MICROBIAL PATHOGENICITY

Detection of haemolytic activity of campylobacters by agarose haemolysis and microplate assay

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Summary. There are several methods for the detection of haemolytic activity in campylobacters. However, we found the haemolytic effect of campylobacters on conventional blood agar plates to be variable, inconsistent and difficult to interpret. Blood agarose plates showed campylobacter haemolytic activity more clearly. The incubation conditions (temperature and gaseous) appear to be important for the expression of this activity. Ninety four percent of the *Campylobacter* isolates examined were found to be haemolytic by the microplate assay with minimal haemolytic units that ranged from 1 to 64. Haemolytic activity was detected only from live bacterial cultures and not from any of the 50 bacterial culture supernates, which suggests that campylobacters may possess a cell-associated haemolysin. The identification of such haemolytic activity in a large number of campylobacters (94 %) suggests its potential role as a virulence factor in campylobacter gastroenteritis.

Introduction

Campylobacter spp. are common causes of human enteritis worldwide and are prevalent in developing countries.^{1,2} They rank as the third commonest cause of acute diarrhoea after rotaviruses and enterotoxigenic *Escherichia coli*.^{1,3} Among the *Campylobacter* spp., *C. jejuni* and *C. coli* are isolated most frequently from human diarrhoea specimens with *C. lari* and *C. fetus* detected less often.

Despite several studies,^{4,5} campylobacters are generally regarded as non-haemolytic.⁶ The reason for this could be the lack of a sensitive method for the detection of haemolytic activity. However, haemolytic changes on blood agar plates have been observed in this study as well as in others.

The aim of this study was to investigate methods for the detection and characterisation of the haemolytic activity of *Campylobacter* strains isolated in Malaysia.

Materials and methods

Bacterial isolates

A total of 100 *Campylobacter* isolates (85 clinical and 15 animal) were used in this study. *C. jejuni* isolates were identified by a specific DNA probe⁷ (kindly provided by V. Krishnapillai, Monash Uni-

versity, Australia) and further characterised by biotyping.^{8–10}

Agarose haemolysis assay

Briefly, agarose 1 % was prepared in PBS, pH 7.2, and erythrocytes of various origins (human, ox, sheep or rabbit) were added to a final concentration of 2 %. The media were poured into petri dishes to a thickness of 1 mm and the agarose plates were left to dry in air.

To determine the optimal conditions for the expression of campylobacter haemolytic activity, a maximum of six bacterial cultures were inoculated in a circular manner on one blood agarose plate, and the plates were incubated for 24 h at two different temperatures (37°C and 42°C) and in two gaseous conditions (aerobic and micro-aerophilic).

To determine quantitatively the haemolytic effect on blood agarose, holes of 1 mm diameter were punched into the plates. Bacterial suspensions of 10⁸ organisms in 10 µl were pipetted into the holes. The plates were incubated aerobically at 42°C with bovine erythrocytes. These were found to be the optimal conditions in the preliminary experiments above.

Microplate assay

The microplate assay was performed as described by Dominguez-Rodriguez *et al.*¹¹ Two-fold dilutions of bacterial suspensions were made in microtitration plates with V-bottomed wells (Linbro, USA) into which 50 µl of PBS had been placed. To each bacterial

Fig. 1. a, Haemolytic activity of *Campylobacter* spp. on a blood agar plate. Activity is most distinct at the initial streaking site. b, clear zones due to campylobacter haemolytic activity on a blood agarose plate.

dilution, 100 μ l of 1% washed bovine erythrocytes were added, and the plates were incubated aerobically at 42°C for 18–24 h. The haemolytic activity titre was expressed as minimal haemolytic units (MHU) which was the highest dilution at which haemolysis was detected. In both assays, sterile PBS was used as a negative control.

Characterisation of haemolytic activity of campylobacters

To determine whether the haemolysin was an extracellular product, campylobacters were cultured in three different media, each of which contained a

high percentage of animal extracts, i.e., CAYE (casamino acid supplemented with yeast extract) medium, CAYE medium supplemented with fetal bovine serum 5% and Mueller Hinton broth. The bacteria (20 isolates) were grown for 48 h in the above media and the resulting cultures were centrifuged at 6000 rpm for 5 min to remove most of the intact cells and cellular debris. These crude supernates were then serially diluted and the haemolytic activity was assayed by the above two methods.

Polymyxin treatment

Campylobacter isolates were suspended in PBS

Table I. Expression of campylobacter haemolytic activity on blood agarose plates under various incubation conditions

Incubation conditions	Number (/20) of haemolytic isolates
Aerobic 42°C	18
Aerobic 37°C	13
Microaerophilic 42°C	14

Note: the haemolytic activity of campylobacters incubated microaerophilically was difficult to determine.

containing polymyxin B sulphate at a final concentration of 2 mg/l. The suspension (10^{10} organisms/ml) was incubated at 37°C for 1 h before centrifugation at 6000 rpm for 5 min. The supernate was collected and the pellet was resuspended in PBS. The haemolytic activity of both polymyxin B-treated supernates and bacterial cell suspensions were determined by the agarose haemolysis and the microplate assay as described above.

