DETECTION OF *NEOSPORA CANINUM* DNA BY PCR ANALYSIS IN BOVINE ABORTED FOETUSES *

Maria Silvia Gennero [1], **Stefania Bergagna** [1], **Marco Pasino** [2], **Angelo Romano** [2], **Davide Grande** [2], **Anna Trisciuoglio** [2] and **Ezio Ferroglio** [2]

RESUMÉ

La néosporose est une infection des bovins reconnue au niveau mondial comme cause primaire d'avortement, aussi bien dans les élevages à viande, que dans les élevages à vocation laitière. Nous avons évalué la présence de ce protozoaire parasite dans les avortons par la méthode PCR dans les élevages de la Région du Piedmont (Italie), puis nous avons comparé les résultats obtenus avec les résultats de la sérologie pour établir la corrélation avec la séroprevalence. L'analyse PCR a été réalisée sur les tissus de 71 fœtus avortés provenant de 34 élevages à sérologie positive pour *N. caninum*. Nous avons mis en évidence une séroprévalence majeure au moment du vêlage dans les élevages à viande. Cette étude confirme que *N.caninum* est une cause importante d'avortement et que la recherche du DNA par PCR est une méthode valide pour détecter *N.caninum*.

Mots-clés : *Neospora caninum,* bétail, PCR.

SUMMARY

Neosporosis is a common infection in cattle and is a major cause of abortions worldwide. *Neospora caninum* abortions have been reported in both dairy and beef cattle. We evaluated the presence of this protozoal parasite in aborted foetuses by mean of PCR analysis in herds of the Piedmont Region (Italy) Subsequently we compared these results with those obtained by serodiagnosis in order to establish a correlation with seroprevalence. PCR analysis was carried out on tissues from 71 aborted foetuses (liver, muscle, kidney, head) collected from 34 selected herds with a prior history of abortion problems and seropositivity for *N. caninum*. Seroprevalence in pregnant beef cattle was higher than in dairy cattle, but in the latter we found a higher prevalence of positive aborted foetuses at PCR analysis. This study confirms that *N. caninum* is an important cause of abortion and that DNA detection by PCR analysis is a valid tool for diagnosis of *N. caninum* infection in aborted foetuses.

Keywords : *Neospora caninum*, Cattle, PCR analysis.

* Texte de la communication affichée lors des Journées AEEMA, 22-23 mai 2008

[1] Istituto Zooprofilattico Sperimentale di Piemonte, Liguria e Valle d'Aosta, Via Bologna, 148-Torino, Italy ; mariasilvia.gennero@izsto.it

[2] Dipartimento di Produzioni Animali, Epidemiologia ed Ecologia, Via Leonardo da Vinci 44 Grugliasco, Turin, Italy

I - INTRODUCTION

Firstly identified in dogs, Neospora caninum is a coccidian protozoan that parasites many species of animals. In bovine species, Neosporosis belongs to the most frequently diagnosed causes of abortion world-wide, both in dairy and beef cattle and it can cause severe economic losses due to abortion, milk losses and early culling. Positivities to N. caninum has been signalled from many regions of the world including Italy.

Bovine neosporosis can be diagnosed in cattle using serology and in foetuses using methods such as histopathology, immunohistochemistry and polymerase chain reaction (PCR) [Dubey et al., 2007].

We deemed interesting to evaluate the presence of N. caninum DNA in aborted fetuses by PCR in the herds considered of our Region in order to correlate positivity with herd seroprevalence, herd aptitude and management. We test different tissues of aborted fetuses at different age to check the main localization of the parasite and to search the most sensitive organ to probe by genomic analysis in making diagnosis of fetal neosporosis.

II - MATERIAL AND METHODS

1. SAMPLING

Seventy-one aborted foetuses were collected from 2002 to 2007 from 34 selected herds with prior history of abortion problems and positivity to serological test for *N. caninum*. Herds are located in Piedmomnt and 18 of them are beef cattle herds while 16 are dairy herds. All of the selected dairy farm rise Italian Holstein Fresian breed and all beef farm were composed by Piemontese beef breed. We collected aborted foetuses from our studied farms within 12-24 hours since abortion, and then we executed the necropsy immediately in the laboratory of our Department.

For every foetus a sample was collected at necropsy, with sterile and single use scalpels of at least 25 mg of tissue from brain (CNS), femoral biceps or gluteal muscle, heart (left ventricle), liver, kidney, lung, spleen and testicle.

