STUDIES ON *BRUCELLA OVIS* (N.SP.), A CAUSE OF GENITAL DISEASE OF SHEEP IN NEW ZEALAND AND AUSTRALIA*

By M. B. BUDDLE

Animal Research Station, Department of Agriculture, Wallaceville, New Zealand

(With Plates 7 and 8)

A genital disease of sheep in New Zealand has been shown to be due to infection with an organism of the genus *Brucella* (Buddle & Boyes, 1953). Infection is apparently widespread in the Dominion and can be responsible for impaired fertility in rams, abortion in ewes and neo-natal mortality in lambs. The principal clinical manifestations are lesions of the epididymes, tunicae vaginales, and testes in the ram and placentitis in the ewe, with abortion or neo-natal death of lambs.

The organism may be recovered readily from the semen of naturally and experimentally infected rams even in the absence of palpable lesions of the genitalia and from the epididymes, tunicae vaginales, testes, accessary sex glands and regional lymph nodes and from the placentae and mammary secretion of infected ewes as well as from the tissues of aborted lambs.

Rams have been infected experimentally by the intravenous, subcutaneous and intratesticular routes as well as *per os* and by the application of infection to mucous surfaces such as the conjunctiva and prepuce (Buddle & Boyes, 1953; Hartley, Jebson & McFarlane, 1954; Jebson, Hartley & McFarlane, 1954; Buddle, 1954, 1955). Natural transmission of infection from ram to ram has been shown experimentally to occur readily during the mating season, and also when rams are run together in isolation from ewes. However, rams grazing pastures contaminated by aborting ewes or mated to infected ewes, or ram lambs from ewes which were experimentally infected during pregnancy and had grossly diseased placentae at parturition, failed to develop active infection.

While experimental infection of ewes by the oral and intravenous routes during early pregnancy has confirmed the susceptibility of ewes, active infection has not been readily induced by experimentally controlled exposure to natural infection. However, active infection has developed in ewes following mating with naturally infected rams (Buddle, 1955). Active infection was not acquired by any ewes running during pregnancy either with a flock of experimentally infected ewes or on contaminated pastures, in spite of obvious opportunities for contact with gross infection from aborted foetuses, diseased placentae and genital discharges. Infection is apparently less persistent in ewes than in rams, a high percentage of infected rams being capable of secreting infected semen for periods in excess of 4 years.

Following the original observations on this organism, a consideration of its characters as they related to those of the classical species, *Br. abortus*, *Br. melitensis*

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and *Br. suis* and of its existence in naturally infected sheep in the non-smooth phase, suggested that it might be described provisionally as a stabilized mutant or new variety of brucella (Buddle & Boyes, 1953). An organism probably identical with the New Zealand ovine brucella strain has been isolated from naturally infected rams in Australia (Simmons & Hall, 1953). A selection of Australian ovine strains isolated in Queensland, New South Wales, Victoria and South Australia, was provided subsequently by courtesy of Australian investigators. In this paper further observations on the characters of New Zealand and Australian ovine strains are reported and the relationship of these strains to the classical species of brucella are discussed. Finally, it is proposed that these strains might be assigned to a new species, *Brucella ovis*.

EXPERIMENTAL PROCEDURES

Source of strains

The fourteen New Zealand ovine strains included in the present study were recently isolated from the semen of naturally infected rams of the Romney, Southdown and Suffolk breeds. Strains isolated in Australia included three from Queensland, two from N.S.W., five from Victoria and three from South Australia. A further two strains had been isolated from ewes following abortion in Scotland.

Representative strains of *Br. abortus* included the FAO/WHO Type strain 544 (Weybridge), the International Standard Antigen strain 99 (Weybridge), six strains recently isolated from cattle in New Zealand and smooth, mucoid and rough clones of strain 6232 kindly provided by Dr W. Braun of Camp Detrick, U.S.A. *Br. suis* strains included the FAO/WHO Type strain 1330 (Minneapolis) and, from the Institut Pasteur, Tunis, seven strains which had been isolated from man and animals in Europe, Asia and America. *Br. melitensis* strains included the FAO/WHO Type strain 16M (Beltsville) and two strains isolated from cattle in Scotland.

Metabolism and cultural characteristics

All cultures were examined morphologically and checked for purity. Growth was observed on the following media: Albimi brucella agar, Albimi agar plus 10% horse serum, veal infusion agar, veal infusion agar plus 10% horse serum, liver infusion agar, potato infusion agar and tryptose agar, incubation being conducted aerobically and in an atmosphere of 10% CO_2 at 37° C. All strains were cultivated in addition on Albimi agar modified to contain 10% horse serum, 2·5% agar, 1% glucose and 5% glycerol for 96 hr. at 37° C. both aerobically and in 10% CO_2. Colonies were examined by obliquely transmitted light, by the rapid microscopical acriflavine test (Braun & Bonestell, 1947) and following staining with 1/2000 solution of crystal violet (White & Wilson, 1951).

Biochemical properties

For studies on the bacteriostatic action of dyes, the basal medium was Albimi agar plus 10% horse serum. The dyes used were thionin, basic fuchsin and methyl violet (National Aniline Division, Allied Chemical and Dye Corp., New York), and were freshly prepared as 0·1% solutions of pure dyes in distilled water. Thionin

and basic fuchsin were added to the medium to make final concentrations of 1/25,000, 1/50,000 and 1/100,000 and methyl violet was added at a concentration of 1/100,000. Inoculated plates were incubated aerobically or in an atmosphere of 10% CO_2 at 37° C. for 4 days.

