THE RELATION BETWEEN SPECIFIC AND NON-SPECIFIC AGGLUTINATION IN THE BRUCELLA GROUP.

By S. R. PANDIT and G. S. WILSON.

(*From the London School of Hygiene and Tropical Medicine.*)

For an understanding of the epidemiology of undulant fever it is desirable that the relations between the different members of the Brucella group should be made clear. The present paper records the results of a preliminary examination, by specific and non-specific agglutination tests, of a number of strains isolated from various sources. A more detailed study by means of specific agglutination and absorption tests is now in progress, the results of which it is hoped to publish at a later date.

Technique.

Altogether 117 strains have been examined. According to their source of origin and other information available at the commencement of the investigation they were preliminarily grouped as follows:

Br. abortus, porcine type	...	12 strains
,, bovine type	...	47 ,,
Br. melitensis	...	47 ,,
Br. paramelitensis ...	...	11 ,,

The strains were derived from various countries including England, France, Denmark, Italy, Malta, Tunis, Algiers, Palestine, India, South Africa, the United States, and Peru. The country of origin of some strains was unknown.

The following tests were employed:

(*a*) *Thermo-agglutination.*

A 48-hour growth on an agar slope was suspended in normal saline, and standardised to an opacity corresponding to about 8000 million *B. coli* per c.c. One cubic centimetre was transferred to a glass tube, 13 × 0·8 cm., which was then immersed in a bath of water kept approximately at 95° C.; the level of the water was just above that of the suspension in the tube. The tube was examined after 5, 10, 15, 30, 45, 60 and 120 minutes. The occurrence of definite agglutination visible to the naked eye against an illuminated dark background was recorded as a positive result. The following notation was used:

Agglutination within 5 minutes	... ...	+++
,, in from 5 to 10 minutes	...	++
,, ,, 10 to 120 minutes	...	+

(*b*) *Salt agglutination.*

A 48-hour growth on an agar slope was suspended in distilled water, heated to 60° C. for 1 hour in a water bath, and standardised to an opacity of 1000 million *B. coli* per c.c. Equal quantities of the suspension and of varying strengths of saline were mixed in Dreyer's tubes, so that the final concentrations of sodium chloride were 1, 2·5, and 5 per cent. The tubes were incubated in a water bath at 55° C., and the results read after 24 hours. The following notation was used:

No agglutination	−
Agglutination in 5 per cent. saline ...	+
,, ,, 2·5 per cent. saline ...	++
,, ,, 1·0 per cent. saline ...	+++

(*c*) *Acid agglutination.*

The same suspension was used as that for salt agglutination. Equal quantities of the heated suspension and of Beniasch's (1912) lactic acid and sodium lactate solutions were mixed in Dreyer's tubes, which were then incubated for 2 hours in a 37° C. water bath. Colorimetric estimations of the H-ion concentration of the nine different solutions employed gave results similar to those theoretically anticipated (Table I). The tube containing the

Table I. *p*H *values of Beniasch's acid solutions.*

Tube	1	2	3	4	5	6	7	8	9
Estimated *p*H	4·77	4·46	4·15	3·85	3·55	3·26	2·96	2·66	2·36
Observed *p*H	4·7	4·4	4·1	3·8	3·6	3·2	2·9	2·6	2·2

most alkaline mixture in which agglutination occurred was recorded, and the following notation was used:

No agglutination, or agglutination in tubes 8 and 9 only	−
Agglutination in tube 7	+
,, ,, tube 6	++
,, ,, tube 5 or below	+++

(*d*) *Agglutination with specific sera.*

Two sera were used obtained from rabbits inoculated with a bovine abortus strain, K 25, of human origin from Denmark, and a paramelitensis strain, Pm 2062, of caprine origin from Tunis. K 25 serum had a titre of 1/5120, Pm 2062 of 1/640. A 48-hour agar growth of the organism to be tested was washed off in 0·5 per cent. formolised saline, heated to 60° C. for 1 hour, and standardised to an opacity of 1000 million *B. coli* per c.c. Equal quantities of the suspension and of varying dilutions of the serum in 0·85 per cent. saline were mixed, and incubated in a 55° C. water bath for 24 hours. The tubes were then examined with a magnifying glass (× 4) against an illuminated

dark background, and the last tube was recorded in which definite agglutination was visible. For purposes of tabulation a positive result was taken as that in which agglutination occurred in a dilution equivalent to a quarter of the titre of the serum or more. With K 25 serum therefore agglutination in dilutions of 1/1280–1/5120, with Pm 2062 serum agglutination in dilutions of 1/160–1/640 was recorded as positive.