2. DNA EXTRACTION AND PCR AMPLIFICATION

DNA extraction was performed from 25 mg of tissue from sampled organs, using the Gen EluteTM Mammalian genomic DNA extraction kit (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer's protocol. DNA extracted from cultured *N. caninum* (NC1) tachyzoites was used as a positive control for the PCR reaction and a sample of distilled water as an extraction negative control. An additional water sample was run together with the foetal samples in the PCR in order to control carry-over contamination. The used primers were Np6 plus

(5'CTCGCCAGTCAACCTACGTCTTCT3')

and Np21 plus

(5'CCCAGTGCGTCCAATCCTGTAAC3'),

as suggested by Muller *et al*. [1996].DNA amplification was performed in 50 µl reaction mix, containing 8 µl of DNA extract, 0.5 mM each primer and 25 µl of RedTaqTM Ready MixTM PCR Reaction Mix (Sigma–Aldrich, St. Louis, MO, USA). Amplifications were carried out in a Bio-Rad iCycler thermal cycler. Samples were initially denatured at 94°C for 1 min, then submitted to 40 amplification cycles at a denaturing temperature of 94°C for 1 min, an annealing temperature of 63°C for 1 min, and an extension temperature of 74°C for 3 min. A final extension step of 72°C for 10 min was performed.

After amplification, 10 µl aliquots from each reaction were analysed by electrophoresis on 2% agarose gel, in comparison with molecular weight markers (DNA Molecular Weight Marker V, Roche Diagnostics, Mannheim, Germany ; PCR 100 bp Low Ladder, Sigma-Aldrich, St. Louis, MO, USA). Gels were stained with MegaFluor kit (Euroclone, Milano, Italy) under the conditions suggested by the manufacturer and photographed on a UV transilluminator using a CCD Camera (Gel-Doc Bio-Rad). Positive controls (DNA extracted from N. caninum NC1 culture) and negative controls

were included in each step. Samples were considered positive when a 337 bp specific Np6plus-Np21plus product [Muller *et al*., 1996] was present.

3. SEROLOGY

Blood samples were collected from 21 of 34 herds. Of these, 8 are beef herds and 13 are dairy herds. Each blood sample was collected in plain Vacutainer tubes, then it was centrifuged and the serum separated, frozen and stored at -20°C until tested. No haemolytic sera were tested. Serum sample was tested for the presence of antibodies to *N. caninum* detected by a commercially available ELISA-kit for bovine serum (HerdCheck Anti-Neospora ; IDEXX Laboratories) according to the manufacturer procedure.

4. STATISTICAL ANALYSIS

Statistical comparison of data was done with EPIINFO (Ver.6) and differences were considered as significant when $p \leq 0.05$.

III - RESULTS

1. SEROLOGY

All the herds we tested had at least one positive animal to ELISA testing. The overall ELISA seroprevalence average of all tested cattle heads was 43% : 376 animals out of 874 tested resulted seropositive. Within-herd prevalences vary from 5% up to 97.2%. In beef cattle we found a higher seroprevalence than in dairies one : 52.6% (182/243) and 36.7% (194/446) respectively. These difference was highly significant by χ^2 test analysis : χ^2=62.56 and p=0,00000. In dairy herds prevalences range from 5% (1/20) up to 63.7% (58/91) while in beef herds range from 38.9 (7/18) up to 97.2% (35/36).

2. PCR

The overall prevalence at PCR analysis of positive foetus coming from the 34 selected herds was 38% : 27 positive foetuses with at least a positive organ on 71 tested. Single organs PCR positivities results are summarized in the table 1.

Table 1

Single organs PCR positivities results

ORGAN	PCR negative	PCR positive	Total tested	percentages
CNS	50	19	69	27%
Muscle	51	14	65	21%
Kidney	51	12	63	19%
Heart	59	6	65	9%
Spleen	16	1	17	6%
Liver	63	3	66	5%
Lung	53	2	55	4%

As regard different tissues analysed belonging to positive foetuses (animal with at least one positive organ by PCR), globally we found 19 positive CNS on 27 tested (70%) ; 14 positive muscles on 24 (58%) ; 12 kidneys on 24 (50%) ; 6 hearts on 24 (25%) ; 1 spleen on 6 (16%) ; 3 livers on 25 (12%) ; 2 lungs on 21 (9%). We also tested the testicles of 2 animals and we found one positive sample. Among these organs the differences in percentage positivity we found are statistically significant χ^2=35.06 ; p=0.000004.

The comparison of percentages of positivity different organs by PCR and the significativity of these differences show that CNS, muscle and kidney are more statistically significant tissues to highlight the presence of *N. caninum* infection.

