**FINAL REPORT**

The project presented for the 2022-2023 edition of Paolo Cordioli scholarship, entitled “Histological lesions in Bubalus fetus and identification of the main abortion pathogen agents” was structured into two work phases. The first phase was carried out at the U.O.S. Histopathology Laboratory, Experimental Zooprophylactic Institute of Southern Italy, Portici. The second phase was carried out at the Pathological Anatomy Laboratory of the DIVAS, Department of Veterinary Medicine and Animal Sciences, Lodi, in collaboration with Professor Paola Roccabianca. On the 06/13/2022 the first meeting took place with Prof. Roccabianca, Dr. degli Uberti and Prof. Fabrizio Ceciliani to define the terms of the stay, the topic, the goals to be achieved and the work plan.
During the first phase, an initial search was carried out on the paper archives of the Histopathology Laboratory, focusing only on cases of buffalo abortions or calves dead in 24-48 hours from birth coming from the autopsy rooms in the Campania region. The adult buffaloes and calves older than one month were excluded. Data extraction was requested from the software in use at the IZSM in order to know the laboratory tests performed and any positivities detected on these samples: in the end, 28 suitable cases were identified to be included in the search for abortigenic infectious agents.

The research was initially carried out on the evaluation of histological lesions on Haematoxylin-Eosin preparations of all organs and tissues embedded in paraffin for each animal. Then, after the morphological diagnosis based on the score of the lesions found, as suggested by Prof. Roccabianca, immunohistochemical techniques were performed on the target organs for the identification of a specific agent.
The abortigenic pathogens evaluated were Bovine herpes 1 (BAHV1), Bovine viral diarrhea/mucosal disease pestivirus (BVDV), Chlamydia spp., Toxoplasma gondii and Neospora caninum. The following antibodies were purchased by DBA Italia srl:

- BHV-1/IBR Mab gB-gI IgG2b
- Anti-BVDV Monoclonal antibody
- Mouse anti Chlamydia LPS
- Neospora caninum MAb gp65 IgG1 Isotype

Furthermore, DIVAS has kindly provided polyclonal anti-Toxoplasma gondii (rabbit polyclonal antibody; 1:5,000 dilution; ViroStat; Westbrook, ME, USA) and anti-Neospora caninum primary antibodies (goat polyclonal antibody; 1:6,000 dilution; Veterinary Medical Research & Development; Pullman, WA, USA).
In order to collect and analyze data, anatomopathological sheets were drawn up according to the procedures of the DIVAS. The sheet was structured in this way:

- A general section to report the anamnestic data (identification number, sex, race, age);
- A section regarding the macroscopic examination (lesions observed for each organ);
- A section about histological examination (description and morphological diagnosis for each organ);
- A section dedicated to report any other laboratory tests positivity (e.g. PCR);
- A section for the results of the immunohistochemical examination for each pathogen investigated.

The sheet is shown below for illustrative purposes.

| CASO N° | SCHEDA ANATOMOPATOLOGICA |
|---|---|

| Numeri riferimento | RAZZA | SESSO | ETA' |
|---|---|---|---|
| | | | |

**ESAME MACROSCOPICO**

| MATERIALE | LESIONI | NUMERO/DIMENSIONI | ALTRE CARATTERISTICHE MACROSCOPICHE |
|---|---|---|---|
| FETO | | | |
| PLACENTA | | | |

**DIAGNOSI *DEFINITVA ISTO CITO FENOTIPO***

**ESAME ISTOLOGICO**

| Sede lesione | Tipo lesione | |
|---|---|---|
| **PLACENTA** | | |
| | | |
| | | |
| | | |
| **ORGANI INTERNI** | | |
| **Milza** | | |
| **Fegato** | | |
| **Pancreas** | | |
| **Intestino** | | |
| **Polmone** | | |
| | | |
| **ALTRO** | | |

**IMMUNOISTOCHIMICA**

| IHC | | | | | PCR | | |
|---|---|---|---|---|---|---|---|
| NEOSPORA | TOXO | BHV-1 | BVDV | CHLAMIDIA | | | |
| | | | | | | | |

Unfortunately, not all tissues were available for each case since the abortion occurred at different gestation phases and the autopsy was performed in different autoptic rooms. For each organ received and preserved in the form of paraffin inclusions in the histotheques of the Histopathology Unit, two different microsections of 3-4 µm were obtained, by using the rotary microtome and then collected on a slide and stained in Haematoxylin- Eosin by Leica Autostainer XL. The dried slides were digitalized using a Pannoramic 250 scanner, including them in the collection of relevant pathological cases.

