Original Report

Diagnosis of Toxoplasma gondii infections in newborn farm foxes by polymerase chain reaction (PCR)

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Summary

The purpose of the study was to confirm congenital toxoplasmosis in dead newborn foxes derived from seropositive vixens. The polymerase chain reaction (PCR) technique was used to identify T. gondii DNA in 24 samples from neonate organs (lung, liver, brain). B 1 gene sequence was amplified using two pairs of primers. Lung, liver and brain samples from 4 newborn foxes (in all 9 samples) gave positive PCR results, The females which had infected neonates came from 3 different farms. Amplification with first primers pair gave more positive results. This may indicate a higher sensitivity of reaction with the use of first pair of primers or unstable conditions of reaction for the second one. The results of this study show that PCR can be a suitable method for finding congenital toxoplasmosis in foxes.

Introduction

Toxoplasma gondii can infect most species of warm-blooded animals, but carnivores seem to be especially disposed for the infection. The high risk is related to more possibilities of ingesting both tissue and cyst of this protozoan parasite in raw meat and oocysts from the environment and food contaminated by cat feces.

Depending on the region where the examinations were performed, and method, 3 - 87% positive serological results were found in dogs (29, 39). The percentage of infected cats is often high and in some regions of Poland up to 75% (41). Various prevalences of infection (3 - 19%) were noted in farmed mink and foxes (2, 16, 20). In Poland the seroprevalence in breeding foxes was found to be 33.6% (36). Among wild animals the highest prevalence was found in carnivores, e.g. the presence of T. gondii-specific antibodies was detected in 84% of red foxes and in 64% of coyotes (38). Seroprevalence of T. gondii infection in pet and farmed carnivores depends on the way of feeding (raw or cooked food), hygienic standards and local climatic conditions. This relationship was determined both in dogs and in fur animals (15, 36, 42).

Apart from members of the cat family (*Felidae*), which are the only known definitive hosts for the sexual stages of T. gondii and are the main reservoir of infection, the other carnivores are not considered to play an important role in epidemiology of toxoplasmosis. However carnivorous fur animals like mink and foxes may constitute a potential risk of acquiring toxoplasmosis for man, when considering the pelting procedure (8, 16, 19).

In carnivores (as in humans) acquired Toxoplasma

infection in immunocompetent individuals is generally an asymptomatic infection. However, it is possible that lack of patognomic symptoms during acute infection results in non-diagnosed cases of toxoplasmosis. The most dangerous acute primary infection by females during pregnancy may lead to reproductive infection. Even asymptomatic infection in pregnant females may cause placenta damage and fetus death or transplacental transmission followed by congenital toxoplasmosis. Early fetus death, abortion, stillbirth and neonate mortality were noted in seropositive bitches or mink and fox females (2, 35, 36). Especially death of cubs in the first few days of life, with the main histological changes and the presence of parasite cysts in the brain were described after both natural and experimental T. gondii infection of pregnant bitches, queens, mink and vixens (9, 10, 11, 17, 18).

The objective of the study reported here was to confirm congenital toxoplasmosis in dead new-born foxes derived from seropositive vixens. The additional purpose was to adapt polymerase chain reaction (PCR) technique to identify T. gondii DNA in samples from neonate organs of carnivores.

Material and methods

30 fox females, which had aborted or lost their litters in the first 5 days after whelping, were examined for the presence of T. gondii-specific antibodies using the latex agglutination test (Pastorex Toxo® Sanofi Diagnostics Pasteur).

Biological samples consisting of lung, brain and liver taken from newborn foxes (in one case from aborted fetus) derived from seropositive vixens. In all 24 samples of organs (collected from 5 farms) were used for examinations by PCR technique.

