CLASSIFICATION OF THE GENUS *BRUCELLA*

Yasuro ISAYAMA*

In the 1910s, Japan became free from the infectious abortion of cattle, but the importation of breeders from abroad caused the spread of this disease. Epizootics of this disease became quite frequent among the stock farms and dairy farms in Tokyo and other districts around the year 1910 causing great financial damage. Brucellosis of bovine was first recognized in our country in 1916 by Okuda and Fukuda, and *Brucella abortus* as *Bacillus abortus* of Bang (1897) were isolated from the cows as well as from the aborted fetuses of the foreign breeds like Jersey and Holstein in more than 10 farms in Tokyo during the period 1913 - 1915.

Twenty years later, Nohmi *et al.*(1936) isolated *Br. suis* from the aborted fetus of a sow imported from the United states. In the same year, Itabashi *et al.*(1936) isolated *Br. melitensis* from the aborted fetus and the organs of ewes.

After the Second World War, the examination of *Brucella* agglutinin in the sera of all dairy cows was compulsory, and many cows were slaughtered when found positive.

To improve dairy farming, in Japan, after the War, more than fifteen thousand Jersey cattle have been imported from the United States, Australia and New Zealand and distributed to the farmers from 1953 till 1960. In spite of severe inspection, it was not always possible to eliminate all contaminated cattle which became the centres of small epidemics in many regions of Japan.

The authors isolated many strains of *Brucella abortus* from imported Jersey cows and home-born Holstein cows, and proceeded to their classification. Between the two groups of strains, there were obvious differences in biological properties, especially in sensitivity to dyes and penicillin. While the strains from the Jerseys presented the typical characters of *Br. abortus (Br. abortus* biotype 1), those from the Holsteins showed a higher sensitivity to dyes and penicillin and were classified as *Br. abortus* Type II(Wilson) (*Br. abortus* biotype 2).

In the 1950s, the Subcommittee on the Taxonomy of *Brucella* was not satisfied with having *Br. ovis* (Buddle, 1956) placed in the genus *Brucella* with regard to *Br. neotomae* (Stoenner and Lackman, 1957). It was recommended that further studies should be carried out before a decision on its position in the genus could be made.

Subsequently, on the basis of taxonomical results reported, the Subcommittee(1966) decided that *Br. neotomae* should be accepted as a new species.

Of great interest was the work on content of (G + C) and on DNA homology reported. At the next Meeting of the Subcommittee (1970) it was decided to incorporate *Br. ovis* and *Br. canis* (Carmichael and Bruner, 1968) into the genus *Brucella,* as shown in Table 1.

Identification of microorganisms isolated from an enzootic case of canine abortion in Japan

Canine brucellosis was first recognized in the United States in 1966. Abortion was first observed in August, 1971 in a beagle colony where 187 dogs had been kept. Since then stillbirth and sterility as well as abortion have been recorded continuously among the bitches. About 10 months later, bacteriological studies were carried out in order to identify the causal agent and in July 1972, a small Gram-negative organism was isolated from a fetus brought to the laboratory.

The first strain to be isolated was named QE13 and was isolated in pure culture from the brain and a mixture of various organs of aborted fetus of a dog. Subsequently 8 and 6 canine strains were isolated from the same colony by Suzuki and Ueda respectively.

* Chief of Bacterial Products Laboratory, Biological Products Division, National Institute of Animal Health, 3-1-1, Kannondai, Yatabe, Tsukuba, Ibaraki 305, Japan.

Table 1 Differential characters of the species and biotypes in the genus *Brucella*.

