SHORT ARTICLES

SOME FACTORS AFFECTING THE HAEMOLYTIC ACTIVITY OF *VIBRIO CHOLERA*E

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CHARACTERS commonly used to differentiate the classical biotype from the El Tor biotype of *Vibrio cholerae* are the Voges-Proskauer test, resistance to Mukerjee's phage IV and to polymyxin B, and haemolysin production. Strains isolated in various parts of the world in the pandemic of the last twelve years have been more like the El Tor biotype than the classical, though they have been reported as haemolysin negative.

Some authors (Liu, 1959; Sakazaki, Tamura and Murase, 1971) have considered that haemolysin-negative strains should not be designated as El Tor biotype. Others (Mukerjee, 1963; Sakazaki *et al.*, 1971) have reported on the variability in the haemolytic activity of cholera vibrios, whereas Sen and Shrivastava (1970) claim that this property is most constant. Many modifications of the original test of Greig (1914) have been employed, and we decided to carry out a limited study on the haemolytic activity of six strains to investigate possible causes of discrepancy in the literature. In the present study, the strains were grown on solid medium and in various liquid media described by earlier workers (e.g., Feeley and Pittman, 1963; Sakazaki *et al.*, 1971), the cultures being tested at various stages of growth for haemolytic activity against the RBC of three different animal species.

MATERIALS AND METHODS

Strains of Vibrio. Six strains of *V. cholerae* were used: two of the classical biotype from culture collections (no. 8021 from the National Collection of Type Cultures, London, and no. 281 from the Manchester University Collection of Bacteria (MUCB)); two of the El Tor biotype (no. 719, isolated in Calcutta in 1957, and no. 283 from the MUCB); and two from the present pandemic (no. 14268, isolated in Singapore in 1969, and no. 41, received from Nigeria in 1971).

Culture media. Three basal media were used, at pH 7.2: Nutrient Broth no. 2 (Oxoid Ltd, Southwark Bridge Road, London SE1); "Bacto" Heart Infusion Broth (HIB) (Difco Laboratories, Central Avenue, East Molesey, Surrey, England), as recommended by Feeley and Pittman (1963) and by Sakazaki *et al.* (1971); and Brain Heart Infusion Broth (BHIB) (Oxoid), because brain heart infusion agar was recommended by Liu (1959). In two of these media, glycerol (1% v/v) was incorporated (HIGB and BHIGB); Liu (1959) and Sakazaki *et al.* (1971) had shown that glycerol enhanced the haemolytic activity of *V. cholerae*.

Blood from three species was used: sheep blood in Alsever's solution (Wellcome), defibrinated horse blood (Oxoid), and freshly collected oxalated human blood. Each was washed three times in 0.85% sodium chloride and a 2% (v/v) suspension prepared by resuspending packed RBC in 0.85% sodium chloride.

Tests for haemolysin. Each of the six strains was grown overnight at 36°C in peptone water and a standard inoculum (c. 0.04 ml) was transferred to 15-ml amounts of each of the five liquid media. After incubation at 36°C for 8, 26 and 72 hours, 1-ml samples of each culture were removed and mixed with 1 ml of a 2% (v/v) suspension of each species of RBC.

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Controls consisted of uninoculated culture medium mixed with RBC suspension. The mixtures were incubated at 36°C for 2 hours and left at 4°C overnight before readings were made. After the initial incubation at 36°C, some haemolysis was detectable, but it was not distinct and the results were difficult to read at this stage.

The degree of haemolysis was compared with a series of standards prepared by mixing 1 ml of each culture medium (uninoculated) with 1 ml of RBC suspension treated as follows: (a) 2% (v/v) RBC in 0.85% sodium chloride (no haemolysis), (b) 2% (v/v) RBC in distilled water (100% haemolysis), (c) dilutions of (b) in distilled water, to correspond to 75, 50, 25 and 12% haemolysis. Each test was repeated and, in most cases of marked disagreement, a third test was performed.

TABLE I

Results of differential tests, for biotype and serotype, of six strains of Vibrio cholerae

Strain number	Result of VP* test	Diameter of zone of inhibition by polymyxin B (mm)†	Sensitivity to phage IV‡	Slide agglutination by stated antiserum		
				Polyvalent	Inaba	Ogawa
8021	—	10	S	+	—	+
281	—	10	S	+	—	+
719	+	>7	R	+	—	+
283	+	>7	R	+	+	—
14268	+	>7	R	+	—	+
41	+	>7	R	+	—	+

* Voges-Proskauer.

† Disk containing 50 international units of polymyxin B; diameter 7 mm.

