Molecular and bioassay examination of *Neospora caninum* infection in bovine aborted fetuses in Khorasan Razavi province, Iran.

Abstract

Neospora caninum plays a significant role in causing abortion and reproductive failure in dairy cattle. The majority of neosporosis-related abortions take place during the 5–6 months of gestation. Fetal death in the uterus, resorption, mummification, autolysis, stillbirth, birth with clinical symptoms, or being born clinically healthy but with chronic infection are all possible outcomes. The objective of the study was to identify *N. caninum* infection in aborted bovine fetuses through molecular analysis and mouse bioassay testing. From 2019 to 2022, 121 bovine aborted fetuses were collected from dairy farms in Khorasan Razavi province. The fetal brain samples were screened for detection of the parasite DNA by polymerase chain reaction assay (PCR). In addition, a portion of PCR-positive brain tissue was homogenized and inoculated into the peritoneum of five BALB/c mice. All mice were sacrificed six weeks post infection and examined using serology, microscopic, and PCR methods. If the mice's brain samples were PCR positive, the mouse bioassay test was repeated two times. The *N. caninum* DNA was detected in 19.8% of brain samples in bovine aborted fetuses. Among PCR-positive brain samples, only ten samples were suitable for mouse bioassay examination. All inoculated mice were seronegative without clinical signs after three times bioassays, although the brain samples of three mice groups were PCR-positive after repeated bioassays. The PCR results showed a moderate frequency of *N.caninum* infection in aborted bovine fetuses. Furthermore, the isolates obtained in this study had low pathogenicity in BALB/c mice. It seems the isolates belong to an avirulent strain

Keywords: *Neospora*; Abortion; Cattle; PCR; Bioassay examination;

بررسی مولکولی و زیست سنجی *نیوسپورا کانینوم* در جنین های سقط شده گاوی در منطقه مشهد، استان خراسان رضوی، ایران.

نیوسپورا کانینوم نقش بسزایی در ایجاد سقط جنین و نارسایی تولید مثل در گاوهای شیری دارد. اکثر سقط های مربوط به نئوسپوروزیس در طی ۵ تا ۶ ماه بارداری اتفاق می افتد. مرگ جنین در رحم، مومیایی شدن، اتولیز، مرده‌زایی، تولد با علائم بالینی یا تولد از نظر بالینی سالم اما با عفونت مزمن، همگی پیامدهای احتمالی این آلودگی هستند. هدف از این مطالعه تعیین آلودگی *نیئوسپورا* در جنین‌های گاو سقط‌شده از طریق آنالیز مولکولی و تست زیست‌سنجی موش بود. از سال ۲۰۱۹ تا ۲۰۲۲، تعداد ۱۲۱ جنین سقط شده گاوی از گاوداری های استان خراسان رضوی جمع آوری شد. نمونه های مغز جنین برای یافتن DNA انگل با روش واکنش زنجیره ای پلیمراز غربالگری شدند. علاوه بر این، بخشی از بافت مغزی جنین ها یی که در آزمایش واکنش زنجیره ای پلیمرازمثبت شده بودند، پس از طی مراحل همگن سازی به روش داخل صفاق به پنج موش BALB/c تلقیح شدند. در پایان تجربه ، همه موش‌ها شش هفته پس از آلودگی به روش انسانی کشته شده و نمونه های خونی و بافتی آنها با روش‌های سرولوژی، میکروسکوپی واکنش زنجیره ای پلیمراز مورد بررسی قرار گرفتند. چنانچه نمونه‌های مغز موش‌ها با آزمایش واکنش زنجیره ای پلیمراز واکنش مثبت نشان می دادند، آزمایش زیست‌سنجی موش جهت جدا سازی بهتر دو باره تکرار می گردید. در این بررسی در ۱۹/۸ درصد نمونه های مغز در جنین های سقط شده گاوDNA *نیوسپورا* شناسایی شد. از بین نمونه‌های مغزی PCRمثبت ، تنها ۱۰ نمونه برای بررسی زیست‌سنجی در موش ها مناسب بودند. همه موش‌های تلقیح‌شده پس از سه بار آزمایش زیست سنجی فاقد علائم بالینی بوده و در آزمایش سرولوژی نیز نتیجه منفی نشان دادند، در صورتیکه آزمایش PCR در تعدادی از نمونه‌های مغزی دو گروه از موش های تلقیح شده پس از دو آزمایش زیستی سنجی مکرر مثبت بودند. نتایج این مطالعه نشان داد که درصد قابل توجهی از جنین های سقط شده گاو

آلوده به *نیوسپورا* بودند . علاوه بر این ، این جدایه ها در موش BALB/C بیماری زایی پایینی داشتند، بنظر می رسد این جدایه ها متعلق به سویه های غیر حاد *نیوسپورا* باشند.

