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Detection of antibodies specific to *Campylobacter fetus* subsp. *venerealis* in the vaginal mucus of Nigerian breeding cows

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Summary

The presence of bovine venereal campylobacteriosis in the Lake Chad Basin of Nigeria was investigated using an enzyme-linked immunosorbent assay (ELISA) for the detection of IgA antibodies specific to *Campylobacter fetus* subsp. *venerealis* in vaginal mucus ($n = 66$). IgA antibodies specific to *C. fetus* subsp. *venerealis* were detected in 7 (11%) vaginal mucus samples. All but one of the IgA-positive samples originated from cows belonging to herds with a history of abortion and infertility which suggested an association between antibody detection and poor herd fertility. It was concluded that bovine venereal campylobacteriosis is prevalent in the Lake Chad Basin of Nigeria and its contribution to reduced reproductive performance in cattle herds may be grossly underestimated in this part of the world.

Keywords

Antibody, Breeding cow, *Campylobacter fetus* subsp. *venerealis*, Cow, Enzyme-linked immunosorbent assay, ELISA, IgA, Lake Chad, Nigeria.

Individuazione di anticorpi specifici per *Campylobacter fetus* subspecie *venerealis* nel muco vaginale di vacche da riproduzione in Nigeria

Riassunto

E' stata studiata la presenza di campilobatteriosi venerea bovina nel bacino del lago Ciad in Nigeria mediante un test immunoenzimatico (ELISA) per la rilevazione di anticorpi IgA specifici per Campylobacter fetus *subsp.* venerealis *nel muco vaginale di vacche da riproduzione. Gli anticorpi sono stati rilevati in 7 campioni di muco vaginale (11%). Lo studio evidenzia come la campilobatteriosi venerea nel bovino sia particolarmente diffusa nel bacino del lago Ciad e il suo ruolo nella riduzione delle prestazioni riproduttive negli allevamenti bovini in Nigeria sia sottostimato.*

Parole chiave

Allevamento bovino, Anticorpo, *Campylobacter fetus* subsp. *venerealis*, ELISA, IgA, Lago Ciad, Nigeria, Vacca.

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Introduction

Cattle production constitutes a tremendous potential for livestock development in Nigeria (2). However, reports show that this resource is plagued by reproductive abortifacient diseases in this country (3, 4, 11, 23, 24, 25). Furthermore, information on the occurrence of bovine venereal campylobacteriosis in Nigeria is scanty and is not properly documented.

Bovine venereal campylobacteriosis, which is caused by *Campylobacter fetus* subsp. *venerealis*, has been reported to be a widespread disease associated with bovine infertility worldwide (21, 27). The most prominent clinical presentation includes embryonic death, mid-term abortion, frequent return to oestrus and extended oestrus cycles (19, 28). It is also characterised by long calving intervals, increased age at first calving and low pregnancy rates (4, 12, 18). The economic losses associated with this disease are well recognised (12, 13, 21, 27, 29).

Bovine venereal campylobacteriosis usually occurs in areas where extensive cattle management and natural breeding are practised (19, 22). In Nigeria, the disease was first reported by Nuru in 1974 (23) when it was diagnosed in a herd of South Devon cattle imported into Kano State (21). By the late 1980s and early 1990s, the presence of the disease were reported from an outbreak in an artificially inseminated herd in Zaria, Kaduna Sate (3) and from a field survey of three northern states from which a prevalence of 2.9% was reported (4). To date, the prevalence of this disease in other parts of the country remains to be established.

Bovine venereal campylobacteriosis is diagnosed widely by culture and isolation of the organism from infected bulls, cows and heifers or aborted foetuses (14, 27, 29), but because of the fragile nature of the causative organism, laboratory diagnosis of the disease by conventional bacteriological technique is difficult (6, 13, 16, 17). Bulls are the main carriers of *C. fetus* subsp. *venerealis* and the main sources of infection to susceptible cows during mating (21, 26). Whilst preputial washings from carrier bulls are the best samples for the isolation of the organisms in culture, serological evidence of infection in a herd can be demonstrated by the examination of genital secretions of cows and heifers (10, 13, 21). An enzyme-linked immunosorbent assay (ELISA) for the detection of IgA antibodies specific to *C. fetus* subsp. *venerealis* which was reported to have a specificity of 98.5% in detecting the organism (15) has been used for the detection of the disease in cattle herds in Australia (13, 15) and New Zealand (18).

