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A UREASE TEST FOR CHARACTERIZING BRUCELLA STRAINS

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Many strains of Brucella, notably the American *Br. suis* types are capable of hydrolyzing urea to form CO_2 and NH_3. The reaction can proceed to the formation of ammonium carbonate.

The methods at present in use for measuring urease activity to Brucella strains are either rather inaccurate or require specialized equipment (Wohlfeil & Weiland, 1927; Püshel, 1936; Ferguson & Hook, 1943; Schneider & Gunderson, 1946; Christensen, 1946; Bauer, 1949; Hoyer, 1950; Pacheco & Thiago de Mello, 1950; Sanders & Warner, 1951; Renoux & Quatrefages, 1951; Huddleson, 1951; Pickett, Nelson & Liberman, 1953; Niznánsky & Krêméry, 1953; Godgluck & Marggraff, 1955). In addition they expose workers to infection with virulent material, e.g. the use of the Warburg apparatus, or Van Slyke & Archibald's (1944) titrimetric method or Conway's (1939) microdiffusion method.

For these reasons the Joint FAO/WHO Expert Committee on Brucellosis feels that if the urease test is to be of real value, it should be improved (1953).

In this report the work carried out at the Onderstepoort centre leading to the institution of a routine pH threshold urease test, will be recorded. This test is reliable and the results can be presented numerically.

Materials and Methods

Strains

(*a*) Reference strains:

16M *Br. melitensis* (Beltsville)
544 *Br. abortus* (Weybridge)
1330 *Br. suis* (American) (Minnesota)

(*b*) Laboratory strains:

S. 19 *Br. abortus* (Beltsville)
Rev. 1. *Br. melitensis* (Elberg, Berkeley)

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(*c*) Field strains: recent isolates from cases including:

man	*Br. melitensis*
	Br. abortus
cattle	*Br. abortus*
goats	*Br. abortus*
sheep (rams)	*Br. ovigenitalium*
sheep (ewes)	*Br. abortus ovis*

Typing

The strains were typed on the following characteristics:

(1) Host
Man, ox, sheep, goat

(2) Staining reactions
Hansen's (Hansen *et al.*, 1939)
Gram's (Van Drimmelen, 1960)
Stamp-McEwen's (Stamp *et al.*, 1950)

(3) Antigenic structure
Monospecific agglutination: *melitensis*, *abortus* (Jones, 1958)

(4) Bacteriophage sensitivity
Lysis by *abortus* phage, *suis* phage (Parnas *et al.*, 1958; Van Drimmelen, 1960; Jones, 1960)

(5) Biochemical properties
Dye sensitivity (Pickett *et al.* 1952)
H_2S production (Huddleson,1927)
Di-ethyl-di-thio-carbamate resistance (Renoux, 1952)

(6) Cultural characters
CO_2 dependence (Huddleson, 1921)
S–R variation: acriflavine test (Braun *et al.*, 1947): heat agglutination

(7) Biological properties
Pathogenicity for guinea pigs

Media

The urea medium of Rustigian & Stuart (1941) was prepared and used as recommended by Pickett, Nelson & Liberman (1953) at pH 6·2.

The modification introduced for the present work was adjustment to six different pH values: 7·0–2·0 (with a difference of one unit from one tube to the next).

Testing

Urease activity was tested by adding 0·25 ml of a dense suspension (about 10^9 organisms from a 48 hour surface culture on albimi agar) in saline to a series of tubes each containing 1·0 ml of medium at the various pH values. After being agitated the tubes were incubated at 37°C. The results were read after four hours and confirmed after 18 hours. Results were recorded as the " pH threshold value " i.e. the lowest pH at which urease activity was indicated by indicator colour change.

Control tests

Tests by the methods of Schneider & Gunderson (urea solution) (1946), Christensen (urea agar) (1946), Pacheco & Thiago de Mello (paper strips) (1950), and titrimetric H_2SO_4 tests (Onderstepoort) and also titrations of soybean urease were carried out for comparative purposes.

Results

On the basis of pH differences in media as recommended by earlier workers, the sensitivity of urease was investigated by first determining the optimum pH for maximum activity of a known amount of urease. The findings are shown in Table 1.