کلمات کلیدی: *نیوسپورا*،سقط جنین، گاو، واکنش زنجیره ای پلیمراز، بررسی زیست‌سنجی

1. Introduction

Neospora caninum is recognized as the primary cause of abortion in cattle worldwide (1). There are two ways that *N. caninum* can be transmitted to cattle. The main method of infection is typically referred to as vertical, congenital, or endogenous transmission. This occurs when the bardyzoites of cysts in the dam's tissues become active and transform into tachyzoites, which then travel through the placenta and into the fetus. The secondary method of infection is known as horizontal or post-natal transmission. This happens when pregnant dairy cattle consume sporulated *N. caninum* oocysts, and the sporozoites transform into tachyzoites, likely spreading through the circulation in cells of the mononuclear phagocytic system and potentially infecting the fetus through transplacental transmission (2). The endogenous (vertical) transmission could occur in approximately up to 95% of infected dairy cattle (1,3). The high seroprevalence of *Neospora* infection has been shown in dairy cattle in Iran (4,5,6,7). Hence, it was found that *N. caninum* infection also plays a role in causing abortion in dairy cattle (8,9). Although there is a high prevalence of *N. caninum* infection in dairy cattle in Iran, only one *N. caninum* isolate was recovered from a bovine fetus that was aborted (10). The present study aimed to detect the frequency of *N.caninum* infection in aborted bovine fetuses and evaluate the pathogenicity of *N. caninum* in BALB/c mice

2. Materials and Methods

The research was conducted in the northeastern province of Khorasan Razavi in Iran, covering an area of over 127,000 km2 and located at coordinates 33°30′-37°41′ N; 56°19′-61°18′ E. This province falls within the northern temperature zone and experiences a semi-arid climate characterized by cold winters and moderate summers. There are approximately 25000 cattle distributed across 110 dairy farms in this region, with herd sizes ranging from 30 to 2000 cattle varying between farms. The predominant cattle breed in the area is Holstein-Friesian.

2.1. Sample collection

From 2022 to 2023, 121 aborted bovine fetuses were collected from different areas of the province. Then, the fetuses were necropsied, and collected the brain tissue for molecular and bioassay examination

2.2. DNA extraction and PCR

Samples were used to extract genomic DNA with the MBST Genomic DNA kit (Molecular and Biological Transmission Systems, Tehran, Iran) following the manufacturer's instructions. After that, we conducted a PCR assay to identify the *N.caninum* gene, following the previously described method by Müller et al 2002(11).

The simple PCR reaction was performed in a 25µl mixture containing 2µl of total DNA, 10µl of commercial premix master mix (Parstous Mashhad), 1µl of each primer, and 11µl of nuclease-free water in a thermocycler. The cycling process began with an initial denaturation step at 95 °C for 5 minutes, followed by 40 cycles of 94 °C for 1 minute, 63 °C for 1 minute, and 74 °C for 3.5 minutes, and concluded with a final extension step at 74 °C for 10 minutes.

The Oligonucleotide primers used were NP21plus (5'CCCAGTGCGTCCAATCCTGTAAC3') and Np6plus (5'CTCGCCAGTCAACCTACGTCTTCT3').

2.3. Bioassay in mice

Six to eight-week-old female BALB/c mice were acquired from the Razi Vaccine and Serum Research Institute (Mashhad Branch). The mice were kept in plastic box cages in groups of 5 and given rodent feed and water ad libitum under standard conditions. A total of 100 grams of brains from PCR-positive aborted fetuses were homogenized in 500 ml of 0.85% NaCl solution (saline) containing antibiotics (100 IU/ml penicillin and 745 IU/ml streptomycin) and then homogenized using an electrical mixer. The mixture was subsequently filtered through two layers of gauze. After an incubation period of three hours at room temperature, the samples were centrifuged at 1500 g for five minutes, and 5 ml of the homogenate deposit was administered intraperitoneally to 5 BALB/c mice (1 ml per mouse).

