COMPARISON OF TWO ASSAYS FOR THE DETECTION OF HAEMOLYSINS OF *AEROMONAS* SPECIES

J Vadivelu, S D Puthucheary, P Navaratnam

ABSTRACT

The haemolysins produced bv Aeromonas species were detected and compared by two assay methods - a modified blood agar plate assay and the rabbit erythrocyte haemolysin method. Both assays showed a high level of agreement (86%). The titres of the rabbit erythrocyte haemolysin assay correlated with the haemolytic zone diameter of the ox blood agar assay. In addition the agar haemolysin assay had simple media requirements, was easy to perform and results were well defined.

Keywords: *Aeromonas spp, haemolysins, a comparative assay.*

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INTRODUCTION

The haemolysins of *Aeromonas* species have been implicated as a major virulence factor in the pathogenesis of human extraintestinal infections often associated with soft tissue necrosis. To date, three types of haemolysins of *Aeromonas* species have been identified, purified and characterized; alpha (α) haemolysin produced optimally at 22°C and causes incomplete haemolysis of ox erythrocytes[1] , beta (ß) haemolysin, a high molecular weight (50-51 KDa) heat-labile protein produced at 37°C with broad specificity causing dermonecrosis of rabbit skin and lethal to mice[2], and the Asao toxin, another high molecular weight (48-50 KDa) heat-labile protein antigenically similar to the ß-haemolysin but functionally causes fluid accumulation in the rabbit ileal loop test[3]. Detection methods of the haemolysin include the ability of the haemolysin to lyse 50% of rabbit erythrocytes[4-6]. More recently, an ELISA has been described for the ß-haemolysin[7] The variety of haemolysins produced requires a broad, rapid, easy to perform and reliable assay for detection.

This study was designed to compare the reliability and ease of performance of a modified agar haemolysin assay with the widely used rabbit erythrocyte haemolysin assay, in the detection of haemolysins of *Aeromonas* species.

MATERIALS AND METHODS

Bacterial Strains

One hundred strains of *Aeromonas* species isolated from clinical specimens (which included faeces, urine, pus, peritoneal dialysis fluid, blood, tracheal secretions and bile) of patients at the University Hospital, University of Malaya, Kuala Lumpur were used in this study. All strains were identified as *Aeromonas spp* by the API 20E system (2019,1985) (bio-Merieux, France), of which 86 strains were *A. hydrophila* and the remaining 14 isolates were not speciated. *Escherichia coli* strain WF5 (UK) of environmental origin was used as the negative control and *Aeromonas hydrophila* strain 9008 of clinical origin was used as a positive control. The bacterial strains were stored at 4°C on tryptone soy agar slants (Oxoid, UK) during the course of the study.

Preparation of cell-free culture filtrates

Each strain was inoculated into 3ml of Brain Heart Infusion (BHI) broth (Oxoid) and incubated overnight at 37°C with agitation at 100 rpm. The cultures were then centrifuged at 12,000 x g for 20 minutes, filter-sterilized (0.45 um filter acrodiscs, Gelman, UK) and the filtrates stored at 4°C for use within two weeks of preparation.

Agar haemolysin assay

The assay method of Honda and Finkelstein[8] as modified from Zen-Yoji *et al*[9] for the detection of the *Vibrio parahaemolyticus* haemolysin was performed. Ox blood was collected in Alsever's solution and centrifuged at 1,500-2,000 rpm. The packed cells were washed twice with phosphate buffered saline and then added to 1% molten agarose at a final concentration of 2% erythrocytes. 20 ml of the agarose was poured into 4 by 4 plates (Sterilin, UK) and following solidification, wells 2 mm in diameter were punched into the agar. To each of the wells, 10 ul of cell-free culture filtrate was added. The diameters of the haemolytic zones were measured following incubation at 37°C for 2 hour and subsequently after overnight incubation at 4°C.

