Seroprevalence of *Aspergillus fumigatus* antibodies in bovine herds with a history of reproductive disorders

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ABSTRACT: Bovine reproductive disorders are of growing interest from both economic and health standpoints. The role that fungi play in these processes is becoming increasingly important. Study of the seroprevalence of anti-*Aspergillus* antibodies in cow sera taken from herds with previous reproductive disorders is therefore of great interest. We used an indirect ELISA technique, that had shown suitable results in previous studies in other animal species. Thirteen percent of the 387 animals tested in the current study were positive, accounting for 41% of herds. When this technique was applied to the sera of two fetuses, that were aborted due to aspergillosis, a significant difference in anti-*Aspergillus* antibody levels was found compared with those in control sera. The ELISA technique was compared with PLATELIA and PCR for 24 ELISA-positive cattle sera; these assays resulted in only one and three positive results, respectively. In conclusion, the ELISA analysis showed a significant seroprevalence of *Aspergillus* antibodies in herds with previous reproductive disorders. There is therefore a need for more attention to be paid to *Aspergillus* in the differential diagnoses of these conditions and to improve preventive methods to effectively control this organism.

Keywords: *Aspergillus*; abortion; fungi; ELISA; PCR; antibodies

Abortion in cattle is a serious problem, which results in considerable economic losses to the industry from reduced productive efficiency.

The different factors influencing reproductive efficiency, and the difficulty in obtaining a definitive diagnosis of infertility, make laboratory investigation necessary in many cases. However, the diagnostic accuracy of the laboratories has traditionally been low for reproductive problems. Various factors contribute to this problem: etiological complexity, samples in bad condition or inappropriate samples, and lack of technical ability. Even when it is possible to detect the presence of an infectious agent in the fetus, caution must be taken before considering its role in the disease process. It must be borne in mind that the presence of a specific microorganism does not necessarily mean a causal association between the microorganism and the abortion.

Bovine mycotic abortion is a worldwide sporadic disease that usually affects a small percentage of animals within a herd. Cows typically abort in their second or third trimester of pregnancy, usually between the fifth and seventh month of gestation (Tell, 2005).

Systemic bovine aspergillosis is difficult to diagnose clinically in cattle because there is no typical clinical picture and therefore most cases are not diagnosed until post-mortem pathology is applied. *Aspergillus fumigatus* infection is predominantly found in the lung, the gastrointestinal tract and,

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following subsequent hematogenous dissemination, also in other organs including the placenta (Jensen et al., 1993).

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 et al., 1996). Therefore, histopathological examination is essential to confirm infection. A tentative etiological diagnosis may be obtained from the morphological details of fungi within tissue sections, but as the appearance of hyphae in sections is altered by a number of factors, this is considered to be difficult (Jensen, 1993).

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.

In the present work, we evaluated the usefulness of 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 or infertility in which the etiology had not been determined. These results were compared with the determination of *Aspergillus* antigens (galactomannan) and *Aspergillus* DNA in serum by polymerase chain reaction (PCR). Concurrently, indirect ELISA methodology was tested in fetal sera previously diagnosed as *Aspergillus*-related abortions.

MATERIAL AND METHODS

Animal sera

(a) Control sera comprised:
- 20 cattle sera from a farm with good hygienic conditions and no history of abortions or fungal diseases,
- 10 fetal sera, obtained from slaughterhouse fetuses with no lesions present.

(b) Sera of 387 cows from 71 herds, with a minimum of five animals per herd. These herds presented previous histories of abortions and/or infertility, with the absence in all cases of a diagnosis of the infectious origin of the problem.

(c) Two sera from fetuses from aspergillar abortions diagnosed using microbiology. One abortion took place in the 7th month of gestation in a herd with a 10% abortion rate, and the other in the 6th month of gestation as an isolated case in the herd.

ELISA methodology

To obtain the antigenic extract, we used a strain of *A. fumigatus* from our culture collection (strain ZG-001). Antigenic mycelial extract from *A. fumigatus* was prepared as described previously (Garcia et al., 1997), with Czapeck-Dox broth medium (Difco Laboratories, Detroit, MI, USA) plus 1% dextrose to encourage fungal growth. The protein content of the antigenic extract was measured by a protein assay based on the Bradford procedure (Biorad, Hercules, CA, USA).

To perform the ELISA assay, each well of a 96-well microplate (Dismalab, Madrid, Spain) 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, 100 µl of a 1/5 000 serum solution was added to the well. For fetal sera, the following serum dilutions 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, and 100 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.