In Nigeria, where over 90% of farmers use natural breeding in cows, the incidence of sexually transmitted disease, such as bovine venereal campylobacteriosis, is likely to be high (25). Therefore, the present study was designed to investigate the prevalence of the disease in cattle herds which use natural breeding using an ELISA for the detection of IgA antibodies specific to *C. fetus* subsp. *venerealis* in the vaginal mucus of cows in the Lake Chad Basin of Nigeria.

Materials and methods

Animals and study area

The study was conducted in the conventional Lake Chad Basin and extended between 10°-14° latitude north and 12°-15° longitude east. The climate is Sahelian with an annual rainfall of between 100 mm and 1 500 mm from north to south. The vegetation is semi-arid with savannah grasslands towards the south.

The animals studied were a mixture of Nigerian indigenous breeds and a few Simmental and Friesians crosses managed under traditional semi-intensive and pastoral-nomadic husbandry systems. The herds managed in the semi-intensive systems have a home base and are normally found in the suburbs of major cities and towns feeding on crop residues as the main dietary energy source for cattle around the harvesting period (November-December). Those managed in the pastoral nomadic system are involved in extensive migration from one area to another in search for quality and good quantities of forage and water.

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Herd and cow selection

The study area was divided into three clusters; one herd each with and without reproductive problems was randomly selected from each cluster. On this basis, six herds were selected for inclusion in this investigation; three each from those with history of reproductive failure and those without. The herds were identified from a list based on reports of a previous survey (20). In all the herds, cows were selected for sampling if they had calved at least once, were pregnant or lactating and if they were on heat and bred by a bull or were served within 4 weeks prior to sampling.

Herds with poor reproductive performance characterised by calving intervals (CI) of 480-600 days, high rates of return to service (2-3 services per conception), low calf crop yield (CCY) (a calf/cow in 24-36 months) and incidence of abortion or stillbirth were identified as herds with fertility problems in one group and the other group which consist of herds with CI (>480 days), CCY (a calf/cow in 12-18 months), with no history of abortion or stillbirths were considered as herds with normal fertility.

Sampling strategy and sample collection

The herds selected had a total of 150 susceptible cows with an average of 23 cows per herd. Susceptible females were defined as those adult cows over 2 years of age and most of which have come on heat and were bred at least once. Depending on the herd size (defined in this case as the number of susceptible females), 6-19 vaginal mucus samples were collected per herd according to standard protocol (15) and despatched to the laboratory in chilled conditions.

A total of 66 samples in herds with a history ($n = 40$) and from those in herds without any history of reproductive failure ($n = 26$) were collected. Negative and positive control samples were kindly supplied by Steven Hum of the Elizabeth Macarthur Agricultural Institute in Camden, Australia. These samples were heat inactivated at 56°C for 30 min, cooled and stored at –20°C and later transported on dry ice to the Department of Veterinary Pathology at the University of Liverpool, for analysis. At Liverpool, the samples were agitated for 1 h, centrifuged at 400 g for 5 min and the supernatant stored at 4°C. The samples were diluted 1:45 in phosphate buffered saline (PBS) before being tested for the presence of IgA antibodies specific to *C. fetus* subsp. *venerealis* using the ELISA (13).

Controls

The negative and positive control samples were used to establish the cut-off points between positive and negative readings in the ELISA.

Antigens

The antigen used for coating the ELISA plates was prepared from a reference strain (ATCC 19438) of *C. fetus* subsp. *venerealis* grown on Skirrow's agar under microaerophilic conditions using a gas generating kit (GENbag microaer, BioMérieux, Marcy L'Etoile) at 37°C for 3-4 days. After checking the culture for purity, the colonies were suspended in 0.5% formal saline for 1 h, centrifuged at 500 g for 1 h, washed twice with PBS (pH 7.5) and then re-suspended in carbonate-bicarbonate buffer (0.05 M, pH 9.6) using syringe and 23G needle. The final concentration of bacteria was adjusted to give an optical density (OD) of 0.155 at 610 nm. Flat-bottom polystyrene microtitre plates were then coated with 100 µl antigen suspension, left overnight at 4°C and then stored at –20°C until use. The plates were then rinsed twice with distilled water, filled with PBS Tween 20, left for 5 min, rinsed twice with distilled water and then tapped gently to remove moisture.

Detection of antibodies

Chequerboard titrations were used to establish optimal concentrations of the conjugate (horseradish peroxidase-conjugated rabbit polyclonal anti-bovine whole immunoglobulin (IgG, IgM and IgA, code ab8530, ABCAM plc, Cambridge) and positive and negative controls The optimal concentrations were found to be 1:1 000 and 1:90 for the conjugate and control samples, respectively.