Hydrogen sulphide production was tested using Albimi serum agar slopes, the 'lead acetate strips' being placed under the cotton wool plugs and changed daily during 4 days' incubation.

Urease activity was assessed using Bauer's medium (Hoyer, 1950) and all strains were submitted to the sodium diethyldithiocarbamate (D.E.D.T.C.) test following the procedure of Renoux (1952*b*).

Some preliminary studies were undertaken on fermentation of carbohydrates according to the procedure of Pickett & Nelson (1955).

SEROLOGICAL TESTS

Antigens for serum agglutination tests were prepared by first washing off 48 hr. growths on Albimi serum agar with carbolized phosphate buffer (M/15) saline, pH 8·4. After filtering through glass wool, suspensions were held at 4° C. for 2 weeks. Suspensions were then centrifuged, the bacteria resuspended in fresh carbolized phosphate buffered saline pH 8·4 and standardized to a density equivalent to B.W. Opacity Tube No. 4. Suspensions of non-smooth strains prepared in this manner remained stable at 37° C. for 3 days and were not agglutinated by normal rabbit or guinea-pig sera. For serum agglutination tests, the serum dilutions were prepared in carbolized phosphate buffered saline pH 8·4 and to these was added an equivalent volume (0·5 ml.) of the standardized bacterial suspension. The tubes were incubated at 37° C. for 18–24 hr. and tests were read in accordance with the Weybridge procedure.

Immune sera were prepared against four N.Z. (NZ 1, NZ 2, NZ 3 and NZ 4) and eleven Australian strains (Q 1, Q 2, Q 3, NSW 1, NSW 2, V 1, V 2, V 3, SA 1, SA 2 and SA 3) by the intravenous inoculation of two adult male rabbits with 1 ml. of suspensions of each strain containing approximately 3×10^{11} viable organisms per ml. Test bleedings were made from the rabbits immediately before and again 10 days after inoculation and the main bleeding was made 20 days after infection.

Rabbits were also hyperimmunized against strains of *Br. abortus* and *Br. melitensis*. The smooth, mucoid and rough clones of *Br. abortus* strain 6232 prepared by Dr W. Braun at Camp Detrick were utilized. The strains of *Br. melitensis* used were an entirely smooth strain, 'Bovine E', and a subculture of 16M which had been handled in a manner conducive to dissociation, so that approximately 50% of the organisms were in non-smooth phases. Suspensions of these strains were prepared containing approximately 15×10^{10} organisms per ml. and these were heated at 60° C. for 1 hr. Two rabbits were inoculated intravenously with 1 ml. of each suspension and a second inoculation of 1 ml. was given 4 days later. Eighteen days after the second inoculation, the rabbits were finally bled. All sera were held without preservative at −20° C.

Immune sera were absorbed at dilutions of 1/5 or 1/10 in carbolized phosphate buffered saline pH 8·4 depending on titre, sera with titres of 1/640 or higher being

diluted 1/10. Suspensions of each strain for absorption tests were standardized to an opacity equivalent to 20 × BW. Tube 4. Equal volumes of serum dilutions and standardized bacterial suspensions were mixed and held in a water bath at 37° C. for 3 hr. with frequent shaking.

Pathogenicity of ovine strains for guinea-pigs and rabbits

(*a*) Groups of two adult and three weanling guinea-pigs were inoculated intraperitoneally and two adult male rabbits were inoculated intramuscularly with approximately 5×10^9 viable organisms of each of four ovine strains, NZ1, NZ2, NZ3 and NZ4. After 6 weeks, guinea-pigs and rabbits were first bled and then killed for autopsy. Spleens were macerated in Griffith tubes and then inoculated on to six Albimi serum agar plates. In addition, livers of the guinea-pigs and livers, testes and epididymes of the rabbits were cultured.

(*b*) The thirty adult male rabbits inoculated intravenously with approximately 3×10^{11} viable organisms of each of fifteen N.Z. and Australian ovine strains for the serological studies were finally bled 6 weeks after infection and then killed for autopsy.

(*c*) Groups of four adult male guinea-pigs were inoculated intramuscularly with doses of tenfold multiples from 15 to 15×10^9 viable organisms of strain NZ2. Groups of four adult male rabbits were similarly inoculated with from 15 to 15×10^4 organisms of the same strain. The guinea-pigs and rabbits were bled 6 weeks later and then killed for autopsy. Spleens, livers, testes and epididymes were cultured on Albimi serum agar plates.

(*d*) Serial passage of ovine strain in guinea-pigs. A suspension was prepared in a Griffith tube of spleens from two guinea-pigs which had been inoculated intraperitoneally 6 weeks previously with 5×10^9 viable organisms of strain NZ4. This suspension of spleen, shown to contain a heavy concentration of brucella organisms by culture on Albimi serum agar plates, was inoculated intramuscularly into two guinea-pigs. These animals were bled and killed 6 weeks later. Suspensions of spleens were again prepared, six plates inoculated from each spleen and the remainder inoculated into a further two guinea-pigs. The second group of guinea-pigs was killed 6 weeks later. The experiment was repeated with a suspension of spleens from two guinea-pigs, infected 6 weeks previously by the intramuscular inoculation of 15×10^9 viable organisms of the same strain.

RESULTS

Cultural and biochemical characters of strains

The N.Z. ovine strains required an atmosphere containing approximately 10% CO_2 for isolation from naturally infected sheep. Following repeated subculture on solid media or in the yolk sacs of chick embryos, strains may grow aerobically. On first examination of the ovine strains isolated in Australia, all strains required CO_2 for growth, with the exception of three strains, Q2, V4 and V5 which grew aerobically.