(e) Agglutination with absorbed specific sera.

K 25 serum was diluted 1/80 with 0·5 per cent. formolised saline, mixed with an equal quantity of a heat-killed suspension of paramelitensis organisms standardised to 3000 million *B. coli* per c.c., and incubated for 2 hours at 37° C. The mixture was then centrifuged, and the supernatant fluid withdrawn. Pm 2062 serum was similarly absorbed with K 25 organisms. These absorbed sera were tested in the same way as the unabsorbed sera. Agglutination with either serum up to one-quarter of the original titre, 1/1280–1/5120 with K 25 serum and 1/160–1/640 with Pm 2062 serum, was recorded as a positive result.

The results obtained with the different groups of organisms are recorded in Tables II–V; Table VI is an abbreviated synoptic table comprising all strains. It will be noted that the primary classification has been based on the thermo-agglutination test.

Table II. *Porcine abortus strains.*

Thermo-agglutination	No. of strains	No. agglutinating with salt				No. agglutinating with acid				Absorbed sera			Unabsorbed sera		
											Pm			Pm	
		Nil	+	++	+++	Nil	+	++	+++	K 25 only	2062 only	Both	K 25 only	2062 only	Both
–	11	11	0	0	0	0	2	5	4	11	0	0	7	0	4
+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
++	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
+++	1	1	0	0	0	0	0	0	1	0	1	0	0	1	0
Totals	12	12	0	0	0	0	2	5	5	11	1	0	7	1	4

Table III. *Bovine abortus strains.*

Thermo-agglutination	No. of strains	No. agglutinating with salt				No. agglutinating with acid				Absorbed sera			Unabsorbed sera		
											Pm			Pm	
		Nil	+	++	+++	Nil	+	++	+++	K 25 only	2062 only	Both	K 25 only	2062 only	Both
–	43	43	0	0	0	10	22	8	3	43	0	0	43	0	0
+	1	1	0	0	0	0	1	0	0	1	0	0	1	0	0
++	1	1	0	0	0	0	0	0	1	1	0	0	1	0	0
+++	2	2	0	0	0	0	0	0	2	0	1	1	0	1	1
Totals	47	47	0	0	0	10	23	8	6	45	1	1	45	1	1

Table IV. *Melitensis strains.*

Thermo-agglutination	No. of strains	No. agglutinating with salt				No. agglutinating with acid				Absorbed sera				Unabsorbed sera		
											Pm				Pm	
		Nil	+	++	+++	Nil	+	++	+++	K 25 only	2062 only	Both	Neither	K 25 only	2062 only	Both
–	26	26	0	0	0	18	3	1	4	15	0	7	4	17	1	8
+	9	9	0	0	0	1	2	2	4	2	1	3	3	2	1	6
++	4	2	0	2	0	0	0	2	2	0	2	1	1	0	2	2
+++	8	4	0	2	2	0	0	1	7	0	8	0	0	0	7	1
Totals	47	41	0	4	2	19	5	6	17	17	11	11	8	19	11	17

Table V. *Paramelitensis strains.*

Thermo-agglutination	No. of strains	No. agglutinating with salt				No. agglutinating with acid				Absorbed sera			Unabsorbed sera		
		Nil	+	++	+++	Nil	+	++	+++	K 25 only	Pm 2062 only	Both	K 25 only	Pm 2062 only	Both
–	1	1	0	0	0	1	0	0	0	1	0	0	0	0	1
+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
++	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
+++	10	2	3	4	1	0	0	3	7	0	9	1	0	7	3
Totals	11	3	3	4	1	1	0	3	7	1	9	1	0	7	4

Table VI. *Abbreviated synoptic table comprising all strains.*

Thermo-agglutination	No. of strains	Percentage agglutinating				
		With salt	With acid +++	With K 25 absorbed only	With Pm 2062 absorbed only	With both K 25 and Pm 2062 absorbed sera or with neither
–	81	0	14	86	0	14
+ and ++	15	13	47	27	20	54
+++	21	57	81	0	90	10