‡ S = Sensitive, R = resistant.

Haemolysis in solid medium was studied by inoculating each strain on layered plates consisting of nutrient agar in the lower layer and nutrient agar containing 5% (v/v) RBC suspension in the upper layer. These were examined after incubation at 36°C for 18, 24 and 48 hours.

Other differential tests for V. cholerae. For the Voges-Proskauer (VP) test, the method of Barritt (1936) was used, with incubation at 30°C.

Sensitivity to polymyxin B was tested on nutrient agar with disks (7-mm diameter) containing 50 international units.

Sensitivity to phage IV (Mukerjee) was tested by spotting phage at routine test dilution on to nutrient-agar plates seeded with lawns of the six strains.

RESULTS

Identification of strains by tests other than haemolysin production

All six strains were curved, motile, Gram-negative rods and were oxidase positive, arginine negative, indole positive and cholera-red positive. They produced acid, without gas, from glucose, sucrose, mannose and mannitol, the action on glucose being fermentative; but acid was not produced from lactose or arabinose within 3 days. Thus all six were vibrios of Heiberg group I and, because all were agglutinated by polyvalent cholera antiserum, they were likely to be strains of *V. cholerae*.

Table I shows that strains 8021 and 281 were VP negative and sensitive to polymyxin and to phage IV—characteristics of the classical biotype. The other four strains resembled the El Tor biotype in these properties. Strain 283 was of the Inaba and the other five of the Ogawa serotype.

Haemolytic activity in liquid medium

Horse and human RBC were found to be too unstable for this test because the controls on occasion showed haemolysis up to 25%. The results have therefore not been tabulated. The only consistent observation with these two species was partial lysis of horse RBC by 8-hour cultures of three strains (nos. 281, 14268 and 41). This was reproducibly greater than in the control tubes, whereas the haemolysis produced by 26-hour and 72-hour cultures of

TABLE II
The haemolytic activity of six strains of V. cholerae against sheep RBC

Strain number	Age of culture (hours)	Percentage of haemolysis* found with cultures grown in stated medium†				
		NB	HIB	HIGB	BHIB	BHIGB
8021	8	<12	<12	<12	<12	<12
	26	<12	<12	<12	<12	<12
	72	<12	<12	<12	<12	<12
281	8	<12	<12	<12	<12	<12
	26	<12	<12	<12	<12	<12
	72	<12	<12	<12	<12	<12
719	8	<12	<12	<12	<12	<12
	26	<12-12	12-50	75	<12	<12
	72	>75	50->75	>75	<12	<12
283	8	12-75	50-75	50-75	12-50	12-50
	26	25->75	>75	>75	50-75	25-75
	72	>75	>75	>75	>75	75->75
14268	8	<12	<12	<12	<12	<12
	26	<12	<12	<12-25	<12	<12
	72	<12	<12	<12-75	<12-12	<12-75
41	8	<12	<12	<12	<12	<12
	26	<12	<12	<12	<12	<12
	72	<12	<12	<12-25	<12	<12

* Each result was determined in duplicate and, in most cases of discrepancy, a third test was performed. In all cases of discrepancy, whether tested in duplicate or triplicate, the range is stated. <12% = negative; or barely perceptible haemolysis. >75% = clear, and usually complete, haemolysis.

† NB = Nutrient broth; HIB = heart-infusion broth; HIGB = heart-infusion-glycerol broth; BHIB = brain-heart-infusion broth; BHIGB = brain-heart-infusion-glycerol broth.

these strains was not obviously greater than that present in controls. This observation confirms the finding of haemolysin in young cultures of recent isolates (Feeley and Pittman, 1963) and could explain the lysis seen around colonies on solid medium containing horse or human RBC.

With sheep RBC the controls were stable, and—except with the glycerol cultures—the haemolysis was reproducible (table II). Four strains (nos. 8021, 281, 14268 and 41) were consistently non-haemolytic, apart from the varying degree of haemolysis by strains 14268 and 41 when grown in the presence of glycerol (HIGB and BHIGB). With the other two strains (nos. 719 and 283), 72-hour NB cultures gave complete lysis, and similar results were obtained with HIB. By contrast, BHIB was a poor medium for haemolysin production, and even 72-hour cultures of strain 719 were completely non-haemolytic; also, with this medium, haemolysis was difficult to read because of a brownish colour change in the haemoglobin, observed also by Sakazaki *et al.* (1971). However, with neither of the two haemolytic strains was complete lysis produced by 8-hour cultures even in NB or HIB; and, at 26 hours

only partial lysis was produced, especially by the more weakly haemolytic strain, no. 719. For distinct results, it seemed best to use 72-hour cultures in NB (or HIB).

