ORIGINAL ARTICLES

Mycological and serological studies of *Aspergillus fumigatus* in cattle with a history of reproductive disorders

Abdulaziz S. Bahobail

Assistant professor, Biology Department, Faculty of Sciences, Taif University.

ABSTRACT

In this study a total of 56 aborted fetus and serum samples were collected from aborted animals. When the aborted fetus subjected to bacteriological and mycological examination revealed that *Escherichia coli* alone have grown up from 6 aborted fetus tissues. *Aspergillus fumigatus* isolated in pure form from 13 fetal skin and placenta samples. Isolation of these fungi with Non-haemolytic, non-pathogenic *Escherichia coli* as mixed infection was recovered from 8 cases. Regarding the serological and virological results, all the blood samples were negative for antibodies against *Leptospira* serovars tested, *Brucella abortus* and *Chlamydophila abortus*. Three mother of the examined fetus were seropositive at low titre for BVDV antibodies. One of the other aborted dams was seropositive for *Coxiella burnetii* at low titre. The indirect ELISA technique for *Aspergillus fumigatus* on aborted cow sera revealed that, all culture positive were serologically positive. Antifungal susceptibility to four drugs, amphotericin B, itraconazole, Voriconazole and fluconazole. Itraconazole and Voriconazole were found to be more active (MIC range 0.5-1 g/mL) antifungal against *Aspergillus fumigatus*. There is therefore a need for attention to be paid to Aspergillus in the differential diagnosis of these conditions and to improve preventive methods to effectively control this organism.

Key words: Mycological studies - Aspergillus fumigatus - reproductive disorders - Serological studies - cattle reproduction - cows reproduction

Introduction

Bovine abortion is defined as an interruption of pregnancy between the 42nd and 260th day (Givens M.D. and S.M.D. Marley, 2008). Among the infectious causes of abortion in cattle are included bacteriological, viral, parasitic and mycotic agents (Anderson M.L., 2007). The knowledge of fungi as abortive pathogens is dated from the last century, and mycotic placentitis is a cause of abortion in cattle worldwide (Knudtson W.U. and C.A. Kirkbride, 1992).

Abortion associated with mycotic placentitis is well known in cattle and causes considerable economic loss all over the world (Hugh-Jonems, E. and K.C. Austwickp, 1967). Occasionally foetal infection occurs but the dam usually shows no ill effects, spontaneously recovers after aborting, and may subsequently conceive normally. Mycotic placentitis is a major worldwide cause of abortion in cattle, generally occurring in the third trimester of pregnancy (Knudtson W.U. and C.A. Kirkbride, 1992). Bovine mycotic abortion is a worldwide sporadic disease that usually affects a small percentage of animals within a herd (Tell L.A., 2005). In the Northern Hemisphere, the incidence of mycotic abortion is highest between November and April, which corresponds to the approximate time when gravid cows are housed indoors and fed hay and/or ensilage (Kirkbride C. A., et al., 1972)

The disease can also occur in beef cattle confined to pens and fed hay as well as those on pasture. Furthermore, cows confined to sheds and fed in cubicles are at greater risk than those fed loose hay in an open barn (Williams B. M., et al., 1977).

Previous reports indicated that > 60% of cases is caused by uncomplicated infection with *Aspergillus fumigates*.

*Aspergillus fumigatus* is a saprophytic mold that thrives in the soil on organic debris. It sporulates readily with conidiophores producing multitudes of conidia. This microbe can also cause disease in humans and animals (Einsele H. and J. Loeffler, 2011).

The etiological diagnosis of *aspergillus* abortion is achieved either by the identification of fungal colonies that grow in culture media or by observation of fungal elements in affected tissues. However, culture as a diagnostic methodology is prone to the appearance of false negatives, primarily due to lack of in vitro growth of some fungi, growth of contaminating microorganisms, and the isolation of fungal species different from those observed in the tissues on histology. Conversely, false positives may occur due to the growth of Aspergillus on
the fetus following post-abortion contamination. In all cases the growth is very difficult to interpret as truly
etiological in the process (Jensen H. E., et al., 1996).

In view of the lack of sensitivity and specificity of current diagnostic methods, research continues in search
of a methodology that would allow an early and effective diagnosis of the disease.

The present study was undertaken to find correlation between microscopic findings, culture and
serodiagnosis using an enzyme-linked immunosorbent assay (ELISA) technique for the detection of anti-
Aspergillus antibodies in the sera of cattle from herds with a history of abortions in which the etiology had not
been determined. We also studied drug sensitivity of isolated strains.