Species	Biotype	Lysis by phage	CO_2 Required	H_2O Produced	Growth on dyes[a]					Agglutination by		Substrate utilization[b]												Most common host reservoir
					Thionin			Basic fuchsin		Monospecific sera		Antirough serum	Amino acids			Urea cycle substrates				Carbohydrates					
		10^4 X RTD			a	b	c	b	c	A	M		L-Alanine	L-Asparagine	L-Glutamate	DL-Ornithine	DL-Citruline	L-Arginine	L-Lysine	L-Arabinose	D-Galactose	D-Ribose	D-Glucose	*i*-Erythritol	
Br. melitensis	1	–	–	–	–	+	+	+	+	–	+	–												
	2	–	–	–	–	+	+	+	+	+	–	–	+	+	+	–	–	–	–	–	–	+	+	Sheep, goats
	3	–	–	–	–	+	+	+	+	+	+	–												
Br. abortus	1	+	+	+(–)[c]	+	–	–	–	+	+	+	–	–												
	2	+	+	+	+	–	–	–	–	–	+	–	–												
	3	+	+	+(–)	+	+	+	+	+	+	+	–	–												
	4	+	+	+(–)	+	–	–	–	+	+	–	+	–												
	5	+	+	–	–	–	+	+	+	+	–	+	–	+	+	+	–	–	–	–	+	+	+	+	+	Cattle
	6	+	+	–	–,+	–	+	+	+	+	+	–	–												
	7	+	+	–	–,+	–	+	+	+	+	+	+	–												
	8	+	+	+	–	–	+	+	+	+	–	+	–												
	9	+	+	–,+	+	–	+	+	+	+	–	+	–												
Br. suis	1	–	+	–	+	+	+	+	–	–	+	–	–	±	–	–	+	+	+	+	+	+	+	+	+	Pigs
	2	–	+	–	–	–	+	+	–	–	+	–	–	–	–	±	+	+	+	+	–	+	±	+	+	Pigs, hares
	3	–	+	–	–	+	+	+	+	+	+	–	–	±	–	±	+	+	+	+	–	–	+	+	+	Pigs
	4	–	+	–	–	+	+	+	+	+	+	+	–	–	–	±	+	+	+	+	–	–	+	+	+	Reindeer
	5	–	–	–	+			+		+	–	–	+	+	+	+	+	+	+	±	–	±	+	+	±	Cattle, sheep
Br. neotomae		–	+	–	+	–	–	+	–	–	+	–	–	±	+	+	–	–	–	+	+	+	±	+	±	Wood rat'
Br. ovis		–	–	+	–	+	+	+	+	+	–	–	+	±	–	+	–	–	–	–	–	–	–	–	–	Sheep (rams)
Br. canis[d]		–	–	–	–	+	+	+	–	–	–	–	+	±	–	+	+	+	+	+	±	±	+	+	±	Dogs

a Dyes from National Aniline Division, Allied Chemical and Dye Co., New York, are recommended. On Albimi or tryptose agar the results shown should be obtained with the following concentrations of dyes: a, 1:25,000; b, 1:50,000; c, 1:100,000. Other concentrations may be preferable with other growth media.

b +, Oxidized (QO_2N value greater than 50) by all strains by manometric methods; –, not oxidized by any strain; ±, oxidized by some strains.

c Usually positive, but negative varieties occur.

d Proposed new species (3).

All the isolates showed the same properties on the various tests (Table 2), but in one of the four strains oxidative metabolism tests showed considerable higher QO_2N values for L-alanin, L-asparagine, L-glutamic acid, DL-citruline and meso-erythritol, but high values for D-ribose and low values for L-lysine were a feature among all four strains.

The results of the morphological and biochemical studies for these 15 isolates were similar to those reported by Carmichael and Bruner; Jones *et al.*, 1968, and they were identified as *Br. canis*.

The pathogenicity and antigenicity of *Brucella canis* strain QE13 for experimental animals

The pathogenicity and antigenicity of strain QE13, the first isolate of *Brucella canis* obtained in Japan, have been determined in mice, guinea pigs and rabbits and comparison made with reference strains of other *Brucella* species.

1 Mice

For LD_{50} and ID_{50} determinations, from 10^1 to 10^{11} organisms were injected intraperitoneally.