The laboratory-adapted strains could be cultivated on Albimi brucella agar (peptone 'M'), veal infusion agar, potato infusion agar, liver infusion agar or tryptose

agar, but growth was improved by the addition of serum, plasma or blood (horse, ox or sheep).

N.Z. ovine strains have been isolated on Albimi agar but the addition of 10% serum to this medium has given the most consistently satisfactory results.

The N.Z. and Australian strains had a similar morphology, being small bacilli with straight axes ranging from 0·7 to 1·2μ in length and from 0·5 to 0·7μ in width, arranged singly and in pairs. All strains were Gram negative, non-motile and non-sporing.

After 48 hr. incubation on Albimi serum agar or veal infusion serum agar in 10% CO_2 at 37° C., colonies viewed by reflected light were 0·5 mm. in diameter, circular, convex, amorphous, smooth, glistening, greyish white with an entire edge. Five-day colonies were up to 2 mm. in diameter and showed no differentiation (Pl. 7, fig. 1). Four-day colonies on Albimi agar modified to contain 10% horse serum, 2·5% agar, 1% glucose and 5% glycerol, examined by obliquely transmitted light were uniformly of a non-smooth type (Pl. 7, fig. 2). All colonies were similarly non-smooth when examined by the rapid acriflavine test (Braun & Bonestell, 1947) and following staining with a 1/2000 solution of crystal violet (White & Wilson, 1951).

The results of the studies on growth in the presence of dyes, production of H_2S and urease activity of the brucellae are summarized in Table 1. All the N.Z. and Australian ovine strains conformed to a characteristic pattern. Growth occurred in the presence of the three concentrations of thionin, although slight depression was observed with some strains at 1/25,000 concentration. All strains grew uninhibited in the presence of basic fuchsin at 1/100,000, growth of all strains was inhibited at 1/25,000, with variable slight growth at 1/50,000. Methyl violet at 1/100,000 completely inhibited all strains. All strains produced little or no H_2S and were negative for urease activity in Bauer's medium over 8 hr. incubation. When submitted to the sodium diethyldithiocarbamate test (Renoux, 1952*b*) the N.Z. and Australian strains reacted similarly to *Br. melitensis* strains. The most characteristic feature of these reactions was the wide outer zone of inhibited growth (Pl. 7, fig. 3).

In preliminary tests of carbohydrate fermentation using the technique of Pickett & Nelson (1955), a selection of recently isolated N.Z. strains reacted similarly to *Br. suis* strains by fermenting glucose, maltose, mannose and trehalose but not inosite and rhamnose.

Two brucella strains isolated from ewes in Scotland differed distinctly from the N.Z. and Australian ovine strains. Both were in the smooth phase, one identified as 'Ovine (Aberdeen)' was biochemically a typical *Br. abortus* and the other, 'Ovine (Caithness)' was a typical *Br. melitensis*. Serologically the former behaved as a typical smooth *Br. abortus* and the latter as a smooth *Br. melitensis*.

The FAO/WHO Type strain 544 behaved characteristically as a *Br. abortus*, but urease activity was very weak. The smooth, mucoid and rough clones of strain 6232 behaved biochemically as typical *Br. abortus* as regards CO_2 requirement, dye sensitivity, H_2S production and urease activity. The six strains isolated from bovine abortions in N.Z. behaved as typical *Br. abortus*.

Table 1. *Biochemical characters of brucella strains*

Strain and origin	Bacteriostatic effect of dyes: Thionin 1/25,000	Thionin 1/50,000	Thionin 1/100,000	Basic fuschin 1/25,000	Basic fuschin 1/50,000	Basic fuschin 1/100,000	Methyl violet 1/100,000	H_2S production	Urease activity
Ovine strains									
14 N.Z. 13 Australian	++ or +++	+++	+++	−	±	+++	−	−	−
Br. suis									
1330 (Minneapolis)	+++	+++	+++	−	−	−	−	+3d	15 min.
D10 Hare (Switz.)	−	−	−	−	−	−	−	−	15 min.
H63 Human (Turkey)	+++	+++	+++	−	−	−	−	+3d	15 min.
S3 (Portugal)	−	−	+	−	−	−	−	−	15 min.
S5 Boar (U.S.A.)	+++	+++	+++	−	−	−	−	+3d	15 min.
S6 P900 Lister	+++	+++	+++	−	−	−	−	+3d	15 min.
S7 '39' (Israel)	+++	+++	+++	−	−	−	−	+3d	15 min.
S6 '6266' (Israel)	+++	+++	+++	−	−	−	−	+3d	15 min.
Br. abortus									
544 (Weybridge)	−	−	−	+++	+++	+++	+++	+3d	−
99 (Weybridge)	−	−	−	+++	+++	+++	+++	+3d	2 hr.
6232, S, M & R (Camp Detrick, U.S.A.)	−	−	−	+++	+++	+++	+++	+3d	2 hr.
6 N.Z. bovine strains	−	−	−	+++	+++	+++	+++	+3d	2 hr.
Ewe Aberdeen (U.K.)	−	−	−	+++	+++	+++	+++	+3d	2 hr.
Br. melitensis									
16M (Beltsville)	+++	+++	+++	+++	+++	+++	+++	−	2 hr.
Bovine E (U.K.)	+++	+++	+++	+++	+++	+++	+++	−	2 hr.
Bovine K (U.K.)	+++	+++	+++	+++	+++	+++	+++	−	2 hr.
Ewe (Caithness, U.K.)	+++	+++	+++	+++	+++	+++	+++	−	2 hr.

−, ±, +, ++, +++ = degrees of growth.