The following observations may be made:

1. With a few exceptions the porcine and bovine abortus strains are non-thermo-agglutinable; on the other hand nearly half the melitensis, and all but one of the paramelitensis strains react positively to the thermo-agglutination test. It will be noted, however, that whereas many of the melitensis strains fall into the one and two plus group, all the thermo-agglutinable paramelitensis strains fall into the three plus group. The thermo-agglutination test, it may be remarked, depends on the presence of salt in the suspension, and is therefore essentially a saline auto-agglutination test. If the organisms are washed three times in distilled water, and finally suspended in this medium, they remain stable, even after being subjected to heat at 95° C. for 2 hours and allowed to stand overnight at room temperature.

2. None of the porcine or bovine abortus strains is agglutinated by salt at 55° C., but six of the melitensis and all but three of the paramelitensis strains show some degree of agglutination. It is apparent that the salt test at 55° C., even with a strength of 5 per cent. sodium chloride, is a considerably less delicate method of detecting differences in the stability of suspensions than the thermo-agglutination test at 95° C. in the presence of normal saline. Thus, taking all groups together, only fourteen out of the total 117 strains are agglutinated by salt at 55° C., while thirty-six respond positively to the thermo-agglutination test.

3. There is a general correlation between the thermo-agglutination test and the acid agglutination test. The notation employed in recording the results of the latter test was of course quite arbitrary. Nearly all strains show some degree of agglutination in the most acid tubes, but few, with the exception of those that are thermo-agglutinable, react in tubes lower than the fifth. From Table VI it will be seen that of the non-thermo-agglutinable strains only

14 per cent. show agglutination in the fifth tube; of the one and two plus thermo-agglutinable strains 47 per cent., and of the three plus thermo-agglutinable strains as many as 81 per cent. react in this tube.

4. Of the non-thermo-agglutinable strains 86 per cent. are agglutinated by an abortus serum absorbed with paramelitensis, but not by a paramelitensis serum absorbed with abortus; on the other hand, 90 per cent. of the strongly thermo-agglutinable strains are agglutinated by an absorbed paramelitensis, but not by an absorbed abortus serum; of the one and two plus thermo-agglutinable strains some agglutinate with an absorbed abortus, and some with an absorbed paramelitensis serum, while about half of them agglutinate with both sera. With unabsorbed sera very much the same results are obtained, but the distinctions tend to be less sharp. That is to say, rather fewer strains agglutinate with either the one or the other serum, while more agglutinate with both sera.

DISCUSSION.

The term "paramelitensis" was introduced by Nègre and Raynaud (1912 *a*, *b*) to denote a strain of melitensis that was sharply distinguished by direct agglutination from the usual type. Burnet (1925), in studying twenty-eight strains of melitensis from human patients and from goats in Tunis, found that by agglutination and absorption of agglutinins they fell into two distinct types, which he termed Type I and Type II. The two types were distinguished in other ways. For example, Type I strains had a greater agglutinogenic power when injected into rabbits than Type II strains. Type II strains proved to be thermo-agglutinable, while Type I were not. Moreover, Type II strains were frequently agglutinated by normal human serum to a titre of 1/100 or 1/150, whereas Type I strains were rarely agglutinated by normal serum. It may safely be deduced that Burnet's melitensis Type II corresponds to the paramelitensis of Nègre and Raynaud. Of recent years several workers (for references see De Antoni, 1929) have found that certain *Brucella* strains are susceptible to agglutination by non-specific agencies, such as heat, acids, formol, peptone, mercuric chloride, alcohol, and aniline dyes, and there has been a considerable measure of agreement that susceptibility to heat is a characteristic of the para strains. The reactions of individual strains, however, are not absolutely constant, and it has been found possible by certain experimental procedures, such as repeated sub-culture in broth containing specific antiserum, to render a strain thermo-agglutinable (Favilli, 1926; Burnet, 1928; Frendzel and Szymanowski, 1930). Hadley (1927) put forward the suggestion, which was endorsed by Ross (1927 *b*), that the normal type of melitensis represents the smooth form, whereas the para type is essentially the rough form.