Table 2 Biological properties

Species, biotypes and strains	Lysis by Tb phage		CO_2 requirement	H_2O production	Growth on dyes					Agglutination in		
					Thionin			Basic fuchsin		Monospecific sera		Anti-rough serum
	RTD	RTD 10^4X			a	b	c	b	c	A	M	
Isolates 15 strains	–	–	–	–	+	+	+	–	–	–	–	+
Br. canis RM6/66	–	–	–	–	+	+	+	–	–	–	–	+
Br. melitensis 16M	–	–	–	–	–	+	+	+	+	–	+	–
Br. abortus 544	+	+	+	+	–	–	–	+	+	+	–	–
Br. suis 1330	–	+	–	+	+	+	+	–	–	+	–	–
Br. ovis 63/290	–	–	+	–	–	+	+	+	+	–	–	+
Br. neotomae 5K33	–	+	–	+	–	–	+	–	–	+	–	–

a = 1:25,000; b = 1:50,000; c = 1:100,000; A = *abortus*; M = *melitensis*

The cause of death was confirmed by culturing heart blood of mice used in LD_{50} determination. For ID_{50} determination, surviving mice were sacrificed 1 month after inoculation and their spleens cultured.

The pathogenicity of *Br. canis* QE13 followed that of *Br. melitensis*, *Br. abortus* and *Br. suis*. The strong infectivity of *Br. neotomae* for mice, as in the case of *Br. canis* for the dogs, is interesting, considering its origin.

2 Guinea pigs

Doses of 10^1 to 10^6 organisms were inoculated subcutaneously. After 1 month the animals were killed by exsanguination. The distribution of bacteria in lymph nodes, organs and blood was determined by culture. The serum agglutination titres were also determined.

Persistence of the microorganism and increasing agglutination titres in the animals tested amounted to a level of 10^1 for *Br. abortus*, 10^2 for *Br. suis*, 10^3 for *Br. melitensis* and were at the same level for *Br. canis*. Antibody titres of animals inoculated with *Br. melitensis*, *Br. abortus* and *Br. suis* were high ranging from 1:320 to 1:1,280, and in the case of *Br. canis* they were almost the same as those listed above.

3 Rabbits

After inoculation of 10^9 microorganisms intravenously the bacterial distribution and agglutination titres were examined 1 and 2 weeks later. High bacterial counts were obtained in various animals infected with *Br. melitensis, Br. abortus* and *Br. suis*. Agglutination titres of rabbits were generally high, especially 2 weeks later. Therefore, *Br. canis* was not exceptional in this respect.

Our results indicated the existence of two *Brucella* groups according to the pathogenicity for experimental animals. The most pathogenic ones were *Br. melitensis, Br. abortus* and *Br. suis* as classical *Brucella* species, and the least pathogenic ones were *Br. neotomae, Br. ovis* and *Br. canis*. Of

the latter *Br. canis* appeared the most pathogenic.

The titre for the homologous *Br. canis* antigen was 1:640, that for *Br. ovis* 1:320 whereas reactions were negative for *Br. melitensis, Br. abortus, Br. suis* and *Br. neotomae*. By absorption with *Br. ovis*, this immune serum lost reactivity against the former but retained a titre of 1:160 for homologous *Br. canis* antigen. Similarly by absorption with the latter, the serum completely lost any titre against the homologous strain. From the above results, serum prepared with *Br. canis* showed reactivity not only to *Br. canis* but also to *Br. ovis* antigen and the presence of a common antigen was suggested.

Special mention of a new species of *Brucella*—*Brucella canis*

A disease associated with abortion and early embryonic death in females and epididymitis and testicular degeneration in males has been observed in dogs among which beagles were chiefly affected.

The organism could be isolated on pure culture from blood or vaginal discharge of affected bitches, and from the fetus and placenta. Prolonged (up to one year) bacteraemia is frequently accompanied by lymphadenitis and splenitis and, on culture, abundant growth of the organism can be obtained; the rate of isolation from semen has been low.

The disease is easily transmitted experimentally by inoculation and by oral, conjunctival and contact exposure; the incubation period can vary from 4 days (after intravenous inoculation) to 3 weeks (oral exposure). Puppies born of infected mothers show bacteraemia with enlargement of the lymph nodes. The infection rate is highest when dogs are housed in groups and the incidence can be as high as 25 - 40%. Diagnosis is based on the recovery of the organism and/or an agglutination test using heat-killed cells and incubation in a water-bath at 50 - 52°C (temperatures above 45°C are important for the agglutination test). Human infection has also been reported.