Table 1.—*Titrimetric determination of the activity of soybean urease at different pH levels: The percentage of urea converted by a constant amount of urease during three hours at room temperature*

Urea used Gm	pH	N titrated Gm	Urea calculated Gm	Percentage recovery
20.....	7·0	8·33	17·8	89
20.....	6·6	9·24	19·8	99
20.....	5·0	7·91	17·0	85
20.....	4·0	7·77	16·6	83
20.....	3·0	7·28	15·6	78

Different dilutions of soybean urease were then tested in sets of tubes at six different pH levels containing urea broth with indicator. The results after four hours and 18 hours were identical and are shown in Table 2.

Table 2.—*Activity of soybean urease contained in one tablet and sufficient to hydrolize 80 mgm urea, diluted serially and then tested at six pH levels in the medium used for the Brucella urease tests*

pH	Urease dilution							
	Undiluted	1:2	1:4	1:8	1:16	1:32	1:64	1:128
7·0.....	+	+	+	+	+	+	−	−
6·0.....	+	+	+	+	−	−	−	−
5·0.....	+	+	+	+	−	−	−	−
4·0.....	+	+	−	−	−	−	−	−
3·0.....	+	−	−	−	−	−	−	−
2·0.....	−	−	−	−	−	−	−	−

The higher dilutions were found to have a limited range of activity. The threshold value for undiluted urease was pH 3·0 whereas for urease diluted 1 in 32 it was pH 7·0.

The urease concentrations could therefore be measured by the lowest pH at which urea was broken down i.e. the " pH threshold value ".

The urease activity of Brucella stock strains was tested at weekly intervals by adding uniform suspensions of successive cultures to sets of urea test media as described. The results are summarised in Table 3.

TABLE 3.—*Urease activity of approximately* 10^9 *organisms of different stock strains of Brucella as shown by the pH threshold test*

Date	Strain					
	16M	544	1330	S19	6024	609
	pH threshold					
4.May.60	6·0	—	4·0	5·0	—	·
12.„.60	6·0	—	4·0	5·0	—	·
28.„.60	7·0	—	4·0	5·0	—	·
2.June.60	6·0	—	4·0	5·0	—	·
16.„.60	7·0	—	4·0	5·0	—	·
20.„.60	7·0	—	4·0	5·0	—	6·0
29.„.60	7·0	—	4·0	5·0	—	5·0
13.July.60	7·0	—	4·0	5·0	—	6·0
21.„.60	7·0	—	4·0	5·0	—	6·0
26.„.60	6·0	—	4·0	5·0	—	6·0
10.Aug..60	6·0	—	4·0	5·0	—	6·0
17.„.60	7·0	—	4·0	5·0	—	6·0
24.„.60	7·0	—	4·0	5·0	—	6·0
31.„.60	7·0	—	3·0	5·0	—	6·0
13.Sept..60	6·0	—	3·0	5·0	—	6·0

— = No activity
· = Not tested

The pH threshold value for each strain was found to be reasonably constant and the results were reproducible. The technique has accordingly been applied to routine testing of South African field strains at Onderstepoort. Characteristics of FAO/WHO reference strains and of new isolates from Southern Africa are compared with each other when arranged in order on the scale of their urease test results as shown in Table 4.

A further record of results obtained by this test, is illustrated in a list of some strains examined. See Table 5.

TABLE 4.—*Types of Brucella organisms found in South Africa as grouped by the urease test compared with Brucella reference strains*

Urease scale pH threshold value	(—3·0—)(—4·0—)(—5·0—)(——————6·0——————)(—7·0—)(—————————)												
Strain	*Br. suis* WHO 1330	*Brucella abortus* S19	S.A. *Brucella abortus ovis* 609	S.A. *Brucella abortus* 6015	S.A. *Brucella abortus* 6016	S.A. *Brucella melitensis* 6021	S.A. *Brucella melitensis* 6022	WHO *Brucella melitensis* 16 M	S.A. *Br. ovigenitalium* 605	S.A. *Br. ovigenitalium* 606	S.A. *Br. ovigenitalium* 6010	S.A. *Br. ovigenitalium* 6024	WHO *Brucella abortus* 544
Host	Pigs	Cattle	Sheep	Cattle	Cattle	Human	Human	Goats	Sheep ram	Sheep ram	Sheep ram	Sheep ram	Cattle
Virulence	++++	+	++	++	++	++++	++++	++++	—	—	—	—	+++
Serology	A	A	A	A	A	M	M	M	—	—	—	M	A
Phage	S	A	A	A	A	—	—	—	—	—	—	—	A
CO_2	—	—	+	+	+	—	—	—	+	+	+	+	+
Dye sensitivity—													
T	—	+++	+++	+++	+++	—	+	—	+	+	—	—	++++
RF	+++	—	—	—	—	+	+	—	++	++	++	++	—
H_2S	++++	+++	+	++	++	+	+	—	—	—	—	—	—

