Identification of Ureaplasmas from Cattle Using Antisera Prepared in Gnotobiotic Calves

By C. J. HOWARD* AND R. N. GOURLAY

*A.R.C. Institute for Research on Animal Diseases, Compton, Newbury,
Berkshire RG16 0NN, U.K.*

(Received 14 April 1981)

Antisera were prepared in gnotobiotic calves to three bovine ureaplasma strains which were selected as representatives of the three clusters of serologically similar, but not identical, strains recognized in previous experiments with rabbit antisera. A further 110 isolates from various organ systems of cattle from six countries reacted by the fluorescent antibody technique with at least one of these bovine antisera and it appears that bovine ureaplasmas can be identified and classified by means of three antisera only. It is suggested that the bovine ureaplasmas could be designated as groups A, B or C, these being further subdivided with rabbit antisera. These findings make possible the screening of cattle for antibody to ureaplasmas with just three antigens and also have implications for immunization.

INTRODUCTION

Micro-organisms which have characteristics of the family *Mycoplasmataceae* and which also possess urease activity comprise a single genus, *Ureaplasma* (Shepard *et al.*, 1974). The first isolation of ureaplasmas was from humans. Subsequently, they were isolated from cattle and from a number of other species of animals (Taylor-Robinson & Furr, 1973).

In 1974 the specific epithet *U. urealyticum* was proposed, with strain T960 (ATCC 27618) as the type strain. This strain was isolated from man. Although other urease-producing mycoplasmas isolated from man were different from the type strain, when tested by a variety of serological techniques, they were not considered sufficiently distinct to be regarded as separate species and eight strains were proposed as representing eight serotypes (I–VIII) of this species (Shepard *et al.*, 1974). Subsequent studies have indicated that other serological varieties of ureaplasmas, besides the types I–VIII, occur in man (Robertson & Stemke, 1979).

Ureaplasmas isolated from cattle are distinguishable from *U. urealyticum* when the guanine plus cytosine content of their DNA and their polypeptides are compared (Howard *et al.*, 1978*b*, 1981), and probably constitute a group that should be regarded as a second species within the genus *Ureaplasma*. Strains isolated from cattle are serologically heterogeneous and when compared by means of antisera raised in rabbits appear to form three clusters of cross-reacting, but not identical, strains that are distinct from the *U. urealyticum* serotypes (Howard *et al.*, 1975; Ogata *et al.*, 1979).

Ureaplasmas have been isolated from a number of diseases in cattle, including pneumonia and keratoconjunctivitis, and cause pneumonia, mastitis, conjunctivitis and vulvitis under experimental conditions (Gourlay & Howard, 1979; Doig *et al.*, 1980; Rosenbusch & Knudtson, 1980). Thus, the development of serological tests for the diagnosis of ureaplasma infections in cattle should help in defining the incidence of such infections and their importance in bovine diseases.

This report concerns experiments with bovine ureaplasmas and antisera raised in calves. The implications of the findings for diagnosis, identification of isolates, cross-protection between strains and possible immunization measures are considered.

METHODS

Media. Strains were grown at 37 °C in U4 broth or on the solid medium described by Howard *et al.* (1978*a*) except that urea was added to a final concentration of 0.01% (w/v) and horse serum (10%, v/v) was substituted for foetal calf serum in the solid medium. Plates were incubated under 100% CO₂ for 6 d.

Antisera. The growth from 2 litres of broth culture containing about 10⁶ colour-change-units ml⁻¹ (Taylor-Robinson *et al.*, 1971) was harvested by centrifugation at 10000 g, washed three times with 0.15 M-phosphate-buffered saline pH 7.2 (PBS), resuspended in 4 ml PBS and stored at -70 °C. Gnotobiotic calves (Dennis *et al.*, 1976) were inoculated subcutaneously four times at 2 week intervals with 5 ml of emulsion comprising 1 ml of ureaplasma suspension, 1 ml of PBS, 200 µl of 20% (w/v) Tween 80 (Sigma) and 3 ml of Freund's incomplete adjuvant (Difco). Serum was collected 2 weeks after the last injection and stored at -20 °C. Fluorescein-conjugated rabbit anti-bovine serum was obtained from Miles Laboratories.