This suggestion receives confirmation from the results recorded in the present paper. Careful examination of the tables seems to indicate that the normal smooth form of *Brucella* is non-thermo-agglutinable, is not agglutinated

by salt, and reacts to about titre with a specific serum prepared against a similar smooth strain, but not by a serum prepared against a para strain. On the other hand, strains, no matter what label they bear, which are readily agglutinated by heat, which are often agglutinated by salt, and which react with a para serum but not with a serum prepared against a non-thermo-agglutinable strain, are to be regarded as rough strains.

If this interpretation is correct it appears that about 3 per cent. of the strains labelled porcine and bovine abortus may be regarded as rough, while another 5 per cent. present characteristics intermediate between those of smooth and rough. To use the terminology of Sangiorgi (1927) and of Ross (1927 *b*), the rough strains of abortus may conveniently be referred to as "para-abortus." On the other hand, it will be observed that 21 per cent. of the melitensis strains examined are definitely rough, while 26 per cent. are of intermediate type. Of the eleven strains labelled paramelitensis, seven are definitely rough, three are intermediate in type though approaching the rough more closely than the smooth form, while one strain is almost entirely smooth.

These results suggest that melitensis strains have a greater tendency to become rough than strains of abortus. How far this is true it is impossible to say. With other groups of bacteria it is known that prolonged cultivation in the laboratory favours the smooth-rough transformation; and it may be that in the collection of strains examined the melitensis type were on the whole of less recent origin than the abortus strains. The higher proportion of rough strains amongst the melitensis group may therefore be merely accidental, resulting from their longer period of cultivation outside the body, and may not indicate that melitensis strains are on the whole less stable than abortus strains. This possibility is difficult to exclude, because the data available are insufficient to afford an exact estimate of the average age of the strains. It does not, however, seem probable, because with the recently isolated strains, about which full information was available, there was a far greater tendency amongst the melitensis group to become rough than amongst the abortus group. Only one of the abortus strains that had been isolated within the last five years showed a slight degree of roughness, while twelve of the corresponding melitensis strains were partially or completely rough. It is moreover the opinion of Favilli (1927) that abortus strains change far less readily to the para type than melitensis strains.

In order to ascertain how readily the smooth-rough transformation could be brought about, an experiment was performed in which three strains of porcine abortus, three of bovine abortus, and three of melitensis, all of completely smooth type, were submitted to serial cultivation in heart extract broth, the cultures being kept continuously in the 37° C. incubator, and sub-cultured into fresh medium every five days.

From time to time they were examined by the thermo-agglutination, acid agglutination, and absorbed serum tests. The results are given in Table VII; in order to economise space, many of the intermediate tests have been omitted.

It will be noticed that two out of the three porcine strains became slightly thermo-agglutinable by the fifth passage, that all three of the melitensis strains developed some degree of thermo-agglutinability, while none of the bovine strains did so. After fourteen sub-cultures one strain of porcine type remained absolutely smooth, while two had become almost completely rough; the three melitensis strains had likewise become rough; of the bovine strains two had become partially rough, while one remained smooth. The experiment was continued till twenty-four successive sub-cultures had been made, but no further change occurred; that is one of the porcine and one of the bovine strains still remained fully smooth. These results indicate that the passage of all three types from the smooth to the rough form may be brought about by simple cultivation in broth. The number of strains tested was too small to allow any definite conclusion to be made as to the ease with which this transformation can be accomplished with the different types, but there is at least a suggestion that the melitensis strains are rather less stable than the abortus strains.

Table VII. *Results of serial passage at 5-day intervals in broth.*

	At start				5th sub-culture				14th sub-culture			
	Thermo	Acid	K 25	Pm 2062	Thermo	Acid	K 25	Pm 2062	Thermo	Acid	K 25	Pm 2062
Porcine 1	−	+	+	−	+	+++	+	+	+++	+++	+	+
Porcine 2	−	+	+	−	−	+	+	−	−	+	+	−
Porcine 404	−	++	+	−	+	+++	+	−	+++	+++	+	+
Melitensis L 1	−	−	+	−	++	+++	−	−	+++	+++	+	+
Melitensis L 3	−	−	+	−	++	+++	−	−	+++	+++	+	+
Melitensis Y 20	−	−	+	−	+	+++	−	+	+++	+++	−	+
Bovine Oxford	−	+	+	−	−	+++	+	+	++	+++	+	+
Bovine Am 1	−	−	+	−	−	−	+	−	−	−	+	−
Bovine RCV 42	−	+	+	−	−	+	+	−	+	+++	+	+

Finally, a few remarks may be made on the value of the different tests employed in this work in distinguishing between smooth and rough strains. It has already been pointed out how much more delicate the thermo-agglutination test is than the salt agglutination test as ordinarily carried out at 55° C. with varying concentrations of salt.