Galactomannan determination

The commercially available technique Platelia Aspergillus (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) was used. Serum samples were processed following the manufacturer's instructions. In the interpretation of the results, the cut-off value (index = 1) was 1 ng of galactomannan per ml of serum. Sera with an index < 1 were considered to be negative, and sera with an index > 1.5 were considered to be positive. Sera with an index between 1 and 1.5 were considered to be "doubtful".

DNA detection

For extraction of DNA from serum, 25 µl of serum was incubated for 3 h at 56°C with 65 µl of lysis buffer, which comprised 2.5 mg proteinase K/ml, 1% so-

Table 1. Results obtained after the application of the ELISA, PLATELIA and PCR techniques on 24 cattle sera

Serum	ELISA		PLATELIA		PCR
	OD	interpretation	index	interpretation	
1	1.439	+	0.451	–	–
2	1.938	+	0.606	–	–
3	1.756	+	0.293	–	–
4	0.830	+	2.776	+	–
5	1.220	+	0.852	–	–
6	0.942	+	0.792	–	–
7	1.141	+	0.351	–	–
8	0.979	+	0.598	–	–
9	0.988	+	0.406	–	–
10	1.714	+	0.431	–	–
11	1.911	+	0.744	–	–
12	1.966	+	0.355	–	–
13	1.729	+	0.756	–	–
14	1.861	+	0.718	–	–
15	1.673	+	0.854	–	–
16	0.649	+	1.263	D	–
17	0.806	+	0.381	–	–
18	0.607	+	0.558	–	–
19	1.007	+	0.616	–	–
20	0.627	+	0.862	–	–
21	0.619	+	0.558	–	+
22	1.461	+	0.992	D	+
23	0.815	+	1.05	D	+
24	0.993	+	1.3	D	–

OD = optical density; D = ???

dium dodecylsulfate, 0.015M Tris-HCl pH 8.0. The lysate was then extracted with phenol-chloroform, by precipitating the DNA with ethanol. Detection of DNA was carried out using a nested PCR with primers previously developed by other authors (Yamakami et al., 1996). These primers amplify the variable region V7 to V9 of subunit 18S of *Aspergillus fumigatus* rRNA. The outer primer set was ASP5 (5' GATAACGAACGAGACCTCGG 3') and ASP8 (5' TGCCAACTCCCCTGAGCCAG 3'), which amplify a sequence of 384 bp; the inner primer set was ASP1 (5' CGGCCCTTAAATAGCCCGGTC 3') and ASP7 (5' CCTGAGCCAGTCCGAAGGCC 3'), which amplify a sequence of 357 bp.

The mix for the first amplification consisted of 50mM KCl, 15mM Tris-HCl pH 8.0, 1.5mM $MgCl_2$, 250μM of each dNTP (dATP, dCTP, dTTP, dGTP), and 0.02 IU/μl Taq Gold polymerase (Applied Biosystems, Madrid, Spain). Each external primer (2 pmol/μl) was combined with 10 μl of solution extracted from serum in a final reaction volume of 50 μl. The amplification was carried out in a GeneAmp PCR Systems 9700 thermocycler (PE Biosystems, Foster City, CA, USA). The reaction cycles were as follows: an initial cycle of 95°C for 10 min followed by 30 denaturation cycles at 94°C for 1 min, hybridization at 50°C for 1 min and extension at 72°C for 3 min, finishing with a cycle of 72°C for 10 min.

The second amplification used 10 μl of the product of the first amplification plus 40 μl of the same reaction mix, except that the primers used in this case were ASP1 and ASP7. The reaction cycles were as follows: an initial cycle at 95°C for 10 min followed by 30 denaturation cycles at 94°C for 1 min, hybridization at 65°C for 1 min and extension at 72°C for 3 min, finishing with a 10-min cycle at 72°C. The presence of *Aspergillus* DNA was verified in 1% agarose gels.

RESULTS

Estimation of the cut-off

With the 20 maternal control sera, we obtained an OD mean of 0.349, with a standard deviation (SD) of 0.050. To establish the cut-off, we used the following criteria: $\bar{x}$ + 3 SD for positive sera, and between $\bar{x}$ + 2 SD and $\bar{x}$ + 3 SD for "doubtful" sera. Sera with an OD lower than $\bar{x}$ + 2 SD were regarded as negative. Thus, positive sera had an OD value > 0.5, "doubtful" sera an OD value between 0.4 and 0.5, and negative sera an OD value < 0.4. The use of a reference value of $\bar{x}$ + 3 SD from the control sera resulted in a specificity of 99.9%, based on the values obtained using only uninfected animals (Jacobson, 1998).