Treatment of samples and preparation of DNA
Extraction of DNA was performed with the use of chloroform/isoamyl alcohol method (5). Each sample was finely chopped with a sterile blade, then powdered by crushing under liquid nitrogen in a mortar. The powdered tissues were centrifuged in RB buffer (0,1 SM NaCl, 0,01 M EDTA, pH 8,0) and the sediment was incubated at 37^0C for 15 minutes in a digestion buffer (Tris, EDTA, pH 8,0, lysozyme 100mg/I ml). After the following incubations on ice: with GES buffer (6 g guanidine thiocyanate salt, 2m1 0,5 M EDTA, 0.1 g Nlauroylsarcosine in

10 ml) and with cold 7.5M ammontum acetate, DNA was isolated with the use chloroform/isoamyl alcohol in a ratio 24:1 v/v. DNA was precipitated by izopropanol and washed twice in 70% ethanol. The extracted DNA was resuspended in 100 µl of TE buffer and incubated with RNA-se.

Polymerase chain reaction procedure:
The B1 gene was amplified using the following oligonucleotide primers pair I:

5'ACCTAGATCGTGCGGCAATGTGCC3',
5'TGGTGCGACGGGAGTGAAAGTCATCC3'
and primers pair II:
5'GGAACTGCATCCGTTCATGAG3',
5'CAGACGAATCAACGGAACTG3' (19, 21).

The amplified product of the primers pair I was a 461 bp fragment and of the primers pair II - a 501 bp fragment, respectively. The 50-µl amplification reaction mixture contained a final concentration of PCR buffer (20mM Tris-HC1, 50mM KC1, 2,5mM $MgCI^2$), mixed dNTP (Gibco) 200µM, 2OpM of each primer, 1 U Taq polymerase (Gibco) and sample DNA in 10µl of TE buffer. The positive control contained 20 ng DNA obtained (by the method described above) from T. gondii RH strain tachyzoites.

Amplification was performed in an automated Thermocycler (UNO-Thermoblock, Biometra) for an initial denaturing step of 4 minutes at 94^0C, followed by 35 cycles of 1 minute at 94^0C, 1 minute at 55^0C, and 1 minute at 72^0C. A final step of 8 minutes at 72^0C was included. Amplification products were electrophoresed on 1.5% agarose gel, stained with ethidium bromide and visualised under UV illumination. The agarose gel was analysed using BIO CAPT programme to indicate the 461 and 501 bp T. gondii-specific amplification prod-ucts.

Results and discussion

In sera of 14 vixens (from 30 tested), which had aborted or lost their cubs, the presence of T. gondii-specific antibodies were found by latex agglutination tests. Samples of neonate or fetus organs were collected from 8 of 14 seropositive females with high titres of antibodies (480 lU/ml). No females had signs of disease. One of them aborted at 46 day of pregnancy. Neonates were weak; they did not suck and lived only up to 5 days.

The specificity of the PCR for T. gondii infection with the use of the same primers as those used in the presented study was confirmed previously (14, 33).

Lung, liver and brain samples from 4 newborn foxes (in all 9 samples) gave positive PCR results.

The females which gave birth to infected neonates came from 3 different farms.

A 461 bp product (after DNA amplification with primers pair I) was found in 9 samples, and a 501 bp product (primers pair II) in 5 samples (agreed with samples positive for primers pair I). This may indicate a higher sensitivity of reaction with the use of first pair of primers or unstable conditions of reaction for the second one.

Asymptomatic infections and lack of patognomic symptoms during the acute toxoplasmosis result in difficulties in clinical diagnosis of the disease. The classic diagnosis of T. gondii infections in humans is mainly based on highly developed serological techniques. However serological methods are insufficient for diagnosis of toxoplasmosis in immuno-compromised patients, infants and developing fetuses. The sensitivity of serological methods may be too low and definitive diagnosis based on in vitro culture or mouse inoculation is time consuming and may last up to 3-6 weeks. Besides in vitro culture is also possible the lack of sensitivity of cells (14, 37).

Thus polymerase chain reaction assays have been developed during the last few years for the diagnosis of toxoplasmosis.