Samples:

Tissue samples:

The aborted fetus and placenta that were collected from 56 aborted animals were chilled and referred to
laboratory within 12 hours from the abortion for microbiological examination.

Animal sera:

(a) Control sera comprised:
Twenty cattle sera from a farm with good hygienic conditions and no history of abortions or fungal
diseases.
(b) Sera of 56 cows from 4 herds, these animals presented previous histories of abortions, with the absence
in all cases of a diagnosis of the infectious origin of the problem.
Abortion took place in the 6th to 7th month of gestation period.

Gross examination:

Examination of fetuses and placentas was done as described by Kirkbride (1986).
For the diagnosis of mycotic infection; a sterile disposable syringe was used to aseptically collect 1-3 ml of
abomasal content. In addition, a piece of placenta with 2 or 3 cotyledons was placed in a sterile plastic bag, and
depending on the availability of fetal tissues, portions of lung, eyelid, and skin (if there was evidence of
dermatitis) were collected.

Bacteriological examination:

Bacteriological examination was performed on fetal spleen, liver, kidney, lung, heart, skin, stomach content
and Portions of placenta on blood agar (5% bovine erythrocytes) and Mc Conkey agar (DIFCO) and incubated
for 24-48 hours at 37°C in air. The fetal stomach content was specifically cultured for the isolation of Brucella
spp. on Brucella agar and incubated in microaerophilic (5% CO2) environment (Quinn P. J., et al., 2002).

Mycologic examination:

Fetal spleen, liver, kidney, lung, heart, skin, stomach content and Portions of placenta were placed in a
beaker, washed in running tap water 1-3 min to remove extraneous debris and then blotted with sterile paper
towels. A sterile scalpel was used to scrape a small amount of examined sample was separately spread onto the
surface of plates of Sabouraud dextrose agar (SDA)a containing 1,000 units/ml of penicillin G Solid media were
incubated at both room temperature (Haley L. D. and Callaway C. A. 1978).

Serological examinations for bacteriological abortive agents:

Serological examinations for the main infectious abortive agents were performed on blood sera from
aborted cows.
Moreover, during the necropsy of the fetus, 1 ml of blood from the atrial chambers of the heart was taken.
The serological examination of the blood samples to investigate the presence of antibodies against abortive
agents was performed for Brucella abortus by card test agglutination (Brucellaslide Test, bioMérieux) (Alton G.
G., et al., 1988), Chlamyphila abortus by ELISA (Civtest Bovis Chlamydia ps., Hipra) (Gokce, H.I., et al.,
2007), Leptospira serovars by microagglutination test (Carole A. and D.V.M. Bolin, 2003), Coxiella burnetii
by ELISA (Hunt J. G., et al., 1983)
(Chekit Q Fever antibodies ELISA test kit, IDEXX). ELISAs were performed following manufacturer
instructions.
Serological examinations for Virological abortive agents:

Virological examination was performed on fetal spleen and lung tissues by direct ELISA (ELISA BVD/MD antigen mix screening, Pourquier) following manufacturer instructions to assess the presence of BVD virus (Brock K.V., et al., 2005).

Serological examinations for Aspergillus fumigatus:

Antigen preparation:

Aspergillus antigenic mycelial extract was prepared using the method previously described by Garcia et al., (1997) The organism was grown in synthetic broth [Czapeck-Dox broth (Difco Laboratories, Detroit, MI) + 1% dextrose] for 96 h in an aerated culture at 37°C. After the incubation period, mycelium was obtained by filtration, washed three times with phosphate buffer solution (PBS), and dried with filter paper. The mycelium was completely macerated in a mortar, covered with 10 ml of PBS, and sonicated. Microscopic examination of the preparation revealed at least 80% breakage of the hyphae. The extract obtained was centrifuged at 12 000 g for 30 min and the supernatant was collected and dialysed extensively against deionized water. The retentate was filtered through a 0.45-lm membrane and passed through an Ultrafree-15 centrifugal filter (Millipore Iberica, Madrid, Spain) with a 10-kDa exclusion membrane, centrifuged at 3000 g for 50 min, and recovered by the ultrafiltration membrane (Garcia M.E., et al., 2001).

The antigenic extract was evaluated for its protein content by the protein assay based on the Bradford procedure (Biorad, Hercules, and CA).