The second phase involved the microscopic observation of the slides of the 28 cases stained in Haematoxylin-Eosin and those in immunohistochemistry to respectively detect pathological characteristics correlated to the pathogens researched and any organ immunopositivity. During microscopic observation it was found that the placental tissue, when received, was poorly preserved, to the point of preventing histological interpretation and consequent immunohistochemistry. This eventuality deprived the study of one of the target organs to be investigated. The immunohistochemical examination for etiological agents was conducted on all target organs, as reported in the literature, regardless of the outcome of the RT-PCR diagnosis.
During the compilation phase of the anatomopathological forms, the RT-PCR positivity in 8 of 28 samples to the various pathogens investigated emerged. In particular, 2/8 tested positive for Chlamydia, 1/8 for BVDV, 1/8 for Neospora c., 3/8 for BAHV1, one sample tested positive both for Neospora c. and BAHV1.

**Chlamydia**

For the two samples positive for Chlamydia in RT-PCR, the Haematoxylin-Eosin stained slides of the following organs were observed: lung, liver, kidney, spleen, abomasum, brain, intestine and heart.
The significant pathological lesions observed and compatible with Chlamydial infection were classified, following Storz (1), into: foci of multifocal necrosis; monocytic-lymphocytic inflammation; neutrophilic inflammation and vasculitis.
In the first case (Table 1) the following findings were observed: monocytic-lymphocytic inflammation in the liver, brain and kidney with mild grading (+); Neutrophilic inflammation in kidney (+) and lung (++). In the second case, (Table 2) moderate neutrophilic inflammation (++) was observed in the brain and severe necrosis in the form of multifocal foci (+++) was observed in the kidney. The liver, lung and brain were chosen as target organs to be subjected to immunohistochemistry (IHC) examination, carried out at the U.O.S Histopathology laboratory.
After having identified the sections with the pathological lesions described, they were again microsectioned (3-4µm) and then subjected to the immunohistochemistry session with Leica Bond III using as the first 1:100 dilution of the Mouse anti Chlamydia LPS antibody and inserting a positive control for Chlamydia. The dilution was found to be optimal when comparing the positive control, while the two samples in question did not show any immunopositivity at the lesion sites.

| * | TYPE OF LESION | | | | IHC | PCR |
|---|---|---|---|---|---|---|
| **TARGET ORGAN** | **MULTIFOCAL NECROSIS** | **MONOCYTIC-LYMPHOCYTIC FLOGOSIS** | **VASCULITIS** | **NEUTROPHILIC-HISTIOCYTIC FLOGOSIS** | **1: 100** | **Chlamidya** + |
| **Liver** | | + | | | - | + |
| **Brain** | | + | | | - | |
| **Lung** | | | | ++ | - | + |
| **Kidney** | | + | | + | | |

Table 1

| * | TYPE OF LESION | | | | IHC | PCR |
|---|---|---|---|---|---|---|
| **TARGET ORGANS** | **MULTIFOCAL NECROSIS** | **MONOCYTIC-LYMPHOCYTIC FLOGOSIS** | **VASCULITIS** | **NEUTROPHILIC-HISTIOCYTIC FLOGOSIS** | **1: 100** | **Chlamidya** **+** |
| **Liver** | | | | | **-** | |
| **Brain** | | | | **++** | **-** | |
| **Lung** | | | | | **-** | |
| **Kidney** | **+++** | | | | | |

Table 2

**Bovine viral diarrhea/mucosal disease pestivirus (BVDV)**

For the only sample tested positive for BVDV in RT-PCR there were the following organs on Haematoxylin-Eosin stain slides: lung, heart, kidney and liver. As reported by Baszler (2) and del Piero(3) the relevant pathological lesions have been classified as: necrosis, lymphocyte inflammation, edema, vasculitis and lymphoid hyperplasia. In this case the heart showed mild edema (+), while at the liver level, mild multifocal necrosis was observed (+) associated with moderate lymphocyte inflammation (++) in correspondence with large portions of parenchyma replaced by dissecting fibrosis and hepatocyte atrophy (Tab 3). The hepatic pattern observed, therefore, was compatible with a severe cirrhosis probably related to a possible toxic-metabolic cause. As reported by Baszler (2), the target organ, for revelation of viral antigens, was the brain or, as previously reported by del Piero (3), the skin, but in the case considered neither the brain nor the skin were available; consequently, it was decided to perform IHC on the section of liver in which the described lesions existed. The Anti-BVDV Monoclonal antibody was used at a 1:100 dilution which was found to be suitable when compared with the positive control. Specifically, positivity to Anti-BVDV Monoclonal antibody was detected in the cytoplasm of dendritic cells in the periportal space surrounding a focus of necrosis observed in Haematoxylin-Eosin.
Furthermore, the IHC examination (conducted on the nervous tissue sections of RT-PCR negative animals) showed in only one case mild cytoplasmatic neuronal immunoreactivity. In the same area severe gliosis was also present.