Rabbit erythrocyte haemolysin assay

A modification of the method of James et al [5] was used to determine haemolytic activity of *Aeromonas* species. A 100 ul volume of culture supernate of each strain was serially diluted two-fold in phosphate buffered saline (PBS), and mixed with an equal volume of 1% suspension of rabbit erythrocytes in microtitre plates. The plates were incubated at 37°C for 1 hour and at 4°C overnight. One haemolytic unit (HU) was defined as the reciprocal dilution of the smallest dose in 100 ul of sample which caused 50% haemolysis. Standards containing varying concentrations of lysed erythrocytes were used as controls.

RESULTS

A total of 100 strains of *Aeromonas* species of clinical origin were used to compare the detection of haemolysins by the two methods. A total of 86 strains agreed on both assays (Table I). Of the remaining 14 strains, four were positive in the ox blood agar haemolysin assay and negative by the rabbit erythrocyte haemolysin method and ten were negative on the ox blood agar haemolysin assay but positive by the other assay.

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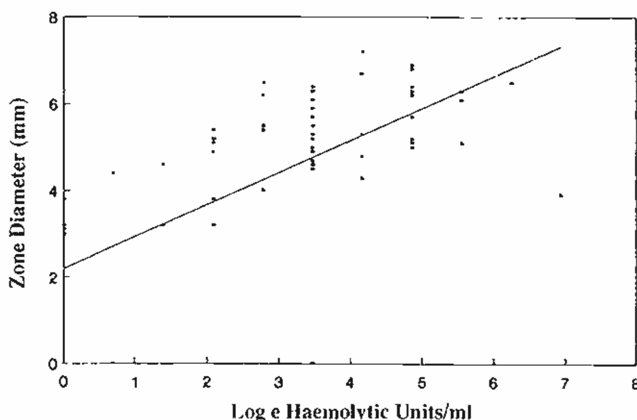
Table I - Comparison of the rabbit erythrocyte and ox blood agar plate haemolysin assays

Haemolysin Assays		Total No.
Rabbit rbc	Ox rbc	
+	+	63
-	+	4
+	-	10
-	-	23
Total number of strains tested		100

The relationship between the zone diameter on the agar haemolysis assay and haemolytic units using rabbit erythrocytes is presented in Table II. On inspection (Fig 1) although there were variations in the sizes of zone diameter for each haemolytic unit, in general there was good agreement between zone diameter and haemolytic units ie the larger the diameter, the higher the units of haemolysins. There was however a poor correlation between 10.24 HU/ml of haemolysins and the related zone diameter of 3.9 mm.

Fig 1 - Correlation of zone diameter and haemolytic activity of culture filtrates of *Aeromonas spp.*



x-Coefficient = 0.742 + 0.123

DISCUSSION

The production of cytotoxins and haemolysins may be an inherent characteristic of isolates of *Aeromonas hydrophila* from intestinal and extraintestinal infections. However we have demonstrated that haemolytic activity was more prominent amongst extraintestinal isolates suggesting its importance in extraintestinal infections[10]. Haemolysin activity due to a range of haemolysins has been significantly associated with *A.hydrophila* and *A. sobria* groups. We have used a modified agar haemolysis assay, described originally for the haemolysin of *V. parahaemolyticus* to ease detection mainly of the ß-haemolysins of *Aeromonas* species and compared its performance with the widely established rabbit erythrocyte haemolysin assay.

There was 86% agreement on both assays (63 strains positive, 23 strains negative) (Table I) amongst the 100 strains of *Aeromonas* species even though a different species of erythrocyte was used in each assay. This high degree of agreement suggests that both types of erythrocytes, ox and rabbit, were sensitive to the haemolysins released by the 63 positive strains. At the same time, in the 23 negative strains, either no haemolysins were produced or the species of erythrocytes used (ox and rabbit) were not sensitive to any other type of haemolysin that may have been produced by the strains. The database used in this study only differentiated between *A. hydrophila* and *A. salmonicida*. Although 86 strains were identified as *A. hydrophila*, the 14 unspeciated strains and some of the 86 *A. hydrophila* strains may indeed have been *A. caviae* or *A. sobria*, which could account for the 23 negative strains. This is highly likely as the new API 20E database groups *A. hydrophila* and *A. caviae* together and *A. sobria* separately.