ELISA methodology:

To perform the ELISA assay, each well of a 96-well microplate (Dismalab, Madrid, Spain) (Garcia M. E., et al, 2004) was coated with 100 μl of the antigenic extract of A. fumigatus at a protein concentration of 5 μg/ml. After incubation for 72 h at 4°C, the plate was washed three times with PBS plus 0.05% Tween-20 (PBS-T). One hundred microliters of blocking solution was added (bovine serum albumin [Sigma, St Louis, MO, USA] 3% in PBS). Plates were incubated at room temperature for 30 min and washed again. After blocking, plates could be used immediately or frozen at −20°C for later use. Following this, 100μl of a 1/5 000 serum solution was added to the well. For fetal sera, the following serum dilution were used: 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280, 1/2560, 1/5120. After incubation for 1 h at 37°C, the plate was washed again, and100 μl of anti-bovine IgG conjugated with peroxidase (Sigma) at a dilution of 1:10 000 was added. After incubation for 1 h at room temperature, the plate was washed again, and 100 μl of a solution of O-phenylenediamine (Sigma) was added. The plate was incubated for 20 min at room temperature in darkness. The reaction was stopped with 50 μl of 6N sulphuric acid, and the optical density (OD) in the well was read in an ELISA reader at 492 nm.

The cut-off was established at 0.2 OD units which was about three times the average OD reading of control uncoated wells.

Correlation of the test result of ELISA and conventional method (C. M.) in terms of sensitivity and specificity:

Sensitivity was calculated as the percentage of Aspergillus fumigatus positive animals that had a positive test result.

\[
\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive} + \text{False negative}}
\]

Specificity was calculated as the percentage of Aspergillus fumigatus negative animals that had a negative test result.

\[
\text{Specificity} = \frac{\text{True negative}}{\text{True negative} + \text{False positive}}
\]

Antifungal susceptibility:

Susceptibility to amphotericin B, itraconazole, Voriconazole and fluconazole was tested by determining minimum inhibitory concentration by agar dilution method (Emmons C.W., et al., 1977).
Results

**Fig. A:** The placenta is thickened, cotyledons appear necrotic and firm. Hyperemia of intercotyledonar areas is also visible.

**Fig. B:** Aborted calf White and irregular lesions localized in the head (periorbital region) are visible in the picture

**Gross lesions on the fetus and placenta:**

Cotyledons appeared thickened, firm and necrotic, whereas intercotyledonary chorioallantois showed brown exudate and moderate hyperemia (Fig. A). Multifocal and coalescing white, raised and dry plaques were present in the skin of the fetus, mainly localized on the head (periorbital regions) and on the back (Fig. B). Small, white, raised plaques were occasionally seen also in the skin of the abdomen, legs and tail. No gross lesions were present in the other organs.

**Isolation of bacteria:**

Non-haemolytic, non-pathogenic *Escherichia coli* alone have grown up from 6 fetal liver, spleen, kidney, heart and lung samples. After 6 days, these plates were still negative for *Brucella* spp. growth.

**Isolation of fungi:**

Colonies with typical morphological features of fungi grew up on Sabouraud agar after 24 hours of aerobic incubation in pure form from 13 fetal skin and placenta samples. Isolation of these fungi with Non-haemolytic, non-pathogenic. *Escherichia coli* as mixed infection were recovered from 8 cases (Table 1). Colonies were fast growing and the texture of colonies varies from wooly to cottony to granular; Surface colony color is smoky gray - green and the reverse is yellow, however, some isolates may show a lavender diffusible pigment; and Color of very mature colonies turn to slate gray while atypical colonies may remain white with slight conidiation. On the colonies a direct microscopic examination with Cotton Blue staining showed Conidial heads are in the form of compact columns in an undisturbed culture; Conidiophores are smooth – walled, often tinted greenish, up to 300 µm long, and terminate in a dome – shaped vesicle with a diameter of 20 – 30 µm long; Hyphae are septate and hyaline; The species is uniseriate producing a closely compacted phialides with size ranging from 5 - 10 x 2 – 3 µm, and only occurring on the upper portion of the vesicle; and Conidia are round to subglobose, smooth to finely roughened, and with diameter size of 2 – 3.5 µm.
Serological examinations for bacteriological and virological abortive agents:

Regarding the serological and virological results, all the blood samples were negative for antibodies against Leptospira serovars tested, Brucella abortus and Chlamyphilia abortus. Three mother of the examined fetus were seropositive at low titre for BVDV antibodies. One of the other aborted dams was seropositive for Coxiiella burnetii at low titre (Table 2).