The acid agglutination test presents certain features of interest. According to Ross (1927 *a*) its main virtue is to separate off melitensis and abortus on the one hand from paramelitensis and para-abortus on the other. This view receives only a limited support from the experimental work recorded in this paper. It is true that the majority of paramelitensis strains, though by no means all, are strongly agglutinated by acid, and it is equally true that the majority of non-thermo-agglutinable abortus and melitensis strains are not; but examination of the tables shows that there are a number of abortus and melitensis strains which are strongly agglutinated by acid, yet which are non-thermo-agglutinable and are not agglutinated by a paramelitensis serum. A strain, therefore, which has none of the other properties of a para strain, may yet be highly susceptible to agglutination by acids. This conclusion seems to accord with the results obtained by Favilli (1926), who found that though the thermo-agglutination and peptone agglutination tests ran parallel, the

lactic acid agglutination test had a wider range, affecting certain strains that did not respond to the other two tests.

Incidentally it may be noted that the majority of the non-thermo-agglutinable strains which agglutinate with acid belong to the porcine and bovine abortus types. This observation is remarkable in view of the results recorded by Vercellana and Zanzucchi (1926) and Zanzucchi and Vercellana (1926). Using lactic acid, they found that strains of abortus were very much less susceptible than strains of melitensis. They went so far as to make the statement that a strain which is agglutinated by lactic acid in dilutions above 1/300 is certainly melitensis, while one which is not agglutinated at all, or which is agglutinated only in low dilutions, is certainly abortus. It is not improbable that many of the melitensis strains with which they worked were rough; if they had confined their observations purely to smooth strains, their results might have been different. Attempts have been made to confirm the conclusions of these workers by Graziosi (1926), Favilli (1926), Cerruti (1926, 1927), and Vidal (1928), but without success. All these authors agree that the acid agglutination test is unreliable for differentiating between strains of abortus and melitensis.

With regard to specific agglutination by smooth and rough sera, the results show a high correlation with those obtained by the thermo-agglutination test. With the abortus and paramelitensis strains the correlation is almost perfect, but with the melitensis strains certain discrepancies are apparent. For example, from Table IV it will be seen that only seventeen of the twenty-six non-thermo-agglutinable strains are agglutinated to one-quarter titre or over by a smooth unabsorbed serum; of the remainder one is agglutinated only by a rough serum, while eight strains are agglutinated by both sera. Such a result indicates that a proportion of non-thermo-agglutinable melitensis strains contain both smooth and rough antigens. These strains are presumably commencing to become rough. If this interpretation is correct, agglutination by a rough serum may be regarded as an even more delicate test of detecting slight degrees of roughness than the thermo-agglutination test.

Summary and conclusions.

1. Altogether 117 strains of *Brucella*, belonging to different types and isolated from different parts of the world, have been examined by the thermo-agglutination, salt agglutination, acid agglutination, and specific serum agglutination tests.

2. The results obtained by the thermo-agglutination and the serum agglutination tests are in close agreement; there is a fairly high degree of correlation between these tests and the acid agglutination test, and a rather lower correlation with the salt agglutination test.

3. Generally speaking, a strain which is highly thermo-agglutinable is frequently agglutinated by salt, is usually agglutinated strongly by acid, and reacts to a paramelitensis, but not to an abortus serum.

4. A strain which is moderately thermo-agglutinable is seldom agglutinated by salt, is frequently agglutinated by acid, and reacts either with an abortus or a paramelitensis serum, or with both sera.

5. A strain which is not thermo-agglutinable is not agglutinated by salt, seldom reacts markedly to acid agglutination, and is generally agglutinated by an abortus, but not by a paramelitensis serum.