The haemolytic activity of strains 719 and 283 was heat labile; it was abolished by heating at 60°C for 30 min.

Haemolysis in solid medium

The results for isolated colonies on sheep-RBC agar (table III) were consistent with those obtained with liquid medium: strain 283 was the most haemolytic, strain 719 gave smaller

TABLE III

*Effect observed with six strains of V. cholerae in agar-plate cultures containing RBC of three different species**

Strain number	Age of culture (hours)	Effect* with RBC of		
		sheep	horse	man
8021	18	Nil	PH	PH
	24	G	PH	PH
	48	G	PH	PH
281	18	Nil	PH	Nil
	24	G	PH	G
	48	G	PH	PH
719	18	C (6)	C (7)	C (6)
	24	C (6)	C (7)	C (6)
	48	C (6)	C (7)	C (6)
283	18	C (10)	C (10)	C (10)
	24	C (10)	C (10)	C (10)
	48	C (10)	C (10)	C (10)
14268	18	G	PH	PH
	24	G	PH	PH
	48	G	PH	PH
41	18	G	PH	PH
	24	G	PH	PH
	48	G	PH	PH

*Effects observed around isolated colonies are indicated by following code: nil = no effect; G = greenish discoloration; PH = partial haemolysis; C = complete haemolysis (diameter of zone given in mm).

With all three species of erythrocyte, clear haemolysis occurred under areas of confluent growth of each of the six strains, even at 18 hours.

zones of lysis, and the other four strains were non-haemolytic, though the haemoglobin was changed to a greenish colour near the colonies.

With horse and human erythrocytes, strains 283 and 719 again gave distinct zones of clear haemolysis, but the other four strains also produced some lysis. This was only partial lysis but was often more complete a few mm from the edge of a colony than immediately adjacent to the colony, thus giving a quite characteristic annular appearance. The partial lysis was present in 18-hour cultures but did not increase on further incubation. This was consistent with the observations on liquid cultures, and could be attributed to a haemolysin that was produced in young cultures but did not persist beyond 24 hours. Moreover, it was a haemolysin to which sheep RBC were barely susceptible.

In areas of the solid medium, however, where growth was heavy and colonies confluent, zones of complete haemolysis appeared with all six strains.

DISCUSSION

Before the present pandemic of cholera, which began in about 1961 in South-East Asia, there was general agreement that the classical biotype of *V. cholerae* was non-haemolytic whereas the El Tor biotype was haemolytic. Many, probably a large majority, of the strains encountered over the last twelve years have conformed to the El Tor biotype except in their production of haemolysin.

In the original test of Greig (1914), a 5% (v/v) suspension of goat RBC was mixed with an equal volume of a 3-day nutrient-broth culture of the vibrio; the mixture was incubated for 2 hours at 37°C and then left overnight at 4°C before the result was recorded. Many attempts have been made in recent years to enhance haemolysin production or to render the test more sensitive, with the intention, sometimes openly admitted, of verifying that the strains of the present pandemic were haemolytic and therefore deserved to be designated as belonging to the El Tor biotype.

There seems to be no objection to replacing goat cells with sheep cells; the latter are more readily available, have been used by several workers, and, in our limited experience, have given distinct and reproducible results. Other species of RBC that have been used include horse, commonly incorporated in blood-agar plates, and human (De, Bhattacharyya and Roychandhury, 1954; Liu, 1959). We have confirmed that *V. cholerae* cultures lyse these cells more readily than sheep cells, but we have found them too fragile for the haemolytic test described here and it seems that their use may well lead to an erroneous result due to non-specific lysis.

There have been several attempts to make the haemolysin test more sensitive by reducing the concentration of RBC to 1% (v/v) (Liu, 1959; Feeley and Pittman, 1963; Sakazaki *et al.*, 1971). However, we have found it difficult enough to estimate degrees of partial lysis after the addition of an equal volume of 2% (v/v) suspensions and quite impossible to do so reliably with 1% (v/v) suspensions. We feel that, if the concentration used by Greig is to be altered at all, 2% (v/v) is the lowest limit consistent with an accurate reading of results, since the test uses a turbid bacterial culture grown in a slightly pigmented medium.

