Isolation of Ureaplasma from Bovine Granular Vulvitis

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ABSTRACT

Cultures for mycoplasmatales, viruses and bacteria were made from bovine vulvar swabs to determine whether ureaplasma was associated with a clinical granular vulvitis observed in 16 Ontario dairy herds.

Ureaplasma was isolated from 23.5% of 34 clinically normal cows, 74% of 27 cows with mild to moderate vulvar hyperemia but no discharge and 100% of 20 cows with acute vulvar hyperemia accompanied by purulent discharge. There were statistically significant differences in rates of isolation among clinical groups. Mycoplasma bovigenitalium was isolated from 7.7% and 20% of cows with moderate or acute vulvitis respectively but not from normal cows.

Haemophilus somnus was isolated from 25% of cows with acute vulvitis. There were no significant differences in isolations of Escherichia coli, Corynebacterium pyogenes and alpha-hemolytic streptococcus between normal and clinically affected animals.

Cultures of 135 repeat samples from 33 cows revealed that ureaplasma persisted in some animals for at least three months. No viruses were isolated from any of the animals in this study.

RÉSUMÉ

Cette étude consistait à rechercher des mycoplasmes, des bactéries et des virus dans des écouvillons vulvaires de vaches, afin de vérifier l'implication possible d'uréaplasme dans l'éruption d'une vulvite granuleuse qui sévissait dans 16 troupeaux laitiers, en Ontario.

On confirma la présence d'uréaplasme chez 23.5% des 34 vaches cliniquement normales, chez 74% des 27 vaches qui ne présentaient qu'une hyperémie vulvaire modérée et chez 100% des 20 vaches qui présentaient à la fois une hyperémie marquée et un écoulement vulvaires. On nota des différences statistiques appréciables relativement au taux d'isolements réalisés chez ces trois groupes de vaches.

On isola Mycoplasma bovigenitalium chez 7.7% des vaches qui présentaient une vulvite modérée et chez 20% de celles qui souffraient d'une vulvite aiguë, mais chez aucune de celles qui paraissaient cliniquement normales.

On isola Haemophilus somnus chez 25% des vaches qui souffraient d'une vulvite aiguë. On ne nota aucune différence appréciable entre les sujets sains et malades, relativement à l'isolement d'Escherichia coli, de Corynebacterium pyogenes et de streptocoques alpha hémolytiques.

La culture de 135 échantillons ultérieurs, prélevés chez 33 vaches, révéla qu'uréaplasme persistait pour au moins trois mois, chez certaines d'entre elles. La recherche de virus à partir des prélèvements effectués chez toutes ces vaches donna toujours des résultats négatifs.

INTRODUCTION

During 1973, a newly recognized granular vulvitis syndrome appeared in Ontario dairy cattle. The disease was characterized by the sudden appearance of a purulent vulvar discharge four to ten days post-breeding. Clinical examination of the vulva during the acute stage of the disease revealed hyperemia with rows of minute granules usually concentrated around the ventral commissure. In approximately

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10% of the cases, raised white nodules 2 to 4 mm in diameter were observed in the dorsal commissure or upper lateral wall of the vulva. Histologically the nodules consisted of epithelial inclusion cysts (Doig, Ruhnke, MacKay and Palmer, unpublished data).

Ureaplasma (19) (T-mycoplasma) was isolated from 59% of 106 vaginal swabs submitted by mail from cows with the disease during 1973.

Experimentally, ureaplasma has been shown to cause mastitis in cows (8), pneumonia in calves (7,9) and ciliostasis in bovine oviductal organ cultures (21). In humans it has been incriminated in reproductive failure and infertility (6). However, its role in reproductive tract disease in cattle has not yet been established.

This investigation was initiated to determine whether ureaplasma was involved in the granular vulvitis syndrome of cows.

MATERIALS AND METHODS

Animals Cultured

Vulvar swabs from 81 cows in 16 dairy herds in Ontario were cultured. The majority of the cows were of the Holstein-Friesian breed. Animals were divided into three groups on the basis of clinical examination: A) Normal — no clinical signs, B) Moderate vulvitis — those with mild to moderate hyperemia and granularity but no purulent discharge and C) Acute — those with severe hyperemia and varying amounts of purulent discharge. An attempt was made to culture both clinically normal and diseased cows from each herd. Of the 81 animals cultured, seven were virgin yearling heifers, 12 were pregnant cows, seven were classified as repeat breeders and the remainder were cows less than 90 days postpartum.

Swabs were taken from the vulva of each cow for mycoplasmal, bacterial and viral cultures. A commercial collection unit[1] consisting of a rayon tipped swab and an ampoule of modified Stuart's transport medium was used. Swabs were either cultured on the day of collection or were packaged with an ice pack, delivered to the laboratory the following day and cultured immediately.

Repeat samples were cultured at various intervals for three months from 33 cows. Cultures were made from two cows positive and eight negative for ureaplasma from group A, 11 positive and seven negative from group B and five positive from group C. The animals originated from eight different herds.