6. There remain, however, a certain number of strains, particularly of the porcine and bovine abortus types which, though non-thermo-agglutinable, inagglutinable by salt, and reacting only with an abortus serum, yet show some degree of acid agglutination.

7. Of the twelve porcine strains examined only one strain was strongly thermo-agglutinable; of the forty-seven bovine strains only two were strongly thermo-agglutinable, a further two showing a milder degree of thermo-agglutinability; of the forty-seven melitensis strains eight were strongly, and thirteen were moderately thermo-agglutinable; while of the eleven paramelitensis strains ten were strongly thermo-agglutinable.

8. These results are taken to indicate, in accordance with the suggestion made by certain previous workers, that those strains which are non-thermo-agglutinable, are not agglutinated by salt, and are agglutinated by an abortus but not by a paramelitensis serum, represent the smooth form, while those strains which are strongly thermo-agglutinable, are frequently agglutinated by salt, and are agglutinated by a paramelitensis but not by an abortus serum, represent the rough form.

9. If this interpretation is correct it will be noticed that the great majority of the porcine and bovine strains examined were of the smooth type, that nearly half the melitensis strains were partially or completely rough, while all but one of the paramelitensis strains were rough.

10. Whether melitensis strains have a greater tendency than abortus strains to undergo the smooth to rough transformation it is difficult to say with certainty, but the reports in the literature and the observations in the present paper render this probable.

11. By serial passage through broth at 5-day intervals, it is possible to transform smooth strains of all three types into the rough form. This transformation appears to occur more readily and to proceed further in a given time with melitensis than with abortus strains; but since only three strains of each type were examined, the results may have been determined as much by chance selection of strains as by any greater inherent tendency of the strains of the melitensis type to undergo variation.

12. It is clear that none of the tests employed suffices to differentiate individual strains of abortus and melitensis. The thermo-agglutination test and the agglutination test with specific smooth and rough sera do, however, enable a differentiation to be made between smooth and rough strains of all types.

13. In the present paper no attempt has been made to distinguish abortus

and melitensis strains by specific agglutination and absorption tests. The general failure of workers hitherto to obtain any clear-cut serological distinction between these types may possibly be due to the fact that many of the strains with which they worked were either partially or completely rough. Since the rough antigen seems to be more or less alike in strains of all types, it is clear that its presence would tend to obscure any difference that might exist between the smooth antigens of the different types. If such a difference does exist it is probable that it will be elicited only by a comparison of absolutely smooth strains.

REFERENCES.

BENIASCH, M. (1912). *Zeitschr. f. Immunitätsf.* **12**, 268.
BURNET, E. (1925). *Arch. Inst. Pasteur Tunis*, **14**, 247.
—— (1928). *Ibid.* **17**, 128.
CERRUTI, C. (1926). *Giorn. di Batter. e Immunol.* **1**, 422.
—— (1927). *Pathologica*, **19**, 216.
DE ANTONI, V. (1929). *Bol. Istituto Sieroterap. Milanese*, **8**, 651.
FAVILLI, G. (1926). *Lo Sperimentale*, **80**, 396.
—— (1927). *Bol. Istituto Sieroterap. Milanese*, **6**, 341.
FRENDZEL, J. and SZYMANOWSKI, Z. (1930). *Zentralbl. f. Bakt.* **117**, 240.
GRAZIOSI, A. (1926). *Nuova Veterinaria*, **4**, 306.
HADLEY, P. (1927). *J. Infect. Dis.* **40**, 1.
NÈGRE, L. and RAYNAUD, M. (1912 *a*). *C.R. Soc. Biol.* **72**, 791.
—— —— (1912 *b*). *Ibid.* **72**, 1052.
ROSS, G. R. (1927 *a*). *J. Hygiene*, **26**, 279.
—— (1927 *b*). *Ibid.* **26**, 403.
SANGIORGI, G. (1927). *Pathologica*, **19**, 3.
VERCELLANA, G. and ZANZUCCHI, A. (1926). *Ibid.* **18**, 247.
VIDAL, J. (1928). *C.R. Soc. Biol.* **99**, 1279.
ZANZUCCHI, A. and VERCELLANA, G. (1926). *Pathologica*, **18**, 395.

(*MS. received for publication* 14. v. 1931.—Ed.)