All strains of *Br. suis* grew well aerobically on Albimi agar. Of the two strains which were susceptible to all dyes tested including thionin, D10 had been isolated from a naturally infected hare in Switzerland.

Serological tests

None of the rabbits used for the preparation of immune sera showed, when tested immediately before inoculation, serum agglutinins for any of the brucella strains. The homologous and heterologous titres of a series of antisera prepared against twenty brucella strains are tabulated in Table 2. A smooth *Br. abortus*

Table 2. *Agglutinin titres of sera prepared against* 20 *brucella strains*

	Antigens					
Unabsorbed sera	A. 6232 S	A. 99 (Weybridge)	M. Bovine E	A. 6232 M	A. 6232 R	17 N.Z. and Aust. ovine strains
Br. abortus 6232, S clone	5,120	5,120	2,560	0	0	0
Br. melitensis bovine E, S	5,120	5,120	10,240	0	0	0
Br. melitensis 16 M, S and non-S	640	640	1,280	320	320	160
Br. abortus 6232, M clone	0	0	0	640	640	320
Br. abortus 6232, R clone	0	0	0	640	640	320
Br. ovis, NZ1	0	0	0	320	320	640
NZ2	0	0	0	160	160	320
NZ3	0	0	0	320	320	640
NZ4	0	0	0	640	640	1,280
Q1	0	0	0	160	160	160
Q2	0	0	0	80	80	160
Q3	0	0	0	320	320	640
NSW1	0	0	0	320	320	640
NSW2	0	0	0	80	80	160
V1	0	0	0	320	320	640
V2	0	0	0	160	160	320
V3	0	0	0	320	320	640
SA1	0	0	0	640	640	640
SA2	0	0	0	320	320	640
SA3	0	0	0	320	320	640

antiserum and a smooth *Br. melitensis* antiserum showed no cross reactions with the antigens of twenty-seven N.Z. and Australian ovine strains and the mucoid and rough clones of *Br. abortus* 6232. Similar results were also observed with two smooth *Br. abortus* antisera (B17 and B403) and two smooth *Br. melitensis* antisera (B31 and B421) prepared by Prof. J. C. Cruickshank and with antisera prepared with entirely smooth suspensions of *Br. abortus* 544 and *Br. melitensis* 16M by Dr Lois Jones (private communication). An antiserum prepared against a suspension of *Br. melitensis* 16M comprising organisms in the smooth and non-smooth phases gave cross-reactions with antigens of the rough and mucoid clones of *Br. abortus* 6232 as well as with the twenty-seven ovine strains.

The mucoid and rough *Br. abortus* 6232 antisera failed to agglutinate suspensions of smooth brucellae but agglutinated all the ovine strain antigens to approximately

half their homologous titres. Each ovine strain antiserum agglutinated all the ovine strain antigens to identical titre and the mucoid and rough *Br. abortus* suspensions to at least half the homologous titre. None of the ovine strain antisera showed cross reactions with the suspensions of smooth brucellae.

Absorption of the *Br. melitensis* 16M antiserum with suspensions of *Br. abortus* 6232M, 6232R or the ovine strains did not reduce the titres for smooth brucellae, but eliminated the cross reactions with the mucoid, rough and ovine strains. Also absorption of this antiserum with suspensions of smooth brucellae did not affect the cross-reactions with mucoid, rough and ovine strain antigens.

Complete absorption of all agglutinins from the 6232M and 6232R antisera was effected using suspensions of the mucoid, rough or ovine strains.

A single absorption of the antisera prepared against the ovine strains with suspensions of either 6232M or 6232R removed all the agglutinins for both 6232M and 6232R and left approximately half the agglutinins for the ovine strains. A second absorption with the mucoid or rough suspensions effected the removal of all or a substantial proportion of the remaining agglutinins for the ovine strains.

Absorption tests with the ovine strain antisera using suspensions of the individual ovine strains permitted the differentiation of these strains into two groups. The first group included the fourteen N.Z., the three Queensland, the two N.S.W., one Victorian and the three South Australian strains. The remaining four Victorian strains comprised the second group. The results of absorption tests with antisera prepared from strains representative of the two groups are summarized in Tables 3 and 4. Single absorptions of the antisera prepared against the first group of ovine strains with suspensions of the second group of strains eliminated the cross reactions with 6232M or 6232R and left up to half the agglutinins for the first group of strains only. Antisera absorbed in this manner were suitable as testing sera, permitting ready identification of strains into either of the two groups. A second absorption of these antisera removed approximately half of the remaining agglutinins. Corresponding results were observed following absorption of antisera prepared against the second group of strains with suspensions of the first group. These absorbed antisera agglutinated only the four strains comprising the second group of ovine strains.

Pathogenicity of ovine strains for guinea-pigs and rabbits

Guinea-pigs inoculated intraperitoneally with 5×10^9 viable organisms of four N.Z. strains showed no macroscopical lesions when slaughtered 6 weeks later. Only one animal died before completion of the experiment, this being a weanling guinea-pig which died 11 days after inoculation with strain NZ 3. Immediately prior to slaughter none of the animals showed serum agglutinins for smooth *Br. abortus* (Weybridge antigen), and their homologous serum titres varied from 0 to 1/20. Brucella organisms were recovered regularly from the spleens and all colonies examined were of non-smooth type.