Biopsies of intraepithelial cysts associated with the disease were cultured from four cows.

Microbiological Techniques

The broth medium described by Taylor-Robinson (23), without thallium acetate, was used for isolation of ureaplasmas. Four tenfold dilutions of each specimen were incubated aerobically and observed for an alkaline pH change. The solid medium was Shepard's A3 agar (18) with the addition of 10% fresh yeast extract and 0.1% urea. Plates were incubated anaerobically[2] and examined after two and five days. The presence of ureaplasma in broth cultures showing color change was always confirmed by subculture to agar medium.

In order to be prepared to process large numbers of specimens simultaneously the complete ureaplasma broth was dispensed in 3 ml volumes and stored at -20°C until required but was generally used within two weeks. Complete ureaplasma agar plates were prepared weekly.

Media for isolation of mycoplasma was slightly modified from that described by Chanock *et al* (4) and was composed of mycoplasma broth base[3] supplemented with 10% fresh yeast extract, 20% inactivated porcine serum, 1000 units per ml penicillin G potassium and 1:2000 thallium acetate. Solid medium was prepared by adding 0.8% Ionagar #2[4] to the broth base. Broth cultures were incubated aerobically at 37°C while agar cultures were incubated

[1]Culturette — Scientific Products, Canadian Laboratory Supplies, Toronto, Ontario.

[2]Gaspack H_2+CO_2 — Baltimore Biological Laboratories.

[3]Difco Laboratories, Detroit, Michigan.

[4]Oxoid, Med-Ox Chemicals, Ottawa, Ontario.

TABLE I. Mycoplasmatales Isolations from Vulvar Swabs from 16 Herds-(1976)

Clinical Classification	Total Cultured	Ureaplasma[a]	*M. bovigenitalium*
Normal	34	23.5[b]	0
Moderate vulvitis	27	74	7.7
Acute vulvitis	20	100	20

[a]Differences in rate of isolation of ureaplasma significant at $p \lesssim 0.05$ with 23.5% < 74% < 100% by Chi-square test
[b]Percent positive

in 8% CO_2 in air. Broth to agar subcultures were made at two days and again at five days.

Isolates were identified as ureaplasma on the basis of metabolism of urea, colony size and morphology. Mycoplasma isolates were identified by the indirect immunofluorescence technique (17) and in some cases confirmed by the growth inhibition test (5).

Swabs were cultured for bacteria on 5% bovine blood in trypticase soy agar with incubation in 10% CO_2 and on MacConkey agar incubated aerobically. A Gram-stained smear of each specimen was also examined.

Attempts to isolate viruses from the swabs were made by inoculation of primary and secondary embryonic bovine spleen cell monolayers.

The intraepithelial cysts were cultured for *Chlamydia* by inoculation via the yolk sac of five day old embryonated hens eggs.

RESULTS

Results of mycoplasmal and bacterial cultures of the first sampling from each cow are given in Tables I and II. No viruses were isolated. Cultures from the intraepithelial cysts from four cows were negative for bacteria, mycoplasmatales and *Chlamydia*.

A chi-square test on the ureaplasma isolations revealed a significant difference ($p<0.05$) among clinical groups. Although 24% of normal herd mates were positive for ureaplasma, this was significantly less than the isolation rate in those with mild to moderate vulvitis (73%) and those with acute vulvitis (100%). The differences among clinical groups in frequency of isolations of *Mycoplasma bovigenitalium* and *Haemophilus somnus* were also significant but isolations of *Escherichia coli, Corynebacterium pyogenes* and alpha-hemolytic streptococci were not significantly different.

Ureaplasmas were always present in large numbers on primary agar cultures from acute cases. The isolations of *M. bovigenitalium* and *H. somnus* were only made from cows positive for ureaplasma. Ureaplasmas were the only organisms isolated from eight of the clinically affected cows. Three of the seven virgin yearling heifers, seven of the 12 pregnant cows and all seven of the repeat breeders were positive for ureaplasma. All but two of these had clinical symptoms at the time of culture.

A total of 135 repeat samples were cultured from 33 cows from the three clinical groups as stated in Materials and Methods. Six cows (two group A, four group B) remained negative and seven cows (one group A, four group B, two group C) remained positive on all cultures. A further six cows (one group A, four group B, one group C) were positive on four cultures but negative on the fifth culture three months after initial isolation. Ten cows (two group A, six group B, two group C) had alternating positive and negative cultures over the test period. Four negative cows from the normal group subsequently developed the disease and were swabbed at the time clinical symptoms began. Ureaplasma was isolated from each cow at that time and on all cultures for two to three months.

No viruses were isolated from the 135 repeat swabs.

