Journal of Veterinary Science & Technology

Marino et al., J Vet Sci Technol 2018, 10:1

Research Article Open Access

Arthrogryposis Multiplex Congenita due to *Toxoplasma gondii* Infection in a Newborn Calf

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Received date: November 12, 2018; **Accepted date:** December 7, 2018; **Published date:** December 14, 2018

Abstract

Arthrogryposis Multiplex Congenita (AMC) includes various conditions of unclear etiology but in general any cause of reduced fetal movement may result in congenital contractures. Neurologic abnormalities appear as the most common causes of AMC (approximately 70% to 80% of all cases). This paper describes a case of clinical congenital toxoplasmosis in a newborn calf and suggests that the protozoan *Toxoplasma gondii* may also be responsible for AMC. In fact, a central nervous system involvement appears to be present in most cases of AMC and in this case the presence of *Toxoplasma gondii* in calf's brain was demonstrated through molecular biology examination and immunohistochemistry analysis.

Keywords: Arthrogryposis; Congenital toxoplasmosis; Cattle; Real time PCR; Immunohistochemistry

Introduction

Arthrogryposis Multiplex Congenita (AMC) is a rare sporadic non-progressive congenital disorder characterized by multiple joint contractures which may include muscle weakness and fibrosis [1,2]. Research has indicated that any cause which inhibits normal joint movement before birth can result in joint contractures since the tendons connecting to joints are not stretched to their normal length [3]. Viruses, neuromuscular diseases, hyperthermia, toxins, insecticides and limb immobilization are responsible for contractures in laboratory animals [2]. Prenatal contractures in farm animals have been correlated to the pregnant animal foraging on plants containing toxic alkaloids [2]. Teratogens identified as causes of arthrogryposis include plants such as lupines (anagyrine being the toxic agent) ingested by pregnant cows between day 40 and day 70 of gestation [2]. Prenatal contracture epizootics in cattle have been correlated to *Akabane* viral infections [4], while individual cases have been correlated to *Schmallenberg* [5] or *Bluetongue* [6] viral infections. Arthrogryposis can be observed in all breeds of cattle, particularly in Charolais [7] and in Angus [8], at birth, the joints of affected calves appear fixed in abnormal positions frequently with scoliosis and kyphosis. The calves are usually incapable of standing or nursing. Muscle changes, in particular atrophy, have also been observed. Neuron necrosis and white matter lesions may be seen in the spinal cord. The arthrogryposis syndrome is due to an autosomal recessive gene with complete penetrance in the homozygous state in Charolais. In Angus cattle, on the other hand, AMC is the result of three gene mutations causing failure of normal neuromotor development *in utero*.

In humans, fetal akinesia is the major cause of AMC and there are multiple and varied intrinsic as well as extrinsic causes for reduced fetal movements. Hall [9] describes the following groups of causes responsible for impaired fetal movement: Neurologic Abnormalities (brain, spine, or peripheral nerve), Muscle Abnormalities (muscular dystrophies or mitochondrial abnormalities), Connective Tissue Abnormalities (diastrophic dysplasia), Intrauterine Space Constraint (oligohhydramnios, fibroids, and uterine malformations) and Vascular Compromise (fetal hypoxia leading to anoxic injury of tissue), Maternal Diseases, and Teratogenic Exposure. A rare case of Arthrogryposis Multiplex Congenita due to *Toxoplasma gondii* infection in a newborn calf was examined. The affected calf, full term male of Brown Swiss breed coming from a farm in eastern Sicily, died shortly after birth and was submitted to the laboratories of Experimental Zooprophylactic Institute of Sicily through the National Veterinary Services. A full necropsy was performed and histopathology samples were obtained from the central nervous systems, lung, liver, spleen, intestine, muscle, heart and kidney. In addition, on the same samples it was performed immunohistochemical examination (IHC) for the detection of *Toxoplasma gondii*.

Materials and Methods

Samples for histopathology analysis (central nervous systems, lung, liver, spleen, intestine, muscle, heart and kidney) were fixed in 10% neutral buffered formalin, processed routinely, sectioned at 2-3 µm and stained with hematoxylin and eosin. Same samples were performed for immunohistochemical examination (IHC) for the detection of *Toxoplasma gondii*. IHC was performed using the EnVision+Dual Link System-HRP (Dako, Denmark). This system is a two-step IHC staining technique based on an HRP labelled polymer, which is conjugated with secondary antibodies. The labelled polymer does not contain avidin or biotin. Consequently, non-specific staining resulting from endogenous avidin-biotin activity is eliminated or significantly reduced. Any endogenous peroxidase activity was quenched by incubating the tissues for 5-10 minutes with Dual Endogenous Enzyme Block. The tissues were then incubated with a ready-to-use primary antibody versus *Toxoplasma gondii* (rabbit polyclonal, Thermo Fisher Scientific-UK), followed by incubation with the labelled polymer using a 30 minute incubation for each. Staining was completed by a 5-10 minutes

incubation with 3,3-diaminobenzidine (DAB+) substrate-chromogen which results in a brown-colored precipitate at the antigen site.