The rabbits inoculated intravenously with 5×10^9 viable organisms of the four N.Z. ovine strains had developed homologous serum agglutinin titres of from 1/160 to 1/1280, 15 days after inoculation, and 6 weeks after inoculation, the titres

Table 3. *Absorption tests with an ovine strain (NZ1) serum*

Antigens	Unabsorbed serum	Absorbed at 1/10 with																	
		6232M	6232R	NZ1	NZ2	NZ3	NZ4	Q1	Q2	Q3	NSW1	NSW2	V1	V2	V3	V4	V5	SA1	SA2	SA3
A6232M	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A6232R	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NZ1	640	320	320	0	0	0	0	0	0	0	0	0	0	320	320	320	320	0	0	0
NZ2	640	320	320	0	0	0	0	0	0	0	0	0	0	320	320	320	320	0	0	0
NZ3	640	320	320	0	0	0	0	0	0	0	0	0	0	320	320	320	320	0	0	0
NZ4	640	320	320	0	0	0	0	0	0	0	0	0	0	320	320	320	320	0	0	0
Q1	640	320	320	0	0	0	0	0	0	0	0	0	0	320	320	320	320	0	0	0
Q2	640	320	320	0	0	0	0	0	0	0	0	0	0	320	320	320	320	0	0	0
Q3	640	320	320	0	0	0	0	0	0	0	0	0	0	320	320	320	320	0	0	0
NSW1	640	320	320	0	0	0	0	0	0	0	0	0	0	320	320	320	320	0	0	0
NSW2	640	320	320	0	0	0	0	0	0	0	0	0	0	320	320	320	320	0	0	0
V1	640	320	320	0	0	0	0	0	0	0	0	0	0	320	320	320	320	0	0	0
V2	640	320	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
V3	640	320	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
V4	640	320	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
V5	640	320	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SA1	640	320	320	0	0	0	0	0	0	0	0	0	0	320	320	320	320	0	0	0
SA2	640	320	320	0	0	0	0	0	0	0	0	0	0	320	320	320	320	0	0	0
SA3	640	320	320	0	0	0	0	0	0	0	0	0	0	320	320	320	320	0	0	0

Table 4. *Absorption tests with an ovine strain (V2) serum*

Antigens	Unabsorbed serum	Absorbed at 1/10 with																	
		6232M	6232R	NZ1	NZ2	NZ3	NZ4	Q1	Q2	Q3	NSW1	NSW2	V1	V2	V3	V4	V5	SA1	SA2	SA3
A6232M	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A6232R	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NZ1	640	320	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NZ2	640	320	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NZ3	640	320	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NZ4	640	320	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Q1	640	320	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Q2	640	320	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Q3	640	320	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NSW1	640	320	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NSW2	640	320	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
V1	640	320	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
V2	640	320	320	160	160	160	160	160	160	160	160	160	160	0	0	0	0	160	160	160
V3	640	320	320	160	160	160	160	160	160	160	160	160	160	0	0	0	0	160	160	160
V4	640	320	320	160	160	160	160	160	160	160	160	160	160	0	0	0	0	160	160	160
V5	640	320	320	160	160	160	160	160	160	160	160	160	160	0	0	0	0	160	160	160
SA1	640	320	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SA2	640	320	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SA3	640	320	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

varied from 1/80 to 1/640. No serum agglutinins for smooth *Br. abortus* were apparent in any animal. The only macroscopic lesion present in the rabbits was diffuse necrosis of a testis in one rabbit inoculated with strain NZ1. Brucella organisms were recovered in heavy concentration from the tails of the epididymes and spleens of the rabbits inoculated with each of the four strains.

The homologous serum titres of the rabbits 6 weeks after intravenous inoculation with approximately 300×10^9 organisms of 15 N.Z. and Australian ovine strains varied from 1/80 to 1/320. One rabbit inoculated with strain NZ2 showed necrosis of one testicle. Brucella organisms could be recovered most consistently in heavy concentration from the tails of the epididymes and spleens.

Histological examination of tissues from infected rabbits was kindly undertaken by Mr W. J. Hartley. Histological changes were more regularly observed in the liver than in the other organs. The lesions in the livers included intrasinusoidal foci of varying size consisting of lymphocytes, epithelioid cells and occasional eosinophiles, the larger foci being associated with limited adjacent hepatocellular necrosis. Similar cellular infiltrations were apparent in and about the portal tracts, sometimes occurring as distinct foci but more usually as diffuse infiltrations extending often into Glisson's capsule. Varying degrees of bile ductule proliferation were observed, associated with an increased mononuclear hypercellularity of the area and early fibroblastic activity.

The spleen showed evidence of apparent lymphoid hyperplasia and mild to moderate epithelioid infiltrations of the pulp and occasionally of the Malphigian corpuscles. Small focal hyaline bodies were observed in some sections. Extensive phagocytosis was apparent in the sinusoids and macrophages contained large quantities of intracytoplasmic nuclear and other debris.

Kidneys showed mild to extensive cortical interstitial infiltration with cellular elements similar to those observed in the liver. Located particularly in the medulla were several large foci of small and large mononuclear hypercellularity, the larger foci showing early central caseation.

One testis showed diffuse degenerative changes, with intensive hypercellularity undergoing caseous necrosis. An acute inflammatory reaction was apparent in the associated epididymis, the tubules showing dilatation, intense cellular infiltration with necrosis, desquamation of the tubule epithelium and spread of the reaction to the interstitial tissues and the presence of many epithelioid cells (Pl. 8, figs. 4, 5).

Guinea-pigs inoculated intramuscularly with doses of from 15×10^3 to 15×10^9 strain NZ4 organisms showed splenic infection when killed 6 weeks later. Homologous serum agglutinin titres of infected guinea-pigs 6 weeks after inoculation did not exceed 1/10. Brucellae were not recovered from the spleens of guinea-pigs inoculated similarly with doses of 15×10^2, 150 or 15 organisms.