Immunofluorescence test. This was basically as described by Rosendal & Black (1972) for unfixed colonies on agar. Blocks of agar with colonies were attached to glass slides with gelatin, bovine antisera were diluted in PBS and the fluorescein-coupled anti-bovine serum was used diluted 1/40. All incubations with sera were at 4 °C for 60 min. After treatment with bovine antisera or anti-bovine sera, blocks were washed by submersion in PBS for 30 min at 4 °C, the PBS being changed three times. Titres were recorded as the reciprocal of the highest dilution of antiserum that produced fluorescence.

Ureaplasma strains. The three bovine strains, designated A417, D48 and T44, used to inoculate calves represented the three serological clusters of bovine ureaplasmas distinguished by rabbit antisera (Howard *et al.*, 1975, 1978*a*) and the three types of polypeptide patterns observed by polyacrylamide gel electrophoresis (Howard *et al.*, 1981). Their origins are listed in Table 1, which also lists the isolates proposed by Ogata *et al.* (1979) as 'serotypes' of bovine ureaplasmas. The remainder of the bovine isolates are listed in Table 2. The strains of *U. urealyticum* serotypes I-VIII are those described by Shepard *et al.* (1974) and were obtained from F. T. Black (Aarhus, Denmark).

RESULTS

Examination of proposed representative bovine ureaplasmas

The antisera were tested against the 11 strains proposed as representatives of the clusters of bovine ureaplasmas by Howard *et al.* (1978*a*) and those proposed by Ogata *et al.* (1979) as possible 'serotypes'. The three antisera appeared specific in that each reacted with homologous and related strains but not with the others (Table 1). The only exception was C13 which reacted with two antisera, although this isolate was clearly more similar to A417 than D48.

Identification of bovine ureaplasma isolates

The 96 strains listed in Table 2 were accordingly tested against a standard 1/40 dilution of the three antisera. All reacted with at least one of them. Many showed very slight fluorescence with a second antiserum in addition to the one with which they fluoresced strongly. One strain, isolated from pneumonia, fluoresced with more than one antiserum: all the colonies fluoresced equally with both antisera, i.e. the result did not appear to be due to mixed antigenic types being present in the culture.

Some differences were apparent in the proportions of the isolates from different anatomical sites that reacted to each of the three bovine antisera. Thus, most of the respiratory isolates reacted with antiserum to strain D48; most strains that reacted with antisera to strain T44 (10/11) were from semen or prepuce. Whether these differences would hold if larger numbers of isolates were examined remains to be seen. No geographical variation in strain type was evident.

Table 1. Reaction of proposed bovine reference strains with bovine antisera

Strain*	Titre by immunofluorescence with antisera to:		
	A417	D48	T44
A417	640	<20	<20
Bu2	320	<20	<20
Mmb167	640	<20	<20
T95	320	<20	<20
T288	640	<20	<20
T45	640	<20	<20
D48	<20	320	<20
T315	<20	80	<20
T71	<20	80	<20
T44	<20	<20	320
T74	<20	<20	320
C13	640	40	<20
C7	<20	80	<20
C12	<20	80	<20
C17	<20	160	<20
C23	<20	80	<20
C24	<20	160	<20

* The strains were all from lungs except T315 and Bu2 (urogenital tract of cows) and T45, T44 and T74 (semen or prepuce of bulls). Strains prefixed C were from Japan (Ogata *et al.*, 1979). The other strains were isolated in England except T95 and T288 (U.S.A.) and T74 (Canada) (Howard *et al.*, 1978 a).

Table 2. Identification of bovine ureaplasma isolates

Anatomical site	Disease†	Country and source‡	No. of strains	No. of strains fluorescing with antisera to:			
				A417	D48	T44	A417 +T44
Lung	Pneumonia	England (a)	28	5	23	—	—
		Denmark (b)	23	3	18	1	1
		U.S.A. (c)	1	—	1	—	—
		Canada (d)	2	—	2	—	—
		<i>Sub-total</i>	<i>54</i>	<i>8</i>	<i>44</i>	<i>1</i>	<i>1</i>
Prepuce	Normal	England (a)	7	1	2	4	—
Semen	Normal	England (a)	6	3	—	3	—
	Unknown	Canada (e)	4	—	1	3	—
Urogenital tract*	Normal	England (a)	6	3	3	—	—
	Unknown	Canada (e)	2	2	—	—	—
	Unknown	Ireland (f)	1	1	—	—	—
	Vulvitis	Canada (d)	2	2	—	—	—
Endometrium	Infertility	England (g)	4	—	4	—	—
		<i>Sub-total</i>	<i>32</i>	<i>12</i>	<i>10</i>	<i>10</i>	—
Eye	Keratoconjunctivitis	England (a)	10	3	7	—	—
		<i>Total</i>	<i>96</i>	<i>23</i>	<i>61</i>	<i>11</i>	<i>1</i>

* Cattle: vagina, urethra or bladder.