The organism is aerobic, forming colonies which, on prolonged incubation (5 - 7 days), are mucoid, and 1 - 1.5 mm in size. Reference has already been made to the work on content of (G + C) and on DNA homology, by which criteria it is indistinguishable from other species of *Brucella*. The organism has an oxidative metabolic pattern similar to that of *Br. suis* biotypes 3 and 4 (although it does not oxidize erythritol); it is not lysed by phage at either RTD or 10^4 RTD and its growth is inhibited by basic fuchsin but not by thionine. Even on primary isolation, the organism is in the non-smooth phase and it does not agglutinate in sera prepared from other (smooth) species of *Brucella*, neither does it agglutinate in mono-specific sera; for confirmation of identity by serological tests, therefore, unabsorbed serum against rough *Brucella* should be used.

A detailed study of the antigenic relationship of *Br. canis* to other species of *Brucella*, showed that *Br. canis* lacked the lipopolysaccharide endotoxin associated with the agglutinogen of smooth types of *Brucella*. By using whole-cell suspensions in agglutination and agglutinin-absorption tests, *Br. canis* was shown to be similar to rough *Br. abortus, Br. melitensis* and *Br. ovis*, e.g. *Br. ovis* antiserum agglutinated *Br. ovis* and *Br. canis* antigens but not (smooth) *Br. melitensis* antigen. By using soluble antigens in immunoelectrophoretic and gel diffusion studies, extensive cross reactions were demonstrated within the genus *Brucella* but not with other Gram-negative bacteria.

It is important to distinguish between this disease caused by *Br. canis* and the occasional recovery of *Br. abortus, Br. melitensis* or *Br. suis* from dogs where dog-to-dog transmission does not occur.

Chemo-taxonomical studies on fatty acids of *Brucella* species

Since the 1974 Meeting of the Subcommittee on Taxonomy of *Brucella*, six species have been registered in the genus *Brucella*. Three of them, *Br. melitensis, Br. abortus* and *Br. suis*, have been important from the beginning of bacteriology and are further divided into three, nine and four biotypes, respectively. The features available for the differentiation of these species are the natural host, biological and biochemical characters, serological specificities, metabolic patterns, and susceptibility to a phage. It is not always easy, however, to apply all these techniques to the dif-

ferentiation, since they are sometimes liable to cause laboratory infection.

This paper describes the results of experiments performed to clarify the effectiveness of fatty acid patterns using gas chromatography for the rapid identification of *Brucella* species.

1 Organisms

Sixteen reference strains of 6 species of genus *Brucella* and 9 field strains of *Br. melitensis*, 43 of *Br. abortus*, 3 of *Br. suis* and 11 of *Br. canis* were used.

2 Methanolysis and extraction of methylesters

Freeze-dried cells (20 mg) were suspended in 3 ml of methanol containing 5% hydrochloric acid in a screw capped glass tube and heated at 85°C for 16 h. After cooling, the mixture was centrifuged at 2,000 G for 10 min. The supernatant was transferred to another tube. The residue was washed with 1.0 ml of methanol. The washings were combined with the supernatant. To remove the hydrochloric acid, N_2 was blown through the combined methanolysate at 40°C. The dry residue was dissolved in 1.0 ml of a mixture of equal volumes of methanol and distilled water. The methylesters were extracted three times with 1.0 ml of n-hexane. The resultant three n-hexane layers were combined, dehydrated with Na_2So_4 and the hexane evaporated to dryness. The residue was dissolved in a small amount of hexane and transferred to a micro-tube. The hexane was again blown off with N_2 to dryness. The residue was dissolved in 60 ml of acetonitrile. The samples obtained were preserved at −20°C until use for analysis by gas chromatography.

3 Gas chromatography

The gas chromatograph used was equipped with a hydrogen flame ionization detector. A column packed with Celite 545 (80 - 100 mesh) and coated with diethylene glycol succinate was used. Analyses were carried out at a constant temperature.

Output of the gas chromatograph was integrated by a digital integrator. This gave the retention time and area of each peak.

The equivalent chain length (ECL) was determined from the logarithm of the retention time using a graph which illustrated the logarithm of the retention time of methylesters of saturated straight-chain fatty acids plotted against their carbon numbers.

The amount of each peak was expressed as a percentage of the sum of all the peak areas.