Serological examinations for Aspergillus fumigatus:

The indirect ELISA technique on cow sera revealed that, all culture positive cases of aspergillosis were also positive while only 2 culture negative cases were positive. From 20 control serum sample one case reacted with A.fumigatus antigen at low titre (1/20) in the present study. As can be seen, differences were found between sera from the aspergillus abortions: were detected up to a dilution of 1:640, and in the other up to a dilution of 1:80.

The indirect ELISA technique on aborted cow sera revealed that, all culture positive were serologically positive (Table 3,4).

Sensitivity of ELISA technique for diagnosis of was aspergillosis 100% while its specificity was 94.28%.

Antifungal susceptibility:

Study was also tested for antifungal susceptibility to four drugs, amphotericin B, itraconazole, Voriconazole and fluconazole (Table 4). Itraconazole and Voriconazole were found to be more active (MIC range 0.5-1_g/mL) (Table 5).

<table>
<thead>
<tr>
<th>Isolated microorganisms</th>
<th>No.</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non hemolytic E.Coli</td>
<td>6</td>
<td>10.71</td>
</tr>
<tr>
<td>Brucella abortus</td>
<td>0</td>
<td>%</td>
</tr>
<tr>
<td>Aspergillus fumigatus</td>
<td>13</td>
<td>23.21</td>
</tr>
<tr>
<td>Aspergillus fumigatus Nonhemolytic E.Coli</td>
<td>8</td>
<td>14.29</td>
</tr>
</tbody>
</table>

Table 2: Serological examination for bacteriological and virological abortive agents in bovine sera

<table>
<thead>
<tr>
<th>Bacteriological and virological agents</th>
<th>No. of positive</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brucella abortus</td>
<td>0</td>
<td>%</td>
</tr>
<tr>
<td>Chlamyphilia abortus</td>
<td>0</td>
<td>%</td>
</tr>
<tr>
<td>Leptospira</td>
<td>0</td>
<td>%</td>
</tr>
<tr>
<td>Coxiiella burnetii</td>
<td>1</td>
<td>1.79%</td>
</tr>
<tr>
<td>B.V.D</td>
<td>3</td>
<td>5.36%</td>
</tr>
</tbody>
</table>

Table 3: Antiaspergillus IgG in blood sera of examined cows

<table>
<thead>
<tr>
<th>Examed sera</th>
<th>NO. of positive</th>
<th>Total positive</th>
<th>Total negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control sera</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Affected animal sera</td>
<td>2</td>
<td>23</td>
<td>33</td>
</tr>
</tbody>
</table>

Table 4: Correlation between Mycological and serological results in examined cows for Aspergillus fumigatus

<table>
<thead>
<tr>
<th>Mycological status</th>
<th>Positive for ELISA</th>
<th>Negative for ELISA</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture Positive</td>
<td>21</td>
<td>0</td>
<td>100%</td>
<td>94.2%</td>
</tr>
<tr>
<td>Culture negative</td>
<td>2</td>
<td>33</td>
<td>94.28%</td>
<td>8%</td>
</tr>
</tbody>
</table>

Table 5: Antifungal susceptibility of Aspergillus fumigatus

<table>
<thead>
<tr>
<th>Antifungal used</th>
<th>No.of positive</th>
<th>Percent</th>
<th>Inhibitory range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>21</td>
<td>100%</td>
<td>0.5-2</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>21</td>
<td>100%</td>
<td>0.125-1</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>21</td>
<td>100%</td>
<td>0.125-1</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>16</td>
<td>67.2%</td>
<td>6.25-12.5</td>
</tr>
</tbody>
</table>
Discussion:

Abortion in cattle is a serious problem everywhere in the world where these animals are reared. The implication of fungi in abortion in cattle has received increasing attention during recent years and it is now recognized that mycotic infection contributes significantly to the annual losses from abortion.

Mycotic abortion, also known as fungal abortion or mycotic placentitis, is caused by different species of fungi and yeasts, *Aspergillus fumigatus* being the most commonly diagnosed casual organism (Jenson H.E., *et al.*, 1993).

Diagnosis of mycotic abortion presents great difficulties because a number of infectious and non-infectious agents are known to cause abortion in cattle. Abortion resulting from various infectious causes must be differentiated from mycotic abortion. Confirmation lies in the isolation of specific etiologic agent.