† Normal, no symptoms of disease; Unknown, disease status of animal unknown.

‡ Sources: (a) isolated by authors; (b) N. F. Friis, Denmark; (c) E. E. Ose, U.S.A.; (d) H. L. Rhunke, Canada; (e) R. B. Truscott, Canada; (f) H. Ball, Ireland; (g) Appleby & Gourlay (1980).

Testing *U. urealyticum* strains

None of the eight serotypes of *U. urealyticum* fluoresced with bovine antisera when tested in a similar manner.

DISCUSSION

The results of earlier studies with bovine ureaplasmas and antisera raised in rabbits indicated that it would probably be necessary to use at least 11 strains as antigens to detect antibodies to all possible variants. However, antisera produced in the unnatural host, e.g. rabbit, are often highly effective in the metabolism-inhibition test (Purcell *et al.*, 1967), while convalescent sera from the natural host, that can be shown to contain antibody by other serological tests, are not. (In fact the three calf sera all had titres to the homologous strain of ≤ 40 , in the metabolism-inhibition test.) It is therefore possible that the natural host recognizes different epitopes on the ureaplasmas to those recognized by laboratory animals. Consequently, antisera were raised in calves against three strains selected as representatives of the three serological clusters of bovine ureaplasmas.

The results using these three sera show that the range of antigens expressed by bovine ureaplasmas can be typified by three strains. It is therefore suggested that bovine ureaplasmas could be regarded as being of one of three serogroups, A, B or C, represented by strains A417, D48 and T44. These three groups correspond to the three groups delineated by analysis of polypeptides (Howard *et al.*, 1981) and also match the three clusters of strains recognized in previous studies using rabbit antisera (Howard *et al.*, 1975, 1978*a*; Ogata *et al.*, 1979). The proposed groups A, B and C are not equivalent to the serotypes of *U. urealyticum*; they are more likely to be equivalent to the groups of *U. urealyticum* formed when comparisons are made by DNA hybridization (Christiansen *et al.*, 1981). Furthermore, the groups are not completely separate, since strains exist (e.g. C13) that possess antigens common to two groups. They are better regarded as clusters of similar strains within a wider spectrum and may be useful for identification purposes.

In comparison to rabbit antisera, bovine antisera appear to unite strains while rabbit sera appear more specific. Possibly the bovine antisera recognize 'group' antigens while rabbit antisera recognized 'type' antigens.

No obvious association between antigenic structure and a particular disease was apparent using these bovine antisera, perhaps because they do not recognize 'type' antigens; rabbit antisera might be more likely to reveal any such relationship. In addition, bovine antisera would not be sufficiently discriminating for following a particular strain in a herd.

These results do indicate some affinity of particular groups for different organ systems. Thus, isolates of group C were most commonly from the male genital tract and isolates of group B from the lung. Further studies will confirm or refute this association.

The implications of our results are that it should be possible to identify bovine ureaplasmas, which are probably a separate species to *U. urealyticum*, by means of three antisera raised in gnotobiotic calves, and it should be possible to screen cattle for serological responses following ureaplasma infections by using just three antigens. Previous studies indicated that there was no cross-protection in cows between strains A417 and O13 which would now be assigned to groups A and B (Howard *et al.*, 1974). However, it is possible that strains within the same group may cross-immunize and that immunization against bovine ureaplasmas could be effected with just three antigen preparations.

We thank Judy Eynon for her assistance and M. Ogata, C. W. Livingston, N. F. Friis, E. E. Ose, H. L. Rhunke, R. B. Truscott and H. Ball for supplying strains.