Legend: A = abortus; S = suis; M = melitensis; T = thionin; BF = basic fuchsin; — = reaction negative; + to ++++ = reaction positive.

TABLE 5.— *Characteristics of some South African strains of Brucella organisms*

Strain reference number	Type or host species	Reference numbers or specimen	Colonies S, I, R or M	CO_2 dependence + or −	Bacteriophage lysis A or S	Monospecific agglutination M or A	Dye inhibition		H_2S production 1, 2, 3 or 4 days	Di-ethyl-di-thio carbamate reaction mm	*Urease activity* by pH threshold method	Virulence for guinea pigs
							Thionine mm	Basic Fuchsin mm				
Control—												
60·12	M	16 M	S	—	—	M	—	—	—	14 (18)	7·0	4
60·13	A	544	S	+	A	A	4	—	—	7	—	3
58·14	S	1330	S	—	S	A	—	3	4	9	3·0	4
60·8	A	S 19	S	—	A	A	5	—	3	11	4·0	1
60·7	A	Parnas	S	—	—	A	2	—	3	12	4·0	—
58·20	M	Rev. 1	S	—	—	M	—	—	—	12	6·0	.
South African strains—												
H. 60·1	Human	blood	I	+	—	A + M	4	8	—	10	7·0	—
O. 60·3	Sheep	semen	I	+	—	—	2	—	—	12	7·0	—
O. 60·4	Sheep	semen	I	+	—	—	—	6	—	8	—	—
O. 60·5	Sheep	semen	I	+	—	—	1	3	—	12	—	.
O. 60·6	Sheep	semen	I	+	—	—	1	4	—	12	—	.
O. 60·9	Sheep	milk	S	+	A	A	8	1	1	7	5·0	2
O. 60·10	Sheep	semen	I	+	—	—	—	6	—	10	—	.
B. 60·14	Cattle	foetus	S	—	A	A	—	12	1	9	5·0	2
B. 60·15	Cattle	foetus	S	+	A	A	5	—	2	6	6·0	2
O. 60·16	Sheep	foetus	S	+	A	A	5	—	2	9	6·0	2
H. 60·21	Human	blood	S	—	—	M	1	4	1	6	6·0	4
H. 60·22	Human	blood	S	—	—	M	3	6	1	6	6·0	4
O. 60·24	Sheep	semen	S	+	—	M	—	3	—	12	—	.

Legend: S = smooth; I = intermediate; R = rough; M = mucoid; A = " abortus "; S = " suis "; M = " melitensis "; − = reaction negative; . = test not completed.

Numerals indicate the size (radius in mm) of the inhibited zone round dye tablets and D.E.D.T.C. tablets; the pH threshold in the case of urease; days in the case of H_2S; and the degree in guinea pig lesions.

Discussion

Urease tests previously described depend on the rate of change of pH. This depends not only on the concentration of urease but also on the composition of the medium, temperature and agitation. The rate of pH change is therefore not a reliable measurement of urease activity.

The pH threshold method gives more reproducible results. As has been shown, the lowest pH at which urease activity can be initiated is directly correlated to the concentration of urease present. Once the reaction has started, the pH rises and conditions become progressively more favourable thus ensuring optimum activity. The pH threshold method therefore gives an "all or none" response which is more or less independent of time and which can be recorded numerically.

All ureases are usually considered to be identical. Should ureases from different sources prove to differ in regard to optimum pH values, the pH threshold test could be used to characterise ureases. In this case it might be found that the urease of *Br. suis* is particularly resistant to inhibition by low pH.

The present work shows that urease activity is an independent character of strains of *Brucella* organisms, not correlated with other characteristics.

It would appear that South African strains of *Br. abortus* display a fairly high, though variable, urease activity, comparable to that of *Br. melitensis* in other parts.

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