Heart, spleen and brain samples were examined for *Toxoplasma gondii* by AF targeted real time PCR. The spleen was also examined for *Neospora caninum* by PCR. Other lung, liver, spleen, intestine, heart, brain and kidney samples were examined for *Adenovirus* (AV) by tissue culture, for *Bovine Herpes virus 4* (BHV4) by tissue culture, for *Bovine Viral Diarrhea virus* (BVDV) by tissue culture and antigen capture Enzyme-Linked Immunosorbent Assay (ELISA), for *Schmallenberg virus* (SBV) by real time PCR, for *Infectious Bovine Rhinotracheitis virus* (IBR) by tissue culture, for *Bovine Viral Diarrhea/Mucosal Disease virus* (BVDV) by real time PCR, for *Parainfluenza 3 virus* (PI 3) by tissue culture, for *Bovine Respiratory Syncytial virus* (BRSV) by tissue culture and for *Bluetongue virus* (BTV) by tissue culture.

The method for virus isolation was performed using confluent monolayers prepared by primary embryonic bovine kidney cell cultures or established cell lines as MDBK or AUBEK, virus strains at a known concentration and specific immunoglobulin FITC conjugated. The cell cultures inoculated with the sample were examined every 24 hours for 3-7 days in order to highlight the cytopathic effect (CPE) of the virus. The control cell cultures, uninoculated, must remain free for the entire incubation period. Positive control cell cultures must present CPE. If CPE occurs in inoculated cell cultures, it is necessary to proceed with the identification of the isolated virus by Direct Immunofluorescence using specific immunoglobulin FITC conjugated. Furthermore, bacteriological examinations were performed on various organs using routine techniques to search for *Staphylococcus* spp., *Streptococcus* spp., *Campylobacter* spp., *Salmonella* spp., *Brucella* spp. and *Chlamydia* spp.

Several exams were performed by serological tests (SN and IIF) on the affected calf's dam. AF targeted Real Time PCR was employed to detect *Toxoplasma gondii*. The chosen DNA target was sensitive enough not to miss any positives because the 529-bp repeated element [10,11] sequence is a common element dispersed in the parasite genome. The real time PCR test was conducted in a 20 µL final volume mix containing: 4 µL master mix 5 × added to water (9.6 µL), 10 pmols of each primer AF1 (CACAGAAGGGACAGAAGT) and primers AF2 (TCGCCTTCATCTACAGTC) 10 × conc. (0.5 µL), probe 529 (6FAM-CTCTCCTCCAAGACGGCTGG-BHQ) 10 × conc. (0.4 µL), 5 µL of DNA template. The amplification protocol was conducted using Light Cycler TaqMan Master (Roche). The protocol used included 10 minute incubation at 95°C for the hot start followed by 40 cycles of 3-step amplification: denaturation 95°C for 10 seconds, annealing 55°C for 20 seconds and extension at 72°C for 30 seconds. Each batch of PCR assays included a DNA isolated from the RH strain of *Toxoplasma gondii* (ATCC® 50174D™) as positive control template and a water negative control templates.

Results and Discussion

Observed lesions were flexure of the carpal and tarsal joints and flexure of the metacarpophalangeal and metatarsophalangeal joints in combination with a moderate lateral rotation of the phalanges causing medial deviation of the fingertips (Figure 1). No limb bone, joint or muscle lesions were found. No others skeletal malformations were apparent. The head of the stillborn calf was normal in size. No secondary palatoschisis, brachygnathia superior or tongue protrusion were found. Congenital toxoplasmosis is rarely reported in cattle despite the possibility of reproductive disorders, such as abortion, being due to the infection [12,13]. In Switzerland, Gottstein et al. detected *Toxoplasma gondii* DNA in 5% of bovine fetuses with viable *Toxoplasma gondii* isolated in two aborted fetuses. It seems clear that *Toxoplasma gondii* can be transplacentally transmitted in cattle, but that this is probably not a common occurrence [11]. Furthermore, there is very little evidence for the correlation between AMC and toxoplasmosis in cattle.