REFERENCES

- APPLEBY, R. C. & GOURLAY, R. N. (1980). Examination of uterine biopsies from infertile cows for the presence of viruses and mycoplasmas. *Research in Veterinary Science* **28**, 223-227.
- CHRISTIANSEN, C., FREUNDT, E. A. & BLACK, F. T. (1981). Hybridization experiments with DNA from *Ureaplasma urealyticum* serotypes I to VIII. *International Journal of Systematic Bacteriology* (in the Press).
- DENNIS, M. J., DAVIES, D. C. & HOARE, M. N. (1976). A simplified apparatus for the microbiological isolation of calves. *British Veterinary Journal* **132**, 642-646.
- DOIG, P. A., RHUNKE, H. L. & PALMER, N. C. (1980).

- Experimental bovine genital ureaplasmosis. I. Granular vulvitis following vulvar inoculation. *Canadian Journal of Comparative Medicine* **44**, 252–258.
- GOURLAY, R. N. & HOWARD, C. J. (1979). Bovine mycoplasmas. In *The Mycoplasmas*, vol. 2, pp. 49–102. Edited by J. G. Tully & R. F. Whitcomb. New York: Academic Press.
- HOWARD, C. J., GOURLAY, R. N. & BROWNLIE, J. (1974). Immunity in experimental T-mycoplasma mastitis. *Infection and Immunity* **9**, 400–403.
- HOWARD, C. J., GOURLAY, R. N. & COLLINS, J. (1975). Serological comparison between twenty-five bovine ureaplasma (T-mycoplasma) strains by immunofluorescence. *International Journal of Systematic Bacteriology* **25**, 155–159.
- HOWARD, C. J., GOURLAY, R. N. & COLLINS, J. (1978*a*). Serological studies with bovine ureaplasmas (T-mycoplasmas). *International Journal of Systematic Bacteriology* **28**, 473–477.
- HOWARD, C. J., POCKOCK, D. H. & GOURLAY, R. N. (1978*b*). Base composition of deoxyribonucleic acid from ureaplasmas isolated from various animal species. *International Journal of Systematic Bacteriology* **28**, 599–601.
- HOWARD, C. J., POCKOCK, D. H. & GOURLAY, R. N. (1981). Polyacrylamide gel electrophoretic comparison of the polypeptides from ureaplasmas isolated from cattle and humans. *International Journal of Systematic Bacteriology* **31**, 128–130.
- OGATA, M., KOTANI, H. & YAMAMOTO, K. (1979). Serological comparison of bovine ureaplasmas. *Japanese Journal of Veterinary Science* **41**, 629–637.
- PURCELL, R. H., WONG, D., CHANOCK, R. M., TAYLOR-ROBINSON, D., CANCHOLA, J. & VALDESUSO, J. (1967). Significance of antibody to mycoplasma as measured by metabolism-inhibition techniques. *Annals of the New York Academy of Sciences* **143**, 664–675.
- ROBERTSON, J. A. & STEMKE, G. W. (1979). Modified metabolic inhibition test for serotyping strains of *Ureaplasma urealyticum* (T-strain mycoplasma). *Journal of Clinical Microbiology* **9**, 673–676.
- ROSENBUSCH, R. F. & KNUDTSON, W. U. (1980). Bovine mycoplasmal conjunctivitis: experimental reproduction and characterization of the disease. *Cornell Veterinarian* **70**, 307–320.
- ROSENDAL, S. & BLACK, F. T. (1972). Direct and indirect immunofluorescence of unfixed and fixed mycoplasma colonies. *Acta pathologica et microbiologica scandinavica* **B80**, 615–622.
- SHEPARD, M. C., LUNCEFORD, C. D., FORD, D. K., PURCELL, R. H., TAYLOR-ROBINSON, D., RAZIN, S. & BLACK, F. T. (1974). *Ureaplasma urealyticum* gen. nov., sp. nov.: proposed nomenclature for human T (T-strain) mycoplasmas. *International Journal of Systematic Bacteriology* **24**, 160–171.
- TAYLOR-ROBINSON, D. & FURR, P. M. (1973). The distribution of T-mycoplasmas within and among various animal species. *Annals of the New York Academy of Sciences* **225**, 108–117.
- TAYLOR-ROBINSON, D., MARTIN-BOURDON, C., WATANABE, T. & ADDEY, J. P. (1971). Isolation of T-mycoplasmas from dogs and squirrel monkeys: biological and serological comparisons with those isolated from man and cattle. *Journal of General Microbiology* **68**, 97–107.