| | TYPE OF LESION | | | | | IHC | PCR |
|---|---|---|---|---|---|---|---|
| **TARGET ORGANS** | **NECROSIS** | **LYMPHOCYTIC FLOGOSIS** | **EDEMA** | **VASCULITIS** | **LYMPHOID HYPERPLASIA** | **1:100** | **BVDV +** |
| **Lung** | | | | | | | |
| **Heart** | | | + | | | | |
| **Kidney** | | | | | | | |
| **Liver** | + | ++ | | | | + | |

Table 3

**Neospora caninum and Toxoplasma gondii**

The expected and schematized lesions for Neospora caninum infection are: myocarditis, encephalomyelitis (perivascular foci), congestion and necrosis as reported by del Piero (3). The two RT-PCR Neospora caninum positive samples did not show significant lesions on histological examination, apart from a mild (+) hepatic congestion in the first sample (Tab 4) and a severe encephalic congestion (+++) in the second (Tab 5), accompanied by widespread gliosis. The target organs selected for immunohistochemical examination are liver, brain and heart. The anti-N. caninum primary antibody (goat polyclonal antibody) was previously tested on a positive sample, used as a control. The tissue sections of the two samples analyzed did not show any positivity. Although lesions such as multifocal necrotic hepatitis and encephalitis, which characterize Toxoplasma gondii infection, were not present, the liver and brain sections of both samples were tested with polyclonal anti-Toxoplasma gondii (rabbit polyclonal antibody; at a dilution of 1: 5,000, without showing any positivity in the IHC test.

| | TYPE OF LESION | | | | IHC | PCR Neospora |
|---|---|---|---|---|---|---|
| **TARGET ORGANS** | **Congestion** | **Miocarditis** | **Encephalomyelitis** | **necrosis** | | + |
| **Brain** | +++ | | | | - | |
| **Heart** | | | | | - | |

| | TYPE OF LESION | | IHC | PCR Toxoplasma |
|---|---|---|---|---|
| **TARGET ORGANS** | **Multifocal necrotic hepatitis** | **Encephalomyelitis** | | - |
| **Brain** | | | - | |

Table 4

| | TYPE OF LESION | | IHC | PCR BAHV1 |
|---|---|---|---|---|
| **TARGET ORGANS** | **MULTIPLE FOCI OF NECROSIS** | **MACROPHAGES (COWDRY'S BODIES INCLUSIONS)** | 1:500 | + |
| **Fegato** | | | - | |

| | TYPE OF LESION | | | | IHC Neospora Toxoplasma | PCR Neospora |
|---|---|---|---|---|---|---|
| **TARGET ORGANS** | **Congestion** | **Miocarditis** | **Encephalomyelitis** | **necrosis** | | + |
| **Liver** | + | | | | - | |
| **Heart** | | | | | - | |

Table 5

**Bovine herpes 1 (BAHV1)**

Among the organs received from the 4 samples that tested positive for BAHV1 there were liver, lung, kidney, spleen, heart and brain. The typical lesions of BAHV1 infection have been schematized as reported by del Piero (3) and Crook (4): multiple foci of necrosis and presence of intranuclear eosinophilic inclusion bodies. In one of the four cases, multiple foci of moderately extensive hepatic and renal necrosis were observed (Table 6). In the same section of the liver there were also layers of perivascular and periductal inflammatory cells (eosinophilic granulocytes). In HaematoxylinEosin sections of brain tissue, microscopic examination highlighted focal necrosis of spheroid neurons, high-grade spongiosis (+++), mild gliosis and severe periventricular inflammation of lymphocytic nature. Intranuclear acidophilic inclusions were not identified in any of the analyzed samples.
IHC was performed on liver, kidney, lung and brain sections with BHV-1/IBR Mab gB-gI IgG2b at a dilution of 1:500. Dilutions of 1:100 and 1:200 were previously tested on the same sections, also including a section of a positive control that showed a correct immunopositivity only with a dilution of 1:500. In the only one sample that showed histological lesions related to BAHV1, the IHC examination highlighted marked immunopositivity in one of the foci of necrosis in the cortex of the kidney. In another of the 4 cases, which did not show any detectable alteration on histological examination in H-E, the immunoreaction occurred in the proximal tubules in the renal cortex (fig1 A B) and in the liver, specifically in the cytoplasm of the periductal macrophages (fig2 A B)

| | TYPE OF LESION | | IHC | PCR BAHV1 |
|---|---|---|---|---|
| **TARGET ORGANS** | **MULTIPLE FOCI OF NECROSIS** | **MACROPHAGES (COWDRY'S BODIES INCLUSIONS)** | 1:500 | + |
| **Liver** | ++ | | | |
| **Lung** | | | | |
| **Kidney** | ++ | | + | |
| **Spleen** | | | | |