Considering the positivity of foetuses coming from low seroprevalence herds (less than 30%) compared with higher seroprevalence level herds, we found a statistically significant difference : only 3 on 22 (14%) were positive foetuses belonging to low prevalence herds while 19 on 32 (59%) came from high prevalence herds (χ^2 =11.30 ; p=0.00077).

As regards PCR tissues testing of fetuses coming from different aptitude and management herds, it seems that there are more positive subjects in dairy, 28 positive on 48 tested (42%), than in beef herds where there are 16 positive on 23 tested (30%). This difference becomes more clear and statistically significant in clustering cattle by global herd seroprevalence : in herds with a seroprevalence higher than 27% we found 2 positive foetuses on 12 tested in beef (17%) and 18 on 32 respectively in dairy (56%) [χ^2 =5.52 ; p=0.019].

IV - DISCUSSION

Our results evidence an high infection rate in piedmontese cattles. All herds tested had at least one seroreactor and 43% of animals tested positives in ELISA. These data confirm previous survey and evidence that Neosporosis is widespread in Piedmontese's herds. Even if some false positive can occours in ELISA, it is noteworthy that even in a dairy herd with low level prevalence (5.3%) a *N.caninum* PCR positive aborted fetus was found. This data confims that the parasite is present in the herd and moreover that abortion can accur also in herds with low seroprevalence.

Regarding herd prevalences, up to 97.2% of cattles tested positive in some herds.

In beef cattle we found a very significantly higher seroprevalence than in dairies ones, 52.6% (182/243) and 36.7% (194/446) respectively. This finding is in contrast with other serological surveys where prevalences in beef cattle were lower than in dairy cattle like in Spain, where more dairy than beef herds were *N. caninum* positive. These results must be interpreted with caution, because the differences observed might have been caused by differences in the production systems used for the different breeds and not by differences in breed-related susceptibility to infection. In fact, in piedmont daiy cattle are mainly keept in intensive farms, while beef cattle are usually reared in extensive systems and graze in open range also in mountain during summer.

PCR results 38% of positive foetus is quite high and highlight the role of *N.caninum* in the abortion in cattle reared in the area.

Totally, 19 out of 69 CNS samples (27%) were positive at PCR. This result confirms that CNS is a major site of localization for *N. caninum*, even if in 2 foetuses, PCR positive in other tissue, PCR was negative in CNS. A lot of studies indicate that brain tissue is the most suitable for the detection of *N. caninum* DNA by PCR [Baszler *et al*., 1999 ; Buxton *et al*., 1998 ; Collantes-Fernández *et al*., 2005 ; Gottstein *et al*., 1998 ; Ho *et al*., 1997]. However, due to tissues sampling procedures, false negative results can be obtained also from SNC tissue. In our analysis CNS (27%), muscle (21%) and kidney (19%) are the organs with a higher PCR positive results. Based on obtained data we suggest that sampling on aborted foetuses must include CNS, muscle and kidney when *N.caninum* infection must be confirmed or excluded.

BIBLIOGRAPHY

Anderson M.L., Blanchard P.C., Barr B.C., Dubey J.P., Hoffman R.L., Conrad P.A. - Neospora-like protozoan infection as a major cause of abortion in California dairy cattle. *J. Am. Vet. Med. Assoc*., 1991, **198**, 241-244.

Baszler T.V., Long M.T., McElwain T.F., Mathison B.A. - Interferon-gamma and interleukin-12 mediate protection to acute Neospora caninum infection in BALB/c mice. *Int. J. Parasitol.*, 1999, **10**, 1635-1646.

Buxton D., Maley S.W., Wright S., Thomson K.M., Rae A.G., Innes E.A. - The pathogenesis of experimental neosporosis in pregnant sheep. *J. Comp. Pathol.*, 1999, **118**, 267–279.

Collantes-Fernández E., Rodriguez-Bertos A., Arnaiz-Seco I., Moreno B., Aduriz G., Ortega-Mora L.M. - Influence of the stage of pregnancy on Neospora caninum distribution, parasite loads and lesions in aborted bovine foetuses. *Theriogenology*, 2005, **65**, 629–641.

Collantes-Fernández E, Arnáiz-Seco I, Burgos BM, Rodriguez-Bertos A, Aduriz G, Fernández-García A, Ortega-Mora LM. - Comparison of Neospora caninum distribution, parasite loads and lesions between epidemic and endemic bovine abortion cases. *Vet. Parasitol.*, 2006, **142** (1-2), 187-191.