Maternal sera

The indirect ELISA technique revealed that 51 out of a total of 387 animals tested were positive

Table 2. Level of anti-*Aspergillus* IgG in blood according to different bovine fetal serum dilutions

Serum dilution	Serum I-246	Serum I-770	Control sera			
			$\bar{x}$	SD	$\bar{x}$ + 2 SD	$\bar{x}$ + 3 SD
1:10	0.336	1.06	0.034	0.027	0.088	0.115
1:20	0.24	0.71	0.030	0.015	0.060	0.075
1:40	0.19	0.602	0.023	0.016	0.055	0.071
1:80	0.12	0.45	0.020	0.020	0.060	0.080
1:160	0.092	0.38	0.025	0.013	0.051	0.064
1:320	0.056	0.31	0.027	0.011	0.049	0.060
1:640	0.041	0.21	0.030	0.015	0.060	0.075
1:1280	0.031	0.12	0.038	0.015	0.068	0.083
1:2560	0.041	0.088	0.036	0.011	0.058	0.069
1:5120	0.024	0.068	0.036	0.020	0.040	0.096

(13%). A total of 29 herds (41%) gave at least one positive result, with the detection of more than one positive animal in 13 of the herds (18%). Table 1 shows a comparison of our ELISA technique with PLATELIA and PCR in 24 cattle sera that tested positive on the ELISA. Using the PLATELIA technique, only one animal tested positive, with four doubtful cases. In the PCR technique, only three animals tested positive, two of which were doubtful in the PLATELIA technique.

Bovine fetal sera

Using the ELISA technique, the control sera values were very similar to the blanks used, revealing almost a total absence of immune response against *Aspergillus* antigens.

Table 2 shows the levels of IgG anti-*Aspergillus* antibodies detected in the different serum dilutions, shown as the average values of the control sera and the values obtained from the two sera from the aspergillar abortions.

As can be seen, significant differences were found between the two sera from the aspergillus abortions: in one fetus antibodies were detected up to a dilution of 1:640, and in the other up to a dilution of 1:80.

DISCUSSION

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. It is estimated that up to 75% of mycotic abortions are caused by the genus *Aspergillus* (Foley and Schlafer, 1987), with *A. fumigatus* responsible for up to 20% of the total number of bovine abortions (Sarfati 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 and Shonheyder, 1993; Jensen et al., 1994; Sarfati 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 et al., 1989). Likewise, infectious bovine rhinotracheitis (IBR) erosions in the forestomachs 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 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 et al., 1989).

Given the ubiquitous nature of this fungus, and with the aim of differentiating between healthy and sick animals, or at least to avoid the appearance of false positives, we use low serum dilutions, 1:5 000, establishing a cut-off point using the statistical formula $\bar{x}$ + 3 SD.

Other authors have applied both the ELISA technique and immunoblotting to the diagnosis of bovine aspergillosis (Jensen and Latge, 1995). These authors explain that both sick and non-infected animals produce antibodies against *A. fumigatus*. However, significantly higher titers against all antigens were detected in sera from infected animals, and only one of the control sera gave a titer above the cut-off levels.

We have already demonstrated, in previous studies carried out in our laboratory, the usefulness of the ELISA methodology in the diagnosis and monitoring of canine (Garcia et al., 2001) and ovine (Garcia et al., 2004) aspergillosis.

It is considered that the PCR technique could lead to early and effective diagnosis of aspergillosis in humans (Kawamura et al., 1999; Skladny et al., 1999; Williamson et al., 2000). This was not the case for the bovine cases studied here, as only three out of the 24 animals analysed tested positive by PCR. We have obtained significantly better results with the PCR technique in previous studies on ovine mastitis (Garcia et al., 2004). One explanation for the results obtained with bovine material could be due to the application of PCR to the study of maternal sera. In these samples and in this type of disease process, it could be considered normal to find an absence of fungal hyphae, and consequently fungal DNA in the dam. Future studies should be designed to evaluate direct application of PCR to the analysis of fetal tissue.

Similar reasoning could be applied to the PLATELIA technique, which detects fungal antigens. In our study, only one of the maternal sera tested positive, and another four were doubtful. In previous studies on other species, we have reported that the PLATELIA technique results in a high number of false positives and negatives, only proving effective in the late stages of the disease (Garcia et al., 2004). In animals infected experimentally, the PLATELIA technique has been shown to be useful for the diagnosis of systemic bovine aspergillosis but not for cases of bovine aspergillar abortion (Jensen et al., 1993).

In conclusion, use of the indirect ELISA methodology demonstrated a significant seroprevelance 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 light of the data obtained, and until a larger study is performed, we consider that it is advisable to use a serum dilution of 1:20, which allows a clear differentiation of the possible positive sera. If these results are confirmed in a larger number of samples, we would have a quick, simple, and economical methodology for diagnosing bovine aspergillar abortion.

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