Other isolations from repeat samples in addition to those listed in Tables I and II were, *M. bovigenitalium* from one cow, *H. somnus* from six, *C. pyogenes* from one, *Pasteurella multocida* from two and *Cory-*

TABLE II. Bacteria Isolated from Vulvar Swabs from 16 Herds

Clinical Classification	Total Cultured	Alpha-streptococcus Not Faecal	Alpha-streptococcus Faecal	*E. coli*	*C. pyogenes*	*H. somnus*[b]
Normal.....	34	55.8[a]	23.5	23.5	0	0
Moderate vulvitis	37	51.8	11.5	40.7	7.7	0
Acute vulvitis...	20	45	5.5	20	16.6	25

[a]Percent positive
[b]Other organisms isolated were: *Geotrichum* — 1, beta-hemolytic streptococcus — 1, *P. aeruginosa* — 1, *P. multocida* — 1, *Klebsiella* — 1

nebacterium renale on four occasions from one cow. Small numbers of other bacteria such as *Neisseria,* nonhemolytic staphylococcus, *Erwinia, Mima polymorpha* and diphtheroids were occasionally found.

DISCUSSION

Granular vulvovaginitis is a common genital disease of cattle recognized since 1887 (11). Prior to 1966, numerous etiologies were proposed including streptococcus, Gram-negative bacillus, protozoa, viruses and nutritional deficiencies (11). Afshar's study in 1966 (1) ruled out streptococcus as an etiological agent and for the first time incriminated a mycoplasma (*M. bovigenitalium*) as a possible cause. However, Afshar hesitated to regard it as the primary cause of the natural cases. Granular vulvitis was observed in seven experimental heifers which were negative for *M. bovigenitalium* and the same mycoplasma could be isolated from only three of 12 natural cases. The study was done before media for isolation of ureaplasmas was included in cultures of bovine specimens and it is possible ureaplasma may have been involved in the condition described.

The epithelial cysts associated with this granular vulvitis syndrome have been previously described on one occasion. Van Kruiningen (25) observed similar cysts in an outbreak of granular vulvitis, palate lesions and respiratory illness in Connecticut dairy cattle. The cause of the syndrome was not determined. Perhaps ureaplasma could have been associated with some of their cases. Ureaplasma would not have been detected in the medium used at that time for culture of mycoplasmas.

Although ureaplasma was present in this study in 24% of cows that showed no symptoms at the time of sampling, the appearance of the organism in four previously negative cows at the beginning of clinical symptoms and its isolation from every cow with acute vulvitis accompanied by purulent discharge is strong evidence that some ureaplasmas can be pathogens of the vulva.

The low isolation rate of *M. bovigenitalium* and H. *somnus* from clinically affected cows in this study indicates that these organisms are probably not primarily responsible for the symptoms observed. However, the possibility exists that they may contribute to a more severe condition if present in association with ureaplasma. Alpha-hemolytic streptococcus, C. *pyogenes* and *E. coli* do not seem to be primarily involved as causative agents since their isolation rates were not significantly higher from diseased than from normal animals. In this study viruses were not involved in the syndrome.

This is the first report showing a definite association between ureaplasma infection and a specific bovine genital disease. Anderson (3) previously reported positive ureaplasma isolations from cows with a postbreeding purulent vaginal discharge, while others have isolated ureaplasmas from the genital tracts of clinically normal males and females (12, 13, 14, 15, 20, 22, 23, 24). Diseases of bovine reproduction associated with other mycoplasmas were reviewed by Afshar (2).

The isolation of ureaplasma from 24% of clinically normal cows in this study is higher than that reported by Taylor-Robinson (11%), Langford (14%) and Panangala (15%). The difference may be due to the anatomical location cultured. Vulvar swabs were sampled rather than cervicovaginal mucus as in previous studies. In a preliminary test, both vulvar swabs and cervico-vaginal mucus of three affected

cows were cultured. Ureaplasma was isolated from all of the vulvar swabs but from only one of the mucus samples.

The difference may also be due to the fact that the normal animals in this study were herd mates of diseased cows and some may have been recovered carriers. From the results of repeated sampling, it appears that the organism persists in some animals for at least three months (the longest time tested).

Previous studies (12, 16) have not shown any association between the recovery of ureaplasma and bovine genital disease or infertility. Both virulent and avirulent strains have been demonstrated by intramammary inoculation (10) and this may account for the apparent discrepancies.

The pathogenicity of ureaplasmas has been demonstrated by experimental reproduction of pneumonia in calves and mastitis in cows (7, 8, 9). Further important evidence of the pathogenic potential of ureaplasmas has been shown in bovine oviductal organ cultures. Five bovine strains including three from vaginal mucus of cows stopped ciliary activity within 48 hours and also caused histological lesions (21). Until such time as strain virulence differences are easily demonstrated the role of ureaplasma in bovine genital disease will no doubt remain controversial.

The data presented here suggests that ureaplasma should be viewed as one of the causes of bovine granular vulvitis. Experimental transmission studies necessary to conclusively establish a cause and effect relationship are currently being carried out. In addition there is a need for investigation into the role of ureaplasma in bovine infertility.

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