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Microsatellite typing and avidity analysis suggest a common source of infection in herds with epidemic *Neospora caninum*-associated bovine abortion

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ABSTRACT

Neosporosis is an important cause of reproductive failure in cattle worldwide. Two different abortion patterns associated with *Neospora caninum* infection have been observed in cattle herds: endemic and epidemic abortion outbreaks. The endemic pattern is characterized by an abortion problem in a herd persisting for several months or years, and is assumed to be caused by reactivation of a chronic infection. In epidemic outbreaks, abortions concentrate within a short period of time, most likely due to a recent point source exposure of naïve animals to *N. caninum*. The aim of the study was to characterize five *N. caninum*-associated epidemic abortion outbreaks in Germany by serological and molecular techniques, including a p38-avidity-ELISA and typing of *N. caninum* in clinical samples by multilocus-microsatellite analysis. DNA extracts from the brain of 18 *N. caninum* infected fetuses from epidemic abortion outbreaks were characterized using 10 *N. caninum*-microsatellite markers. Nested-PCR protocols were developed to amplify the marker regions MS1B, MS3, MS5, MS6A, MS6B, MS7, MS12 and MS21 from clinical samples for subsequent analysis by capillary electrophoresis. Microsatellites MS2 and MS10 were analyzed by previously reported sequencing techniques. Most dams which had aborted showed a low-avidity IgG response to the *N. caninum* p38-antigen, and in three of the five studied herds, the majority of the dams at risk, which had not aborted, had also low-avidity responses suggesting that infection with *N. caninum* had recently occurred in most animals. A common microsatellite pattern prevailed in all fetuses from each individual epidemic outbreak. This pattern was unique for each herd. Although the number of epidemic abortion outbreaks analyzed was limited, the observation of a common microsatellite pattern, accompanied by a low-avidity IgG response against *N. caninum* in the dams, supports the hypothesis of a recent infection from a common point source. The genetic diversity of *N. caninum* observed among these outbreaks may indicate that not a particular *N. caninum* genotype but the horizontal infection route determines the occurrence of epidemic abortions.

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1. Introduction

Neosporosis is regarded as an important cause of reproductive failure in cattle worldwide. The main clinical manifestations are abortions and neonatal mortality. Bovines can become infected vertically (transplacentally), or horizontally by the ingestion of feed and water contaminated with oocysts shed by definitive hosts. Dogs are the only known definitive hosts of *Neospora caninum* in Europe. Foxes have so far not been confirmed as definitive hosts in experimental studies (Schares et al., 2002b). Transplacental infection of the fetus may result in abortion, stillbirth, or in the birth of an asymptomatically infected calf (Dubey et al., 2006).

Two types of transplacental infection (TPI) have been defined: endogenous and exogenous TPI. Endogenous TPI is caused by the reactivation of a pre-existing infection of the dam during pregnancy, acquired either vertically or horizontally, whereas exogenous TPI is the result of an infection of naïve dams from an external source during pregnancy, presumably by ingestion of *N. caninum* oocysts (Dubey et al., 2007; Trees and Williams, 2005). This classification is epidemiologically important, as endogenous and exogenous TPI have been associated with two different abortion patterns in cattle herds, i.e., endemic and epidemic abortion. Endemic abortions persist in the herd for several months or years at a low rate; the occurrence of abortions depends on the reactivation of a pre-existing parasite infection as a result of the individual hormonal and immunological status of the dam and on the gestational stage (Dubey et al., 2007; Guy et al., 2001; Paré et al., 1997; Stenlund et al., 1999; Weston et al., 2005). In epidemic abortion outbreaks, often $\geq$10% of the cows at risk in a herd abort within a relatively short period of 4–8 weeks (Dubey et al., 2007). Abortions typically concentrate within a short period of time, most likely due to a point source exposure of the herd to *N. caninum* (Dubey et al., 2007; McAllister et al., 2000; Schares et al., 2002a). The finding of low-avidity immunoglobulin G (IgG) responses supports the hypothesis of recent infections in herds with epidemic abortions (Björkman et al., 2003, 2006; Schares et al., 2002a). After ingestion of infected intermediate host tissues, dogs shed oocysts for a relatively short period of time (reviewed in Schares and Losson, 2007). If the assumption of a point source exposure is correct, *N. caninum* parasites present in an epidemic abortion outbreak situation would be likely to belong to a single strain of the parasite. However, mixed infections would be possible in oocyst-shedding dogs, if the dogs had ingested material from an intermediate host infected with more than a single strain, or if the dogs had eaten two hosts infected with different strains at the same time. At present, it is not known how often mixed infections occur in nature.