Splenic infection was demonstrated in rabbits 6 weeks after the intramuscular inoculation of doses of 15×10^3 and 15×10^4 organisms of the same strain, infection not being apparent following the inoculation of 15×10^2, 150 and 15 organisms. Homologous serum agglutinin titres of the infected rabbits at slaughter varied between 1/10 and 1/20.

In the two experiments in which suspensions of infected guinea-pig spleens were inoculated intramuscularly into guinea-pigs, ovine strain brucellae could not be recovered from the spleens of these animals when killed 6 weeks later.

DISCUSSION

The present observations indicate that the N.Z. and Australian ovine strains could be classified in the *Brucella* genus on the basis of cell morphology and staining, colonial morphology, cultural characteristics, biochemical properties, pathogenicity for laboratory animals and their association with genital disease in the natural host. The organisms have never been observed in other than the non-smooth phase. These strains react similarly to *Br. suis* in carbohydrate fermentation and dye-sensitivity tests. The ovine strains require CO_2 for primary isolation, grow sparsely on media which are adequate for *Br. suis* strains, have weak urease activity and produce little or no H_2S. In the D.E.D.T.C. test (Renoux, 1952*b*), the ovine strains have reacted similarly to *Br. melitensis*. Further, these strains are significantly less pathogenic for guinea-pigs and rabbits than *Br. suis*.

The antigenic relationship of the ovine strains to the smooth and non-smooth variants of other brucella species is of particular interest. Naturally infected sheep or experimentally infected sheep and laboratory animals have shown no agglutinins for smooth brucellae. Also in the present study, suspensions of entirely smooth brucellae have not been agglutinated by antisera prepared against mucoid and rough cells of a *Br. abortus* strain or the N.Z. and Australian ovine strains. The sheep strains, however, apparently share a common 'rough' antigenic factor with non-smooth brucella strains and possess in addition two antigenic factors which may vary quantitatively in individual ovine strains. It appears that these factors are also present, but in significantly smaller quantities, in the non-smooth variants of the other brucella species. Thus the ovine strains lack the specific antigens of smooth brucellae but are antigenically closely related to the non-smooth brucella variants. Renoux & Mahaffey (1955) have also reported on the recognition of a specific 'Z' antigen in N.Z. ovine strains and explained the serological behaviour of the various 'varieties' of brucella by a concentric representation of the antigens in the micro-organisms.

Wilson (1933) discussing the classification of the brucella group, observed that besides the existence of the three main groups 'there exists within each group a number of sub-groups containing transitional strains, which frequently are associated with some particular geographical location. The suggestion is that members of the brucella group are relatively labile and respond readily to environmental changes'. A greatly increased number of naturally occurring 'atypical' or 'aberrant' strains have been recognized in recent years and these have presented taxonomical problems. Renoux & Carrère (1952) have shown that a spontaneous variant of *Br. abortus* was indistinguishable from *Br. melitensis* following passage through either guinea-pigs or sheep. Also the investigations of Braun & Oglesby (1953) have indicated that many spontaneously occurring aberrant types are not eliminated in competition with the more typical parent type in guinea-pigs. Consequently, it has been suggested that under certain environmental conditions, or even in

certain hosts, these aberrant types may attain an even higher selective value and replace their normal parent type.

Of considerable epidemiological interest has been the recognition of brucellosis of hares occurring in Germany (Witte, 1941), in Switzerland (Burgisser, 1949), in France (Jacotot & Vallée, 1951), and in Denmark (Bendtsen, Christiansen & Thomsen, 1954). Six of eight brucella strains recovered from hares in France had the characters of the Danish variety of *Br. suis* (Jacotot & Vallée, 1954). Swine brucellosis does not exist in France and *Br. suis* has not been isolated from domestic animals. Jacotot & Vallée (1954) have suggested that these strains may have developed by mutation in hares following infection originally with *Br. abortus* or *Br. melitensis*. The strains recovered from hares in Denmark have the biochemical characters of the Danish type of *Br. suis* and have been shown to be pathogenic for swine and pregnant cattle (Bendtsen *et al.* 1954). Hares are presumed to have been the source of three epizootics of swine brucellosis in that country.

Renoux (1952*a*) has suggested that the currently accepted species of brucella may represent merely varieties of a single species as their characterization depends on quantitative rather than qualitative differences. Consequently Renoux has proposed a new species, *Brucella brucei*, with five varieties, *melitensis*, *abortus*, *suis*, *thomseni* and *lisbonnei*. However, for practical purposes other workers (Braun & Oglesby, 1953; Pickett, Nelson & Liberman, 1953) would prefer 'to retain the so-called species of *Br. abortus*, *Br. suis* and *Br. melitensis* as a reference standard, even though they may merely represent the most common biotypes of one species'.

The N.Z. ovine strain has been provisionally described as a stabilized mutant or new variety of brucella in view of its existence in the non-smooth phase in naturally infected sheep and in consideration of the characters which distinguished it from the three classical brucella species (Buddle & Boyes, 1953). The desirability of presenting strains for classification in the smooth phase is generally recognized, as such characters as urease activity and dye sensitivity of non-smooth variants may be depressed as compared to the smooth parent organism (Pickett *et al.* 1953). Luria (1947) has also emphasized that 'character differences that can be brought about by a single mutational step are of little value for classification and should not be made the basis for taxonomic differentiation'. However, the N.Z. and Australian strains are sufficiently stable and distinguishable from *Br. abortus*, *Br. suis* and *Br. melitensis* in a number of independently variable characters to justify their constituting a new species, *Brucella ovis*. In the recently proposed classification system of Renoux (1952*a*), these strains would constitute a new variety, *Brucella brucei* var. *ovis australasiae*.