The mice were observed daily for any clinical signs indicating neosporosis. Blood samples were obtained from the mice's tails six weeks after they were inoculated, and their serum samples were analyzed using an Elisa kit (ID screen ® N. caninum indirect Multi-species, ID. vet, Montpellier, France) to detect *Neospora* antibodies. All the mice that were inoculated were euthanized 42 days after being infected using chloroform inhalation. Brain impression smears were prepared and examined under a microscope to detect cysts. A portion of the brain tissue was tested for *N.caninum* DNA using PCR. The PCR-positive brain samples from each group of mice were combined and then inoculated intraperitoneally into five BALB/C mice. These inoculated mice were observed daily and then euthanized seven weeks after being inoculated. During the necropsy, blood and brain samples were collected and analyzed

using the serology and PCR methods mentioned earlier. If the brain samples from the mice tested positive via PCR, the mice bioassay examination was repeated to once again find a viable cyst.

3. Results

In the present study, *N.caninum* DNA was detected in 19.8% (24/121) of brain samples of bovine aborted fetuses (Fig1). Among 24 PCR-positive brain samples, only 10 samples were suitable for mouse bioassay examination. In the first bioassay round, All inoculated mice in ten groups were normal without clinical signs and the serology and microscopy results were also negative after 42 days post-infection, but, *N.caninum* -DNA were detected in five brain samples of two mice groups by PCR. Similar results were obtained after two rounds of mice bioassay examination in BALB/C mice that were inoculated with PCR-positive mice brains of infected groups. (Table 1).

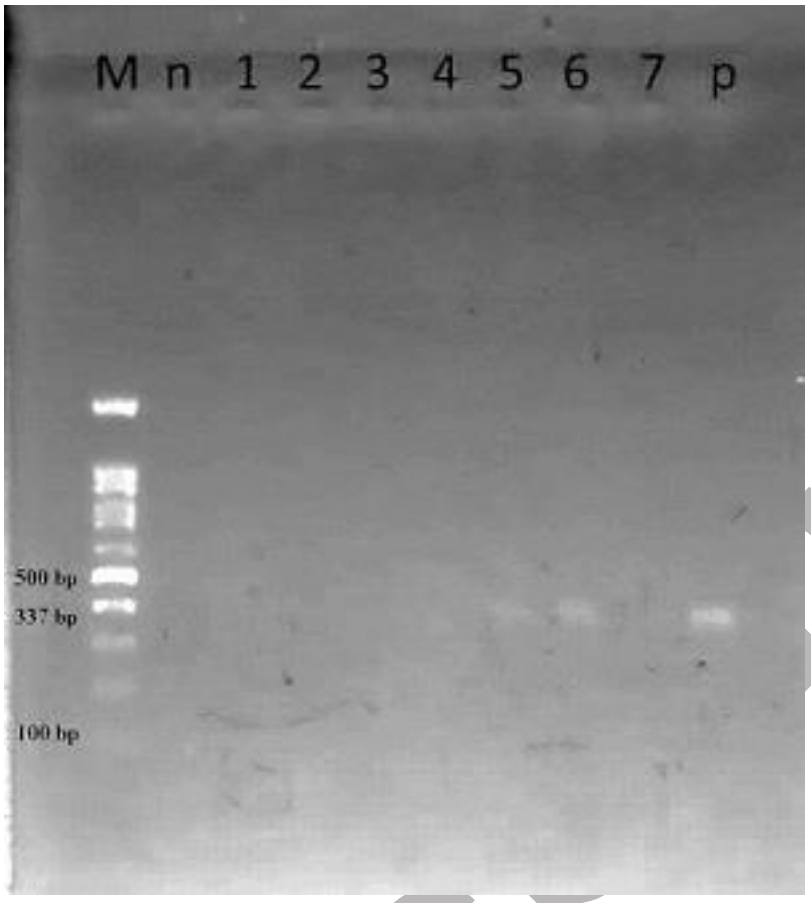

Fig.1. PCR amplification products of *N. caninum* in brain samples Lanes: M: molecular weight marker (between 1000 and 100bp); p: positive control (*Neospora* tachyzoites; 337 bp); n: negative control; 5, 6: positive samples.

١٣٢ Table1- The results of first, second and third round of mouse bioassay examination on PCR -positive bovine and mice brain samples.

Results	**First round**			**Second round**			**Third round**		
Group	bovine aborted fetuses (homogenized brain)			Mice			Mice		
(5 mice)	ELISA	Microscopy	PCR	ELISA	Microscopy	PCR	ELISA	Microscopy	PCR
1	N	N	N	nd	nd	nd	nd	nd	nd
2	N	N	N	nd	nd	nd	nd	nd	nd
3	N	N	P	N	N	P	N	N	P
4	N	N	P	N	N	N	nd	nd	nd
5	N	N	N	nd	nd	nd	nd	nd	nd
6	N	N	N	nd	nd	nd	nd	nd	nd
7	N	N	P	N	N	P	N	N	P
8	N	N	N	nd	nd	nd	nd	nd	nd
9	N	N	P	N	N	N	nd	nd	nd
10	N	N	P	N	N	P	N	N	P