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The ELISA was conducted according to the method described by Hum *et al.* (13) with minor modifications. Briefly, 100 µl of a blocking solution of 0.2% normal rabbit serum in Tris ethylenediaminetetraacetic acid (EDTA) (TE) buffer (0.5M Tris-HCl pH 7.4 and 1 mM EDTA) was added to each well. The plates were incubated at 37°C for 1 h. They were then rinsed with distilled water followed by a 5-min wash twice and two additional water rinses. All washes were tipped out and residues then 'banged out' on paper tissue pad.

The vaginal mucus samples and the positive and negative control samples were all diluted to give a final concentration of 1:90 in PBS Tween 20. A total of 100 µl of each sample were then added to wells in triplicates. In addition to the positive and negative controls, each plate also contained three blank wells, which were filled with PBS Tween 20. The plates were incubated for 2 h at 37°C, before sample tipping and washing as described above. Then 100 µl of the diluted conjugate anti-bovine whole IgA was added to each well and the plates incubated at 37°C for 1 h. The plates were washed again before adding 100 µl of the substrate (containing 0.4 mg/ml each of O-phenyldiamine dihydrochloride and urea hydrogen peroxide (Fast OPD, P9187 Sigma, Aldrich; in 0.05 M phosphate-citrate buffer pH 5.0) to each well, followed by 20 min incubation at room temperature in the dark. The reaction was stopped by adding 50 µl of 4N sulphuric acid. The OD value for each sample was then established at 490 nm. The OD measurements yielded by the test samples were corrected for the OD measurement of positive and negative controls and the results were expressed according to the formula: ELISA value (EV) = OD of sample – OD of Blank.

Results

Of the 66 vaginal mucus samples tested, 7 (11%) were positive, 2 samples gave borderline readings and were considered suspects, while 57 were negative. Six of the seven (86%) cows that tested positive were from two of three herds with a history of reproductive failure. In contrast, only 1 of 26 samples obtained from three herds without a history of abortion/ stillbirth was positive, suggesting an association between antibody detection and poor herd fertility. Table I shows the relationship between frequency of herd reproductive failure and detection of IgA antibodies against *C. fetus* subsp. *venerealis* in the vaginal mucus.

The overall prevalence of IgA antibodies against *C. fetus* subsp. *venerealis*, irrespective of type of herd, was 7 in 66 (11%). However the prevalence was higher in herds with a history of reproductive failure, with 6 of 40 (15%) being positive for *C. fetus* subsp. *venerealis*, compared to only 1 in 26 (4%) of the cows belonging to herds without histories of abortion and stillbirth (Table II).

Table I
Relationship between frequencies of abortions/stillbirths and detection of IgA antibodies against *Campylobacter fetus* subsp. *venerealis* in the vaginal mucus

Herd	Frequency of abortions/stillbirths	Number of cows		Percentage of positives
		Tested	Positive	
1(a)	1	7	0	0
2(a)	14	19	4	57
3(a)	8	14	2	29
4(b)	0	10	0	0
5(b)	0	6	1	14
6(b)	0	10	0	0
Total	23	66	7	100

(a) herds with a history of abortions/stillbirths
(b) herds with no history of abortions/stillbirths

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Table II
Prevalence of antigen-specific IgA antibodies against *Campylobacter fetus* subsp. *venerealis* in cattle herds, with and those without histories of abortions/stillbirths, in the Lake Chad Basin area of Nigeria

History of abortions/stillbirths	Number of cows tested	Number positive	Prevalence (% positive)
A	40	6	15
B	26	1	4
Total	66	7	11

A herds with a history of abortions/stillbirths
B herds with no history of abortions/stillbirths

Discussion and conclusions

The main objectives of the present study was to determine the prevalence of bovine venereal campylobacteriosis and to evaluate the possible use of the IgA ELISA in the detection of antibodies specific to *C. fetus* subsp. *venerealis* in the vaginal mucus of cows for the diagnosis of the disease in the Lake Chad Basin of Nigeria. The results of the study showed that IgA antibodies specific to *C. fetus* subsp. *venerealis* are prevalent in the vaginal mucus of cows in this part of the country. Field reports showed that infertility characterised by abortion are common occurrences amongst livestock in Nigeria, but detailed investigations are not performed (25); therefore, the involvement of bovine venereal campylobacteriosis in lowered fertility in cattle herds across the country is not known.