Little is known about the genotypes of the *N. caninum* strains which may be associated with epidemic and endemic abortion outbreaks in cattle. It is even unclear whether a relationship between the genotype and the occurrence or frequency of epidemic abortions exists. This question can be addressed using highly variable markers which allow identifying different genetic populations of the parasite. Recently, microsatellite-DNA sequence analysis has proved as a valuable tool to study genetic diversity in *N. caninum*. A high degree of heterogenicity among the isolates was observed (Al-Qassab et al., 2009, 2010; Basso et al., 2009a, 2009b; Beck et al., 2009; Pedraza-Diaz et al., 2009; Regidor-Cerrillo et al., 2006, 2008; Rojo-Montejo et al., 2009). Microsatellites or simple sequence repeats (SSRs) are highly variable loci which consist of tandemly repeated units of 1–6 base pair (bp) length, present in the genome of eukaryotic and prokaryotic organisms. The polymorphisms in these sequences result from the gain and loss of single repeat units. Multilocus-microsatellite typing can be achieved either by sequencing or by assessing the length of amplified microsatellite-containing fragments by capillary electrophoresis, using fluorescent-labeled primers. *N. caninum*-microsatellite amplification by nested-PCR showed a higher sensitivity than by conventional PCR (Basso et al., 2009b; Pedraza-Diaz et al., 2009).

The aim of this study was to test the hypothesis that bovine epidemic abortion associated with *N. caninum* is caused by recent infection with a single parasite strain, i.e., by a common source of infection. To this end, the avidity of the IgG response to the parasite was measured and *N. caninum* parasites were typed in clinical samples by multilocus-microsatellite analysis.

2. Materials and methods

2.1. Bovine fetal samples and herd data

Brain material was obtained from 20 bovine fetuses derived from five *N. caninum*-associated epidemic abortion outbreaks in Germany. The outbreaks were recorded in the districts of Fürth, Bavaria (Herd No. 1), Cuxhaven, Lower Saxony (Herd No. 2), Rendsburg-Eckernförde, Schleswig-Holstein (Herd No. 3), Uckermark, Brandenburg (Herd No. 4) and Hochsauerland, North Rhine-Westphalia (Herd No. 5) between 2000 and 2009. Brain samples or whole fetuses (4, 4, 3, 3 or 6 from each outbreak, respectively) were submitted refrigerated to the Institute of Epidemiology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Wusterhausen, Germany, directly by the farmers or via regional diagnostic laboratories (Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit, Landeslabor Berlin-Brandenburg and Staatliches Veterinäruntersuchungsamt Arnsberg) for further analysis. Data collected from the affected farms included geographic localization, number of dams >1.5 years in the herd, date of the onset of the abortion outbreak and its duration, number of dams that had aborted and gestational age at the time of abortion, and number of dogs kept on the farm.

2.2. Serum samples and serological examination

Serum samples were collected from all dams >1.5 years in the herd, including the dams that had aborted, and were tested by ELISA to detect IgG antibodies against p38, a surface antigen (NcSRS2) of *N. caninum* tachyzoites (Schares et al., 2000) and by p38-avidity-ELISA (Schares et al., 2002a), to measure the avidity of bovine IgG directed against this antigen. Samples which tested positive using an ELISA index of 0.04 as the cut-off (von Blumröder et