Table II - Relationship between haemolytic unit by rabbit erythrocyte assay and zone diameter by agar haemolysis assay

Haemolytic	Zone Diameter (mm)						
unit/ml	Enteric	Bile	Throat	Blood	PD fluid	Urine	Wound
0.00	0.0(15) 3.0 (1) 3.1 (1) 3.2 (1)	—	0.0 (3)	3.8 (1)	0.0 (3) —		0.0 (2)
0.02	0.0 (3)	0.0 (1)	—	0.0 (1)	—	—	4.4 (1)
0.04	4.6 (1)	—	—	0.0 (1)	—	—	3.2 (1)
0.08	4.9 (1) 5.1 (2)	—	—	0.0 (1)	3.2 (1) 3.8 (1)	3.8 (1) 5.2 (1)	4.9 (1) 5.4 (1)
0.16	0.0 (1) 4.0 (1) 5.5 (1)	—	6.2 (1)	—	5.4 (1)	—	6.5 (1)
0.32	4.5 (1) 4.6 (2) 4.9 (2) 5.2 (1) 5.5 (3) 5.7 (1) 6.3 (1)	0.0 (1) 5.3 (1) 5.5 (1)	—	5.5 (1) 5.9 (1)	4.6 (1) 4.7 (1) 4.9 (1) 5.2 (1)	—	0.0 (1) 5.0 (1) 5.5 (2) 5.9 (1) 6.1 (1) 6.4 (1)
0.64	0.0 (1) 4.8 (1) 5.3 (1)	6.7 (1)	4.3 (1)	—	—	—	7.2 (1)
1.28	—	—	—	—	5.0 (1) 6.3 (2)	—	5.1 (1) 5.2 (1) 5.7 (1) 6.3 (1) 6.4 (1) 6.8 (1) 6.9 (1)
2.56	6.1 (1) 6.3 (1)	—	—	—	—	—	5.1 (1)
5.12	—	—	—	—	—	—	6.2 (1)
10.24	—	—	—	3.9 (1)	—	—	—

Numbers in parenthesis refer to the number of data points for a particular value.
Total number of different data points — 56.

The results obtained on both groups were discrepant on 14 strains. Various workers have demonstrated that erythrocytes of different species of mammals vary in their sensitivity to haemolysins from different strains of *A. hydrophila*[11,12]. Brenden and Janda[13] demonstrated that erythrocytes of mouse and monkey were most sensitive for detection of haemolysin. In addition it was shown that ox rbc was less sensitive than rabbit rbc. In this study, of the 73 positive strains using rabbit erythrocytes, 10 were negative by the ox erythrocytes and of the 67 strains positive by ox erythrocytes, 4 were negative

using rabbit erythrocytes (Table I). In general, the zone diameters produced using ox erythrocytes related well with haemolysin units (Fig 1) except for one strain producing 10.24 HU/ml. In general, the observation of results was more clearcut on the agar plate assay as it was a definite lysis versus no lysis, whereas on the rabbit erythrocyte haemolysin assay, one had to read a 50% lysis titre which even with the use of standards, may be subjective. This factor may have contributed to the higher number of positives on the rabbit erythrocyte assay as compared to the agar haemolysin assay (71 and 68 respectively).

We have demonstrated that the agar plate method is a suitable alternative to the rabbit erythrocyte assay as shown by the high level of agreement between the assays. Moreover, the agar haemolysin assay is easy to perform using widely available reagents and allows the testing of up to 16 strains per agar plate as compared to 8 strains per microtitre plate used in the rabbit erythrocyte haemolysin assay. Once prepared, the plates can be kept refrigerated whereas the rabbit erythrocyte suspension has to be prepared fresh each time due to autolysis; the standards for lysis have to be prepared and controlled for each assay. Furthermore, as the zone diameter correlates well with the titre, the agar haemolysin assay may be used as a semiquantitative method. No dilution series is required in this method, thus reducing the tediousness of the assay procedure.

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