Van Drimmelen (1953) has discussed the possible proposal of a new species '*Brucella ovis*', for strains isolated from Karakul sheep in South West Africa, which have the biochemical characters of *Br. melitensis* but produced H_2S. This suggestion was not adopted, and the organism was given the designation '*Br. melitensis* var. Karakul'. Obviously the strains for which the designation *Br. ovis* (n.sp.) is now proposed, are quite different from *Br. melitensis* var. Karakul, and this nomenclature should not be the cause of confusion.

Two strains isolated from sheep in the United Kingdom were included in the

present study, one being a *Br. melitensis* and the other a *Br. abortus*. *Br. melitensis* infection of sheep has not previously been recognized in the U.K. This strain was isolated by Mr G. A. M. Sharman, the report of the infection being in course of publication (P. L. Shanks, private communication). Ewe abortion in Scotland due to infection with *Br. abortus* has been reported by Young (1953).

SUMMARY

1. The characters of twenty-seven strains isolated from sheep in New Zealand and Australia were examined and compared with those of representative strains of *Brucella abortus*, *Br. suis* and *Br. melitensis*.

2. These strains could be classified in the brucella genus on the basis of cell morphology and staining, colonial morphology, cultural characteristics, biochemical properties, pathogenicity for laboratory animals and association with genital disease in the natural host.

3. The ovine strains have never been observed in other than the non-smooth phase. Consequently, these organisms lack the specific surface antigens of smooth brucellae but are antigenically closely related to the non-smooth variants of other brucella species. Three antigenic factors were identified and their presence confirmed in all twenty-seven ovine strains.

4. The N.Z. and Australian ovine strains appear sufficiently stable and distinguishable from other established groups of the brucella genus in a number of independently variable characters to justify their constituting either a new species, *Brucella ovis*, or a new variety, *Brucella brucei* var. *ovis australasiae*.

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EXPLANATION OF PLATES

PLATE 7

Fig. 1. Five-day colonies of *Br. ovis* (Strain NZ 1) on Albimi serum agar viewed by reflected light.

Fig. 2. Colonies of *Br. ovis* (Strain NZ 1) showing non-smooth character when examined by obliquely transmitted light.

Fig. 3. D.E.D.T.C. test with *Br. ovis* (Strain NZ 1) showing wide outer zone of inhibited growth.

PLATE 8

Fig. 4. Section of testis and epididymis of rabbit infected with *Br. ovis* (Strain NZ 2), showing extensive hypercellularity and necrosis of testes and acute inflammation with dilatation of tubules of epididymes.

Fig. 5. Section of epididymis of infected rabbit showing dilated tubules containing cellular debris and cellular infiltration of the interstitial tissues.

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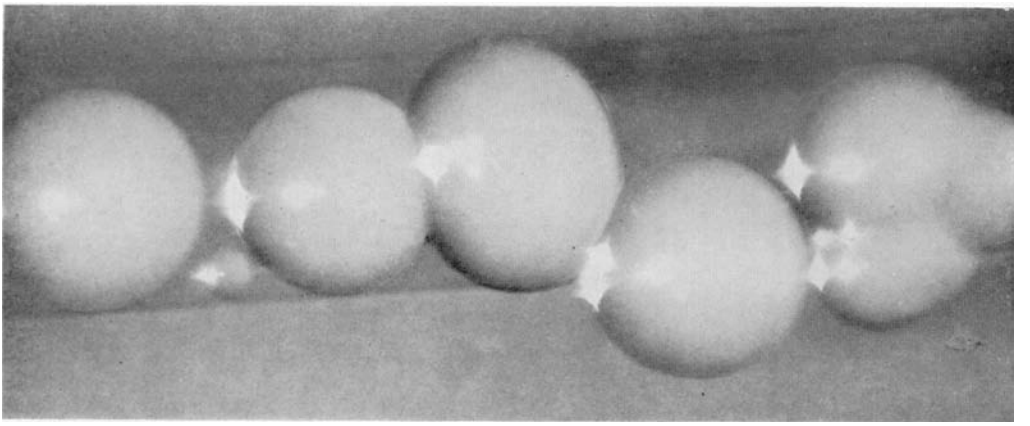