The culture medium has also been varied by different workers. We do not wish to recommend any change from nutrient broth, which was used originally, though we agree with Feeley and Pittman (1963) and with Sakazaki *et al.* (1971) that heart-infusion broth is satisfactory. On the other hand, brain-heart infusion, recommended by Liu (1959) for use in solid medium, was found unsatisfactory, because it supported poor haemolysin production and because it caused a brownish colour-change in the haemoglobin as pointed out by Sakazaki *et al.* (1971). We agree with Liu (1959) and with Sakazaki *et al.* (1971) that the addition of glycerol apparently enhances the production of haemolysin, though this may be due to the haemolytic effect of glycerol itself. Whatever the explanation, we found—like Sakazaki *et al.* (1971)—that glycerol-containing media were unsatisfactory as they gave variable results, and we would support Sen and Shrivastava (1970) in condemning their use.

A further important reason for discrepancy amongst the findings of earlier workers concerns the age of the broth culture to which the RBC are added. Feeley and Pittman (1963) have shown that strains vary in the persistence of the haemolysin that they produce in broth. Whereas the El Tor biotype produced a persistent haemolysin that could be detected in a 3-day culture, there was a lack of persistence in strains from the current pandemic which sometimes gave positive results in 8-hour and even in 24-hour cultures but negative results with cultures tested at 72 hours.

Similarly, three of our strains showed haemolytic activity only in young (8-hour) cultures, but also only when tested with the more fragile RBC of the horse: two of these strains (nos. 14268 and 41) were from the present pandemic and the other (no. 281) was of the classical biotype. This demonstration of haemolytic activity only in young cultures, even by the classical biotype of *V. cholerae*, makes it imperative that tests for the more prolonged haemolytic activity characteristic of the El Tor biotype should be done on 3-day broth cultures as in the original Greig method. The occurrence of less prolonged haemolytic activity, with strains of the classical biotype or from the present pandemic, may however explain our observations with

solid medium, in which complete lysis of horse, human and sheep cells occurred in regions of heavy growth whether the strains were El Tor, classical, or from the current pandemic. It also renders invalid any attempt to differentiate between the haemolytic activities of the classical and El Tor biotypes by including RBC in the broth in which the vibrios are grown.

A further cause of equivocal results in the detection of haemolysin in young cultures is illustrated by strain 719. This is a genuine El Tor strain but produces haemolysin only slowly, so that negative or weakly positive results were obtained with 1-day cultures but, with 3-day cultures, they were distinctly positive.

Comparison of the VP, polymyxin-sensitivity and phage-sensitivity results (table I) with those of haemolysin production (table II) reveals three distinct biotypes: strains 8021 and 281 are of the *classical* biotype in all four characters; strains 719 and 283 are of the El Tor biotype; but the two strains (nos. 14268 and 41) from the *present pandemic* are haemolysin negative though otherwise similar to El Tor strains, and they may well be designated *V. cholerae* (*intermediate* biotype). This would support the suggestions of Haddadin *et al.* (1973) who studied two peaks (7α and 7β) produced in pyrolysis-gas-liquid chromatograms of *V. cholerae*. With strains 8021 and 281 and eight other strains of classical biotype, 7α was distinctly smaller than 7β . With strains 719 and 283 and three other strains of El Tor biotype, 7α was distinctly larger than 7β . With strains 14268 and 41 and fifteen other strains from the present pandemic, all of which were non-haemolytic but similar to El Tor in the other three characters, peaks 7α and 7β were of approximately equal size. Thus the strains of the present pandemic belong to neither the classical nor the El Tor biotype, and attempts to manipulate the haemolysin test will not make them conform to the characteristics of the El Tor type.

Our findings support the view of Sen and Shrivastava (1970) that the haemolysin test is reliable for the differentiation of the biotypes of *V. cholerae*, provided that Greig's method is not modified extensively. They also support the conclusion of Feeley and Pittman (1963), on p. 355 of their paper, that it "remains an excellent epidemiological tool". This applies particularly to the identification of sources of infection in the present pandemic, even though it is now generally accepted that the measures for quarantine, treatment and control of cholera should be the same for all biotypes.

SUMMARY

Three-day nutrient-broth cultures of *Vibrio cholerae* gave distinct and reproducible results when tested for haemolysis of sheep RBC. As a medium for detecting haemolysis, heart-infusion broth was no better, and brain-heart-infusion broth was less satisfactory. Lack of reproducibility, and difficulty in interpreting results, were experienced when medium containing glycerol was used and when attempts were made to detect haemolysin in young (8-hour or 1-day) cultures. Horse and human RBC were found too fragile for this test.

Strains of *V. cholerae* could be separated into three biotypes: classical, El Tor, and an intermediate biotype to which many strains of the present pandemic belong. The latter strains are similar to the El Tor biotype but haemolysin negative. Hence, the haemolysin test, if performed by a reliable method, is a valuable epidemiological tool.

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