Davison H.C., Otter A., Trees A.J. - Significance of Neospora caninum in British dairy cattle determined by estimation of seroprevalence in normally calving cattle and aborting cattle. *Int. J. Parasitol.,* 1999, **29**, 1189-1194.

De Meerschman F., Speybroeck N., Berkvens D., Rettignera C., Focant C., Leclipteux T., Cassart D., Losson B. - Fetal infection with Neospora caninum in dairy and beef cattle in Belgium. *Theriogenology*, 2002, **58**, 933-945.

Dubey J.P., Lindsay D.S. - A review of Neospora caninum and neosporosis. *Vet. Parasitol.*, 1996, **67**, 1-59.

Dubey J.P. - Neosporosis in cattle : biology and economic impact. *J. Am. Vet. Med. Assoc.*, 1999, **214**, 1160-1163.

Dubey J.P. - Review of Neospora caninum and neosporosis in animals. *Korean J. Parasitol.*, 2003, **41**, 1-16.

Dubey J.P., Schares G., Ortega-Mora L.M. - Epidemiology and Control of Neosporosis and Neospora caninum. *Clin. Microbiol. Rev.,* 2007, **20**, 323-367.

Ferroglio, E., Rossi, L. - , Prevalence of Neospora caninum antibodies in wild ruminants from the Italian Alps. *Vet. Rec.*, 2001, **148**, 754-755.

Ferroglio, E., Trisciuoglio A. - Antibodies to Neospora caninum in European brown hare (Lepus europaeus). *Vet. Parasitol.*, 2003, **115,** 75-78.

Ferroglio E., Bassano B., Trisciuoglio A., Rossi L. - Antibodies to Neospora caninum in Alpine ibex from the Italian Alps. *Zeitschrift fur Jagdwissenschaft*, 2003, **47**, 226-228.

Ferroglio, E., Guiso, P., Pasino, M., Accossato, A., Trisciuoglio, A. - Antibodies to Neospora caninum in stray cats from north Italy. *Vet. Parasitol.,* 2005, **131**, 31-34.

Ferroglio, E., Pasino, M., Ronco, F., Bena, A., Trisciuoglio, A. - Seroprevalence of Antibodies to Neospora caninum in Urban and Rural Dogs in north-west Italy. *Zoonoses Public Health*, 2007, **54,** 135-139.

Gottstein B., Hentrich B., Wyss R., Thür B., Busato A., Stärk K.D.C., Müller N. - Molecular and immunodiagnostic investigations on bovine neosporosis in Switzerland. *Int. J. Parasitol.*, 1998, **28**, 679–691.

Ho M.S.Y., Barr B.C., Tarantal A.F., Lai L.T.Y., Hendrickx A.G., Marsh A.E., Sverlow K.W., Packham A.E. P.A. - Conrad, Detection of Neospora from tissues of experimentally infected rhesus macaques by PCR and specific DNA probe hybridization. *J. Clin. Microbiol.,* 1997, **35,** 1740–1745.

Packham, A.E., Sverlow, K.W., Conrad, P.A., Loomis, E.F., Rowe, J.D., Anderson, M.L., Marsh, A.E., Cray, C., Barr, B.C. - A modified agglutination test for Neospora caninum : development, optimization, and comparison to the indirect fluorescent-antibody test and enzyme-linked immunosorbent assay. *Clin. Diagn. Lab. Immunol.*, 1998, **5**, 467-473.

Pare, J., Thurmond, M.C., Hietala, S.K. - Congenital Neospora caninum infection in dairy cattle and associated calfhood mortality. *Can. J. Vet. Res.*, 1996, **60**, 133-139.

Razmi GR, Maleki M, Farzaneh N, Talebkhan Garoussi M, Fallah AH. - First report of Neospora caninum-associated bovine abortion in Mashhad area, Iran. *Parasitol. Res.*, 2007, **100** (4), 755-757.

Rinaldi, L., Fusco, G., Musella, V., Veneziano, V., Guarino, A., Taddei, R., Cringoli, G. - Neospora caninum in pastured cattle : determination of climatic, environmental, farm management and individual animal risk factors using remote sensing and geographical information systems. *Vet. Parasitol.*, 2005, **128**, 219-230.

Varcasia, A., Capelli, G., Ruiu, A., Ladu, M., Scala, A., Bjorkman, C. - Prevalence of Neospora caninum infection in Sardinian dairy farms (Italy) detected by iscom ELISA on tank bulk milk. *Parasitol. Res.*, 2006, **98**, 264-267.