Fig. 1

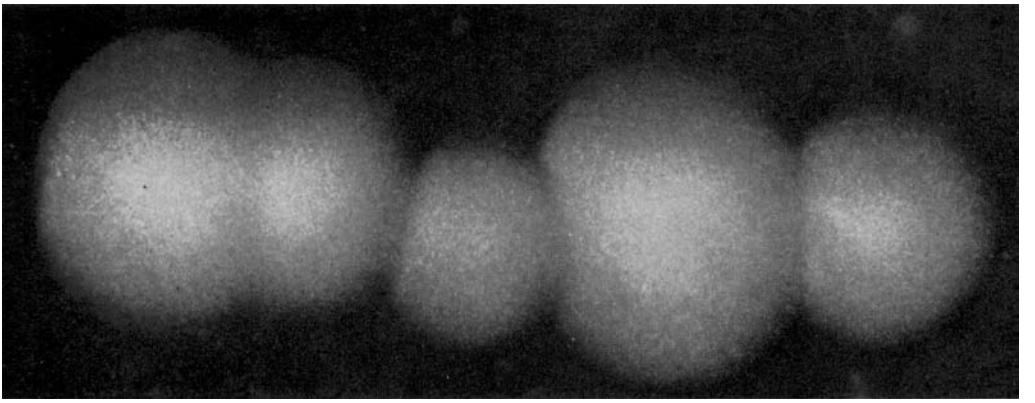

Fig. 2

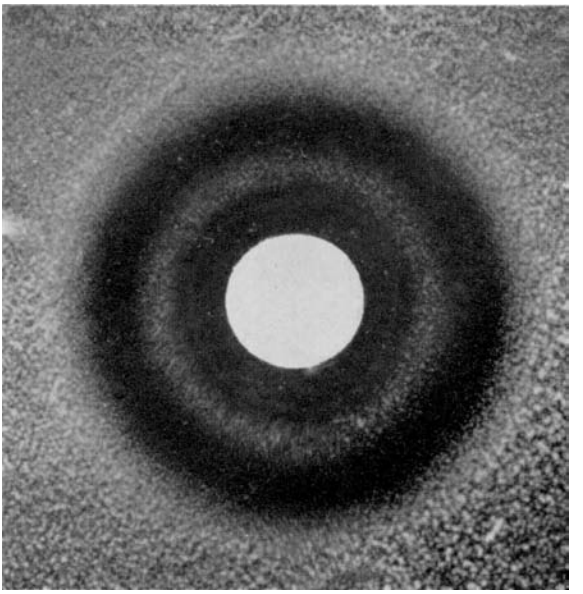

Fig. 3

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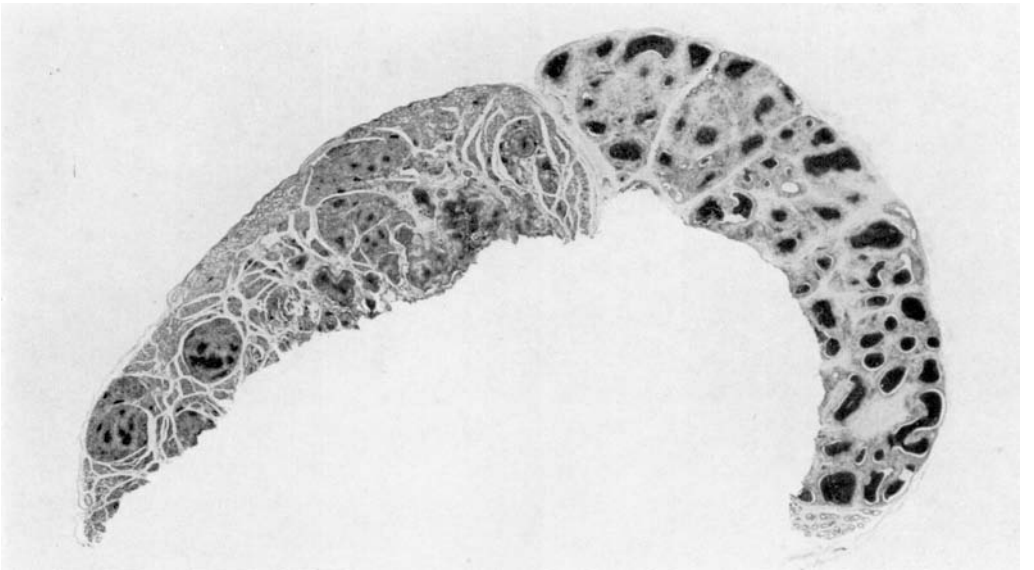

Fig. 4

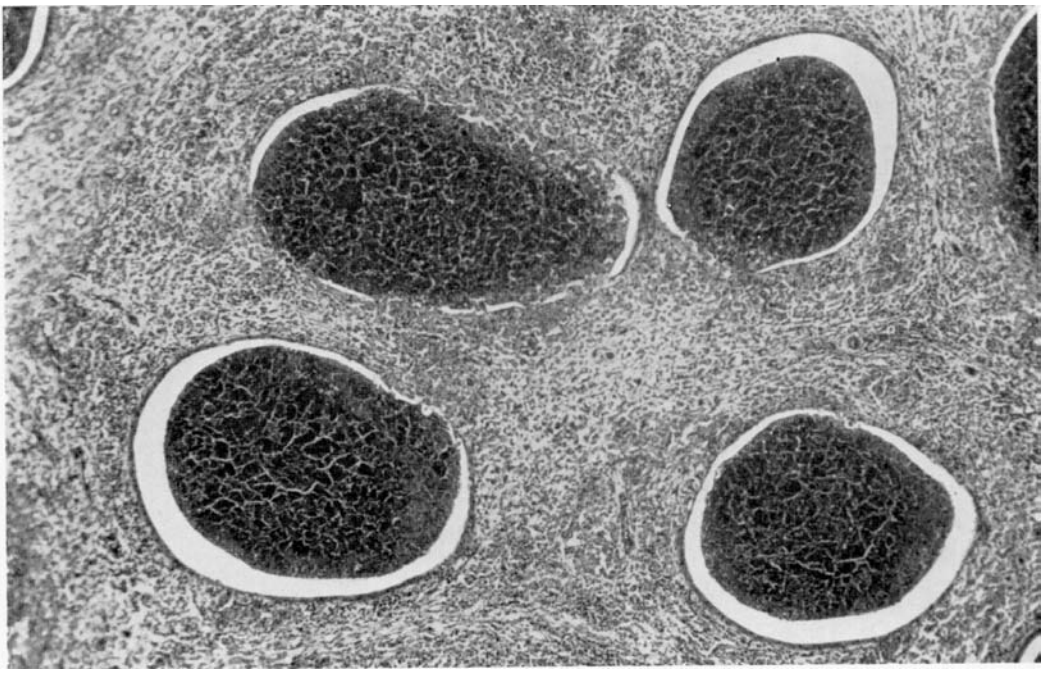

Fig. 5

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