as stillbirth, abortion or infertility in cattle and a neuromuscular disease in congenitally infected dogs[14]. While the immune system of the normal adult appears to be able to cope with the parasite the immature immune system of the developing foetus is particularly vulnerable. In this article we will review current knowledge of the immune response to *N. caninum* in both ruminants and mouse models of infection and discuss the changes to the immune system that occur as a result of pregnancy and how this may influence the delicate host-parasite relationship resulting in disease or death of the foetus. The development of natural immunity to *N. caninum* and the prospect of immunological intervention to control disease will also be discussed.

4.1.2. Natural immunity to neosporosis: observations from the field

Observations of naturally infected cattle have shown that there is a very high rate of vertical transmission of the parasite from dam to foetus [43]. Therefore immunity generated as a result of primary exposure to *N. caninum* is insufficient to prevent vertical transmission. However the main disease manifestation is abortion and limited data from several studies have suggested that the rates of repeat abortion due to neosporosis is relatively low (<4%) [46]. This would suggest that cattle are developing a degree of protective immunity against abortion and that this may be a realistic target for vaccine development similar to the commercially available vaccine, Toxovax®, which prevents abortion in sheep due to toxoplasmosis [123].

It is generally accepted that abortion due to neosporosis is a result of a lethal infection to the foetus (or embryo) following a maternal parasitaemia and/or damage to the placenta, thus compromising the pregnancy. This may occur if the mother becomes infected for the first time through the ingestion of oocysts or if she has a recrudescence of a previous infection. Infection with *N. caninum* is thought to lead to persistent infection with the bradyzoite stage of the parasite contained in tissue cysts [80]. Factors which influence the outcome of *N. caninum* infection are:

- the timing of the parasitaemia during gestation;
- the quantity and duration of the parasitaemia;
- the effectiveness of the maternal immune response; and
- the ability of the foetus to mount an immune response.

If transmission of the parasite to the foetus occurs early in gestation the effects are almost always lethal to the foetus [82,112]. Experimental infection of pregnant cattle at 70 days of gestation with an i.v. inoculation of *N. caninum* tachyzoites resulted in foetal death in five/six animals [124]. At this early stage in gestation the usual outcome is resorption of the dead foetus which may present in the field situation as infertility in the dam. Experimental infection of cattle in late gestation results in the birth of congenitally infected but otherwise clinically normal calves [124] (Innes, Esteban-Redondo, Swales et al. Local and systemic immune responses in pregnant cattle infected with *Neospora caninum*. COST820 annual workshop, Nov 11–14 1999, Interlaken, Switzerland); experimental infection of cattle in mid-gestation resulted in transmission of the parasite to the foetus in all four experimental animals resulting in one stillborn calf. In human congenital toxoplasmosis there is an inverse relationship between incidence of foetal infection and severity of foetal damage at different stages of gestation. Infection occurring in mothers early in gestation results in approximately 17% of the foetuses becoming infected, followed by severe disease and sometimes death. Infection in the third trimester of pregnancy results in approximately 65% of foetuses becoming infected and the severity of the disease is much reduced [125]. A similar phenomenon was observed when groups of cattle were experimentally infected with *N. caninum* at early, middle or late gestation; a group of cattle infected early in gestation did not transmit the parasite to their foetuses whereas groups of cattle