Figure 1: Affected calf with congenital contractures due to congenital *Toxoplasma gondii* infection.

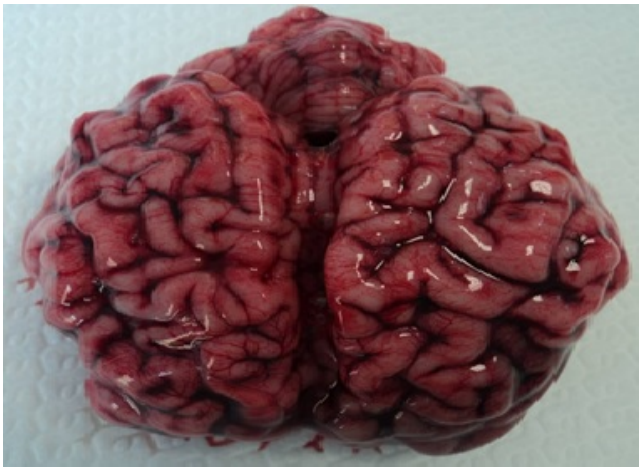

Figure 2: Meningeal vessel congestion was the only lesion observed in the central nervous system (CNS) of the affected calf.

In our case, congestion of meningeal vessels was the only lesions found in the central nervous system (CNS) (Figure 2). No cerebral hypoplasia, lissencephaly, holoprosencephaly, hydrocephalus, porencephalic defect or other grossly visible lesions were observed. Histopathologic features were characterized by microglial nodules scattered throughout the brain and other nonspecific and restricted occasional findings, such focal hemorrhages. All performed virological, bacteriological and parasitological exams were negative. *Akabane virus* was excluded because of the sporadic nature of the case and because of

the absence of the virus in the region. The ingestion of plants such as lupines by pregnant cows was excluded as a result of the type of fixed post housing with use of selected feed. This notwithstanding, the brain and spleen were found positive for *Toxoplasma gondii* by AF targeted real time PCR and only the brain by IHC (Figure 3). With regard to the serological tests performed on the affected calf's dam, the only positivity was for *Toxoplasma gondii* with Enzyme-Linked Immunosorbent Assay (ELISA).

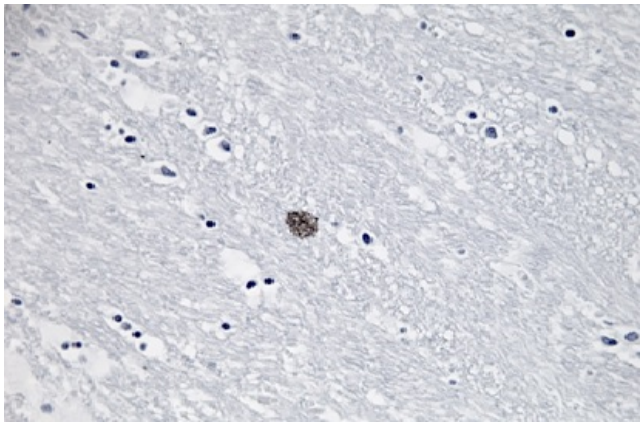

Figure 3: Immunohistochemically the cysts and trophozoites of toxoplasma can be identified within the cerebral tissue (IHC, 400 ×).

Conclusion

A central nervous system involvement appears to be present in most cases of AMC and in this case the presence of *Toxoplasma gondii* in calf's brain was demonstrated through molecular biology examination and IHC. In affected calf, infection with *Toxoplasma gondii* had resulted in a microglial cell response at various stages of activation with formation of nodules. This response was not associated with any other evidence of acute inflammation and the only histopathological manifestation of Toxoplasmic encephalitis was the presence of microglial nodules scattered through the brain. No vascular proliferation, endothelial hyperplasia, perivascular inflammatory infiltrate or necrosis was found. The brain lesion due to congenital toxoplasmosis infection can therefore be considered the cause of the multiple joint contractures and consequent AMC. This paper demonstrates how congenital toxoplasmosis, in addition to well-known fetal abnormalities (hydrocephalus, intracranial calcification, chorioretinitis, etc.) may also be responsible for AMC.

Authors and Contributions

AMFM and AS designed the study, collected, analyzed and interpreted data and wrote the paper. MVB, RG, FS collected and analyzed data and wrote the paper. TA and MP analyzed data.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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