Various gene sequences of T. gondii genome were amplified, such as: unique sequence of P30 gene, anonimous TGRIE or multicopy ribosomal small sub-unit rRNA gene (6, 13, 26). The best results can be obtained after amplification of B1 gene sequence, which is repeated 35 times, conserved in all tested strains of the parasite and specific to T. gondii (4, 31, 33, 40). The PCR with the use of primers pair designed from B 1 gene allows detection of less

than 10 tachyzoites of the RH strain of T. gondii (4, 30). The amplification of the B1 gene may have a big role in diagnosis of congenitally acquired toxoplasmosis by detection of the parasite in amniotic fluid samples. Furthermore, examinations of cerebrospinal fluid by PCR allow early diagnosis of acute toxoplasmosis followed by monitoring of therapy efficacy (14, 31).

Serologic diagnosis of toxoplasmosis in most species of animals is not as developed as in humans. Only in some carnivores is it possible to determine the phase of infection. The increase of T. gondii-specific 1gM concentration was observed in experimentally infected cats early in the course of clinical disease and maintained up to a few months, while specific IgG concentration remained high for years (24). Correlating clinical symptoms with T. gondii specific antibodies titer can lead to a presumptive, but not definitive diagnosis (25). Some animals do not mount an antibody response despite infection with T. gondii (12).

Similarly, correlating reproductive disorders in females with high T. gondii specific antibodies level can indicate, but not prove that toxoplasmosis was a real cause of the reproductive losses (2, 36).

Definitive diagnosis of toxoplasma infection by PCR technique was obtained based on examinations of various biological samples (cerebrospinal fluid, aqueous humor, serum) from some species of animals including dogs and cats (23, 27, 33). Positive PCR results, which were confirmed by mouse inoculation were also found after amplification of DNA extracted from aborted lamb fetus organs. The sensitivity of PCR was similar to that of mouse inoculation and the results of both these methods for most tissues were similar, however, mouse inoculation failed, if parasites were present but were not viable. This fact may indicate that PCR should be applied especially if the tissues are autolysed or contaminated and hence unsuitable for mouse inoculation (30). In the presented study the parasite DNA was detected in the same organs (brain, lung, liver) of newborn foxes as in those of lamb fetuses in the work mentioned above. Both in experimental and natural infection the main changes and the presence of T. gondii cysts were found in brains of congenitally infected dog and fox neonates (3, 9). The results of the presented work mean that for diagnosis of T. gondii infection by PCR other organs as lung and liver are identically useful.

Abortions, stillbirths and neonatal mortality are a major clinical problem in dog kennels and may cause high losses in carnivorous fur animal farms. Multiplex etiology of reproductive disorders make difficult the effective prevention of reproductive losses. Apart from bacterial infections (Escherichia coli, Staphylococcus aureus, Streptococcus sp., Corynebacterium sp. and other), viral infections such as canine herpesvirus in dogs and Toxocara canis infections, toxoplasmosis should also be taken into consideration to establish the real or the most important cause of occurring abortions and newborn deaths in carnivores (7, 21, 28, 34). Positive results of serological examinations can not prove that reproductive losses have been due to toxoplasma infection and diagnosis based on in vitro culture or mouse inoculation may be impossible (because of agents mentioned above) or be too time-consuming. Direct methods of parasite detection such as various staining techniques as well as histological examinations may fail and not allow differentiation of T. gondii and Neospora caninum. Both of these protozoan parasites give similar clinical symptoms and pathologic changes, especially meningoencephalitis, abortions and early cub death can occur both in toxoplasmosis and neosporosis. Cysts of the parasites are morfologically identical (1, 32). It was confirmed that amplification of B1 T. gondii gene gives all the negative results for N. caninum (33). It means that PCR is an adequate method for differential diagnosis of neosporosis and toxoplasmosis.

The results of this study have shown that the PCR can be a suitable method for proof of congenital toxoplasmosis in foxes. Detection of T. gondii DNA in newborn foxes confirmed a potential use of PCR as a diagnostic test, which allows definitive diagnosis and makes it possible to start an early appropriate treatment and prevention in fox farms. Development of the PCR protocol may also lead to diagnosis of congenital toxoplasmosis in dogs by detection of T. gondii DNA in amniotic fluid.

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