Table 6

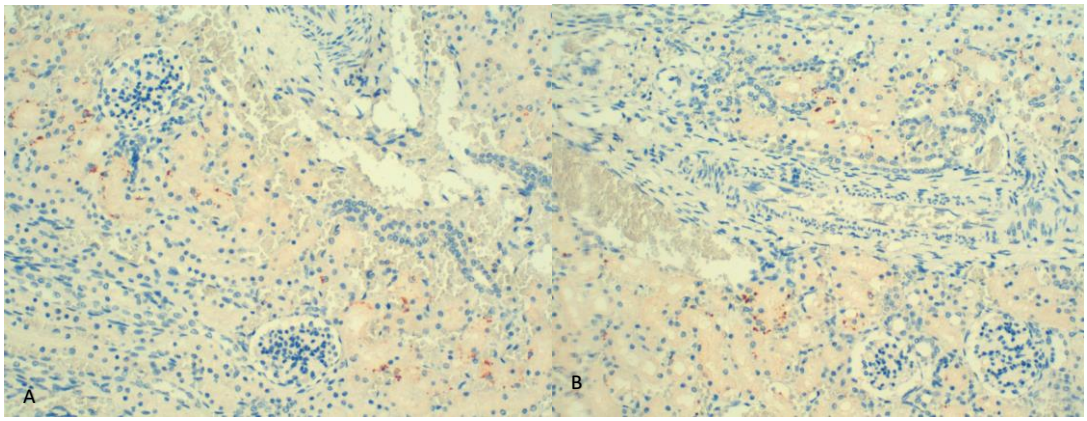


Figure 1 A-B. Kidney, specific reaction for Bovine herpesvirus 1 (BAHV1) in the cytoplasm of the cells of the proximal convoluted tubules. (IHC BHV-1/IBR Mab, 20x)

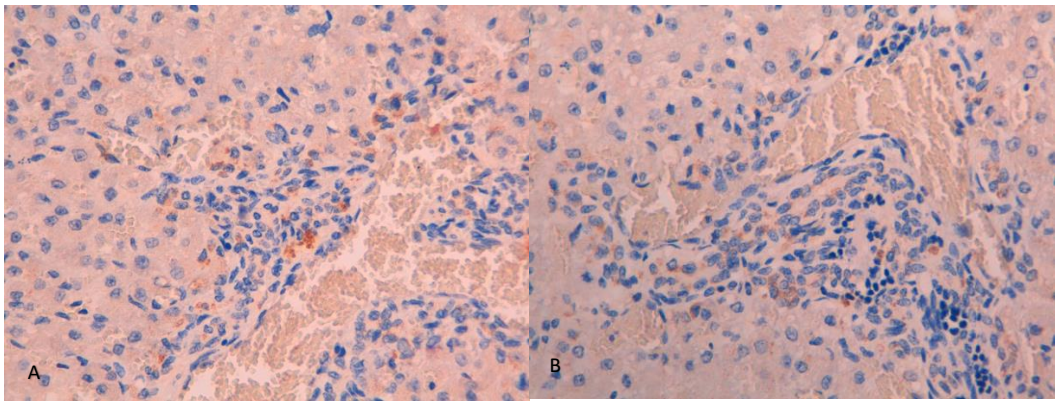


Figure 2 A-B. Liver, specific reaction for Bovine herpesvirus 1 (BAHV-1) in the cytoplasm of macrophages in inflammatory foci in the portal spaces. (IHC, BHV-1/IBR Mab, 40x)

Samples that tested negative for the pathogens investigated showed non-specific lesions in H-E. These lesions could be hypothetically related to coliform infections with septicemic spread. The study underlined how the association between PCR and immunohistochemical examination are essential to reach an etiological diagnosis in cases of abortion. The failure in detection of some

pathogens such as Chlamydia and Neospora in IHC could be related to the conservation status of the samples that was not always optimal.
Tissue autolysis significantly affects the outcome of the test by preventing the correct antigen-antibody reaction and, consequently, its detection. This condition was observed in the placentas examined which would certainly have provided a notable contribution and support to the study if they had arrived in good condition.
The results obtained in the search for pathogens such as BVDV and BAHV1 were encouraging, despite the fact that the target organs were not always collected at autopsy. In this regard, it would be essential to always collect the same organs in order to standardize the process that leads to a correct etiological diagnosis. The study is a pioneer in the histological diagnosis of the causes of abortion in the Mediterranean buffalo (Bubalus bubalis), and, for this reason, it requires in-depth studies to improve the selection of the matrix to be taken and the use of an antibody panel that allows the screening of the suspected pathogens depending on the gestation phase in which the abortigenic event occurred.

References


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