The isolation of non-pathogenic E. coli alone from fetal tissues, associated to the absence of gross lesions in the fetal organs, as well as the BVDV and Coxiella burnetti seropositivity at low titre of the dam have to be considered not meaningful to demonstrate the involvement of these pathogens in the abortive event by Brock *et al.*, (2005). In addition, necrotizing placentitis is rarely associated to BVDV, and in our case BVD virus was not detected by direct diagnostic procedures on fetal spleen and lungs.

In the area where the abortions occurred, the nutrition of dairy cattle is regulated by strict rules and the administration of silage is forbidden. Consequently, dairy cows are fed with a major amount of hay. In our case, heifers were usually fed with grass and hay, while during the pregnancy the ration was supplemented with hay stored the year before in a moist place, exposed to bad weather conditions.

The risk factors associated to the administration of mouldy hay were present. Moreover, the macroscopic lesions on the fetus and placenta were consistent with mycotic abortion, confirmed by cultural findings (Schlafer D.H. and R.B. Miller, 2007). The morphological aspect of the colonies on Sabouraud agar, the microscopic aspect of the hyphae, sporangia and sporangiospores directed the diagnosis to *Aspergillus fumigatus*.

While a large number of bovine abortions can be accurately diagnosed, the etiology of a significant percentage of them continues to be unexplained, many of which are suspected of being caused by fungal agents.

Since the first case of bovine mycotic placentitis, described by Smith in 1920, there have been many studies showing the fungal etiology of this kind of disease (Sarfati J., *et al.*, 1996). According to research based on experimental studies with pregnant mice and cows, the placentitis and pneumonia observed in abortions of fungal etiology are the result of the hematogenous spread of moulds from primary gastrointestinal lesions, more specifically from the omasum in beef cattle (Jensen H.E. and H. Shonheyder 1993; Jensen H.E., *et al.*, 1994) and (Sarfati J., *et al.*, 1996). This spread may be exacerbated by the application of antibiotics, which produce an alteration in the normal flora of the animal (Jensen H.E., *et al.*, 1989). Likewise, infectious bovine rhinotracheitis (IBR) erosions in the fore stomachs have been assumed to be the portal of entry for mycotic invasions. This erosive viral disease could act as a predisposing factor for mycotic invasion, either by producing erosions in the mucosal lining of the forestomachs or due to its immunosuppressive effect (Jensen H.E., *et al.*, 1989). Any immunodeficient or immunosuppressive condition of the host, whether from corticosteroid therapy, infection, metabolic disturbance or stress, might facilitate the establishment of the mycotic infection (Jensen H. E. and J.P. Latge, 1995).

In the present study we have applied both the ELISA technique to the diagnosis of bovine aspergillosis. Sensitivity of ELISA technique for diagnosis of was aspergillosis 100% while its specificity was 94.28%.

Garcia *et al.*, (2004) and Garcia *et al.*, (2001) proved the usefulness of the ELISA methodology in the diagnosis and monitoring of canine and ovine aspergillosis.

From the results reported in this study we concluded that the, use of the indirect ELISA methodology demonstrated a significant seroprevalence of anti-Aspergillus antibodies in herds with a history of reproductive disorders. In view of the results obtained, aspergillosis should be included in the differential diagnosis of bovine abortion.

Furthermore, we recommend the application of the indirect ELISA methodology to the diagnosis of Aspergillus abortion in fetal sera, in a larger number of samples; we would have a quick, simple, and economical methodology for diagnosing bovine aspergillar abortion. However, seropositivity detected in the present study suggests that serological tests have an edge over routine culture for diagnosis of aspergillosis. In the present study the results of in vitro susceptibility testing of 21 isolates of Aspergillus species show that itraconazole and Voriconazole were more active than amphotericin B (MIC range 0.5-2 g/mL) and fluconazole (MIC 6.25-12.5 g/mL) (Chakrabarti A., K. Singh and M. Jtana 1998). There have been no convincing demonstrations that treatment failure in animals with aspergillosis can be attributed to the development of amphotericin B resistance. (Khan S., *et al.*, 2006)
Conclusion:

Our findings support the conclusion that *Aspergillus fumigatus* is the causative agent of the abortion. It is useful to stress that in case of abortion the routine diagnostic procedures should include the mycotic component and assess the fungal species. In accordance to the assumption that mycotic abortion is characterised by the presence of mycotic elements associated with placentitis, confirmation lies in the isolation and identification of the specific mycotic agent. Serological tests have an edge over routine culture methods for the diagnosis of aspergillosis. Itraconazole is more effective than amphotericin B and fluconazole in the treatment of aspergillosis. Thus a combination of culture and serology can be ideal to confirm the diagnosis of aspergillosis.

References