This is the first time an ELISA has been applied in an investigation of bovine venereal campylobacteriosis in Nigerian cattle. The detection of these *C. fetus* subsp. *venerealis* specific IgA antibodies further demonstrates the presence of bovine venereal campylobacteriosis in the Lake Chad Basin of Nigeria and supports the reports of previous workers (3, 4, 23) who observed that the disease is prevalent in Nigeria. These workers diagnosed the disease and isolated *C. fetus* subsp. *venerealis* from cases of abortion in cattle herds in north-central areas and certain parts of north eastern Nigeria.

The present study has also confirmed an observation by Australian workers (13) that the detection of specific IgA antibodies in the vaginal mucus can be an important tool in investigating the prevalence of bovine venereal campylobacteriosis. From their report, high levels of IgA antibodies were detected in response to natural cases of abortion caused by *C. fetus* subsp. *venerealis* in 88% of cows tested but none of the vaccinated animals produced IgA antibody in vaginal mucus, suggesting that false-positive results may not be revealed by this ELISA in vaccinated animals (13). Therefore the positive detection of antibodies to this organism in this study is an indication of natural infection in herds in this area. The application of this tool on a wide scale will help in the elucidation of the epidemiology of bovine venereal campylobacteriosis in developing countries such as Nigeria.

Some studies on the immune responses to *C. fetus* subsp. *venerealis* infection in the genital tract of cows subsequent to experimental intravaginal infection found that specific IgM antibodies appeared between 1 and 2 weeks, IgA antibodies between 3 to 5 weeks and IgG (1, 2) antibodies at 8 weeks post infection (7, 8, 9, 10). Most importantly, IgA antibodies persisted for up to 40 weeks, while IgM was transient, lasting only for 8 to 18 weeks (13), thus making IgA antibodies an ideal target for epidemiological studies.

Although IgA antibodies specific to *C. fetus* subsp. *venerealis* were only present in the vaginal mucus of a small number of cows in the Lake Chad Basin of Nigeria, it is significant that the detection rates of these antibodies were higher (15%) in those herds with a history of reproductive failure than in those without any history (4%). This concurs with the findings of Hum *et al.* (15), who also showed a significant relationship between antibody detection and lowered herd fertility in Australian cattle. The high percentage of

cows testing positive in herds with a history of abortion and lowered fertility is in keeping with the pathogenesis of *C. fetus* subsp. *venerealis* which has been associated with early embryonic death and poor fertility (18).

Our study documents the first demonstration of IgA antibodies specific to *C. fetus* subsp. *venerealis* in Nigerian cattle. The prevalence of this disease in this area may be attributed to the natural breeding practices commonly engaged in almost all the herds found in this area and the problem of diagnosis of field cases (12, 25). The lack of vaccines and of a surveillance programme against bovine venereal campylobacteriosis, the failure of herd owners to report outbreaks of abortions and infertility (1) and the problem of effective control of mass cattle movements across the borders in Nigeria could also be major risk factors to the increased prevalence of this disease in the country.

From data available, the Nigerian cattle population was estimated at 14 million in 1992 (5) and by the mid-2008 the conservative market price for average beef cattle in Nigeria was in the region of US$1 000 per head. Based on this data, the current monetary value of the cattle resources in this country is worth over US$14 billion. If each BVC cow infected with bovine venereal campylobacteriosis at the current level of infection losses a calf as consequence of abortion or infertility each year, it is our hypothesis that the economic impact of this to the Nigerian livestock economy could be significant. It would therefore be interesting that an extensive assessment of the threat posed by campylobacteriosis to the Nigerian livestock industry be undertaken to ascertain the level of the disease nationwide

Since the isolation of the organism from field samples is difficult to achieve (12, 13, 21), the IgA ELISA technique used in this study (13), could provide an appropriate tool for the diagnosis of bovine venereal campylobacteriosis and the prediction of infertility in a cattle population in endemic herds, especially in sub-Saharan Africa.

From the findings in the present study, it was concluded that bovine venereal campylobacteriosis is prevalent in the Lake Chad Basin of Nigeria and its contribution to lowered reproductive performance in cattle may be grossly underestimated in this part of the world. To date, the epidemiology of the disease is unclear, especially in north-west Africa where over 90% of cattle are bred by natural mating (25). The pleomorphism within field strains of *C. fetus* subsp. *venerealis* has not been fully investigated worldwide. Therefore, considering the potential of the African livestock industry, it is imperative that further studies be focused on the isolation of clonal field strains of *C. fetus* subspecies circulating in African cattle. The characterisation of the genomic properties of such isolates will help to develop vaccine strains that will protect animals on the African continent. Clearly, this will require high profile techniques which may not be available in developing countries. Therefore, to achieve faster results, collaboration in campylobacter research between investigators in developing and the developed countries is highly recommended.

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