١٣٣ P=Positive, N=Negative, nd= not done

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4. Discussion

In this research, N. caninum DNA was identified in 19.8% (24 out of 121) of brain samples taken from aborted bovine fetuses using the PCR method. The prevalence of N. caninum infection in aborted bovine fetuses across various provinces in Iran has been reported to range from 12% to 67%, as determined by PCR techniques (Table 2). A meta-analysis indicated that the prevalence of *N. caninum* in aborted fetuses was greater in studies with fewer than 50 samples compared to those with more than 50 samples (23). The author concluded that the pooled estimate for the subgroup with a sample size of 50 or more could provide a more accurate, conservative, and reliable representation of the overall infection rates in aborted bovine fetuses in Iran (23).

Table2- List of different studies frequency of *N. caninum* infection in brain tissue of aborted bovine fetuses in different areas of Iran

province	Study Year	Method	Number examined	Number infected	Frequency	References
Khorasan Razavi	2007	PCR	6	4	67%	(12)
Khorasan Razavi	2007	PCR, IHC	100	13	13%	(8)
Khorasan Razavi	2010	PCR	151	18	12%	(13)
Khorasan Razavi	2013	PCR	200	23	12%	(14)
East Azerbijan	2013	PCR	14	6	43%	(15)
Charhar mahal & Bakhtiiari	2013	PCR	100	11	11%	(16)
Tehran	2014	PCR	16	12	75%	(17)
Qazvin	2014	PCR	128	39	31%	(18)
East azerbiajan	2018	PCR	82	34	41%	(19)
Markazi	2018	PCR	38	10	26%	(20)
Mazandaran	2019	PCR	9	2	22%	(21)
Mazandaran	2021	PCR	78	16	20.5%	(22)

The brain tissue of bovine aborted fetuses is the main source of *N.caninum* isolation (24) but some studies showed that most *N.caninum* cysts in brain tissue were probably non-viable due to autolysis effect (25, 26). In this study, many positive brain samples were autolyzed after abortion and only ten PCR-positive brain samples were suitable for bioassay examination. All inoculated mice in ten bioassay groups showed no clinical signs and no *Neospora* cysts in mice brain tissue, but *N.caninum* DNA was detected in the brain samples of five mice in three bioassay groups. The same results were obtained after conducting two repetitive mouse bioassay examinations with PCR-positive brain samples of mice. It seems that these *N.caninum* isolates from aborted bovine fetuses were avirulent strains in BALB/C mice. So far, virulent to avirulent strains of *N. caninum* have been reported by mice bioassay method (24, 27).

The BALB/C models have been used to determine of pathogenicity of *N. caninum* isolates in bovine aborted fetuses. Some isolates had low virulence and were shown no clinical symptoms and no detectable *N.caninum* cysts in mice brains (28-31).

The PCR results indicated the presence of N.caninum infection in the brain samples of aborted bovine fetuses and in two groups of inoculated mice. However, it remains unclear why no antibodies against N. caninum were found in inoculated mice after three rounds of bioassays. Some studies also reported seronegative results for *Toxoplasma gondii* or *N.caninum* infection in different laboratory animals when inoculated with infected mice or rat brain tissue (32,33). They suggested that the lack of clinical signs and detectable antibodies against to *T.gondii* or *N.caninum* infection may be due to the rapid death of parasites in the neural tissue of mice, but had DNA intact present. The duration between the extraction and analysis of brain tissue might have been excessive to support the viable tissue cysts in the

brain tissue (33). To sum up, this research demonstrated the existence of N.caninum infection in brain-aborted bovine fetuses in the Mashhad region. The result strongly suggests the high frequency endogenous transmission among dairy cattle in Iran. In addition, the bioassay examination also provides further evidence that *N.caninum* isolates might be an avirulent strain and needs to more investigation.

Declarations and statements

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Conflict of interests:

The authors declare no conflict of interest.

Data availability:

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics and animal experimentation

The mice were housed and maintained in the animal care facility at Ferdowsi University of Mashhad. All animal experiments were performed in strict accordance with the guidelines approved by the Animal Ethics Committee of our faculty IR.UM.REC.1399.063.

Author contribution

Study concept and design: G.R.R. Conduction: G.R.R. Molecular, Serological and Bioassay examination:A.K., M.S., Formal analysis and investigation: G.R.R.,A.K., M.S., Writing - original draft preparation: G.R.R., Funding acquisition: G.R.R.

Consent to participate:

Not applicable

Consent for publication:

Not applicable

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