4 Numerical analysis

There are several ways of calculating the similarity. The following formula (Rozett and Petersen) was adopted:

$$\cos = \frac{\Sigma xy}{\sqrt{\Sigma x^2}\ \sqrt{\Sigma y^2}}$$

Calculation was carried out by using a programmable desk-type calculator.

5 Fatty acid composition of *Brucella*

1) Fatty acid composition of reference strains of *Br. abortus*

A gas chromatogram of methylesters of fatty acids of *Br. abortus* 544 demonstrated 15 peaks within 20 minutes. Their ECL and contents are tested together with those of fatty acid methylesters of Gram-negative bacteria. Under such experimental conditions 23 peaks were demonstrated. The fatty acid pattern of *Br. abortus* is similar to that of bacteria belonging to the *Enterobacteriaceae*. The characteristic acids are those of ECL 19.7, 21.3 and 22.4.

2) Fatty acid composition of reference strains of *Brucella*

The patterns of 16 strains studied were very similar to one another. All of them were composed of 15 main fatty acids, except *Br. suis* biotype 3 and *Br. canis*. The difference bet-

ween *Br. canis* and others was quantitative rather than qualitative. The *Br. suis* biotype 3 contained 2 fatty acids shorter than ECL 16.0, but other components were the same as those of most of the other strains.

The *Br. canis* were very different, and composed of 10 esters. The esters of ECL 19.7, 21.3 and 22.4, which are characteristic of *Brucella*, were very scarce. The remaining peaks were similar to those of the *Brucella* strains qualitatively.

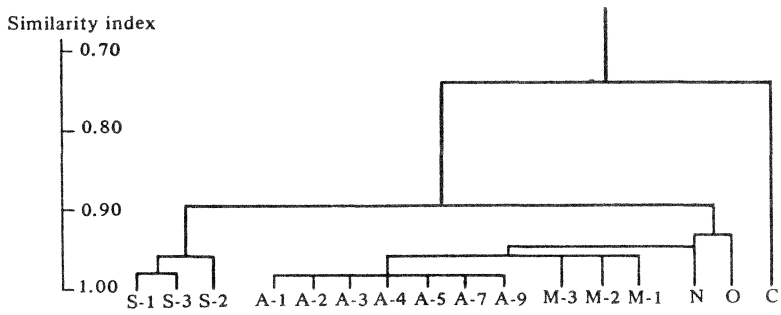
Br. ovis is a well-known rough type *Brucella*, but it is so similar to the smooth type *Brucella* in the fatty acid composition that it is impossible to differentiate it from any smooth type organism at a glance.

3) Fatty acid composition of *Brucella* isolates

Brucella organisms (66 strains of 4 species) isolated in the field were subjected to analysis. The mean contents and standard error were calculated for every species. The mean composition of each species was similar to that of the reference strains.

6 Analysis

The composition of *Br. canis* was obviously different from that of any other species of *Brucella* quantitatively. A low similarity (S = 0.4 - 0.7) was observed between these species. On the basis of fatty acid composition, such species of *Brucella* showed clustering. Similarity and homogeneity of such species were demonstrated in the dendrogram (Fig. 1).



S= *Br. suis;* A= *Br. abortus;* M= *Br. melitensis;* N= *Br. neotomae;*
O= *Br. ovis;* C= *Br. canis*

Fig. 1 Dendrogram of the genus ***Brucella*** based on their fatty acid composition

Analysis of fatty acid methylesters of the whole cell may be useful to determine whether an unknown isolate belongs to the genus *Brucella* or not. The gas chromatographical technique is a rapid and not very dangerous method which can be performed by using equipment indispensable to a modern bacteriological laboratory.

Discussion

Gupta, B.K. (India): Could you describe the clinical symptomatology of the cases from which *Brucella canis* strains were isolated?

Answer: The clinical symptoms observed in the experimental dogs consisted of abortion in females, early embryonic death and epididymitis as well as testicular degeneration in males.

Joseph, P.G. (Malaysia) Comment: Although the oxidative metabolic tests are important for the identification of *Brucella abortus* and *Brucella suis*, they are difficult to carry out and may give rise to laboratory accidents.

Koh, J.G.W. (Singapore): Have you performed any pathogenicity tests of *Brucellas suis* in pigs with different isolates and strains?

Answer: No. We haven't.