Haemolytic activity of bacterial lysates

Bacterial suspensions of 10 isolates that had demonstrated haemolytic activity in the agarose haem-

Fig. 2. **a**, Haemolysis of *Campylobacter* spp. on an ox blood agarose plate. **b**, microplate assay for the quantitation of campylobacter haemolytic activity. Lanes 1–10, *Campylobacter* test isolates; 11, negative control strain in sterile PBS; 12, haemolytic *E. coli* isolate.

olysis assay were lysed by sonication (Branson Sonifier Cell Disruptor B15, USA) at 20 kHz for 10 min. The supernates were collected by centrifugation and assayed for haemolytic activity by the agarose haemolysis assay.

Heat stability

Bacterial suspensions of 20 isolates that had demonstrated haemolytic activity in the agarose haemolysis assay were heated at 100°C for 30 min and the remaining haemolytic activity was determined by the agarose haemolysis assay.

Results

Agarose haemolysis assay

Haemolytic activity of *Campylobacter* isolates was observed on conventional blood agar plates at 37°C and 42°C after incubation for 48–72 h in micro-aerophilic conditions. However, it was always necessary to scrape the colony off the agar in order to see the haemolysis. A more marked haemolytic activity was observed at the initial streaking line where a large inoculum of bacteria was normally present. The haemolytic activity of campylobacters on the agar plate was generally variable, inconsistent and difficult to interpret (fig. 1a).

However, with blood agarose plates, clear and transparent zones could be seen beneath the inoculum after 24 h without scraping off the colonies (fig. 1b). Haemolytic and non-haemolytic isolates could be differentiated on agarose plates. Incubation at 42°C under aerobic conditions enhanced the haemolytic activity (table I). The assay also allowed quantitation of haemolytic activity (fig. 2a and table II).

There was no significant difference in haemolytic activity between human and animal erythrocytes (data not shown).

Microplate assay

The microplate assay of Dominguez-Rodriguez *et al.*¹¹ was simple and rapid, required small amounts of reagents and was more sensitive than agarose haemolysis. It allowed haemolytic activity to be expressed in minimal haemolytic units (MHU; fig. 2b). The results are shown in table III. Haemolysin production was not correlated with biotype (table IV).

Characterisation of haemolytic activity

In this study, haemolytic activity of campylobacters was demonstrated only by intact bacterial cells. Although complex media containing animal extracts which are known to stimulate the production of haemolysin were used,^{12,13} no haemolytic activity was detected in the bacterial culture supernates.

Bacterial lysates of 10 *Campylobacter* isolates obtained after sonication and polymyxin B-treated

Table II. Haemolytic activity of campylobacters determined by the agarose haemolysis assay

Zone diameter (mm)*	Number of isolates	
	Untreated†	Polymyxin B-treated‡
2	42	56
3	25	32
4	23	6
5	3	6
6	6	0
7	1	0
Total	100	100

*Haemolysis was considered positive when the zone was ≥ 3 mm in diameter.

† 10^{10} campylobacters/ml were used.

‡ 10^{10} campylobacters/ml were treated with polymyxin B 2 mg/ml at 37°C for 1 h before the assay.

Table III. Haemolytic activity of campylobacters determined by the microplate assay

MHU*	Number of isolates	
	Untreated†	Polymyxin B-treated‡
1	6	13
2	17	29
4	22	33
8	35	20
16	14	2
32	5	2
64	1	1
Total	100	100

*MHU ≥ 2 was considered positive.

† 10^{10} campylobacters/ml were used.

‡ 10^{10} campylobacters/ml were treated with polymyxin B 2 mg/ml at 37°C for 1 h before the assay.

Table IV. Biotypes of campylobacters and haemolytic activity

Isolates	Haemolytic activity (number of strains)	
	+	–
Clinical		
<i>C. jejuni</i> biotype I	39	2
<i>C. jejuni</i> biotype II	25	1
<i>C. coli</i>	10	1
Untypable isolates	6	1
Total	80	5
Poultry		
<i>C. jejuni</i> biotype I	7	1
<i>C. jejuni</i> biotype II	4	0
<i>C. coli</i>	2	0
Untypable isolates	1	0
Total	14	1

The haemolytic activity of the bacterial suspension was determined by the microplate assay. A minimal haemolytic unit (MHU) of ≥ 2 was considered as positive.

supernates of all isolates were all negative for haemolytic activity. The heat-denatured suspensions of 20 strains showed no haemolytic activity.

Discussion

There are at present several methods for the detection of haemolytic activity.^{4,5} In this study, the haemolytic effect on conventional blood agar plates was variable, inconsistent and difficult to interpret, which is probably why it has not been reported for many years. Therefore, we attempted to develop a new assay for the detection of campylobacter haemolytic activity and to gain an insight into its role as a possible virulence factor of campylobacters.

In contrast to conventional blood agar plates, clear and distinct haemolytic zones were detectable on blood agarose plates (fig. 1b). The conditions of incubation (temperature and gaseous) appear to be important for the expression of this activity. Aerobic incubation at 42°C was optimal for the expression of this activity, since most haemolytic isolates were detected in these conditions (table I).

Haemolytic activity was not detected in the bacterial supernates of 50 isolates tested by both the agarose plate and the microplate assays suggesting that campylobacters may possess a cell-associated haemolysin. Arimi *et al.*⁴ postulated the existence of a cell-bound haemolytic factor that is released intracellularly. However, the supernates collected after polymyxin B-treatment and the bacterial lysates obtained after sonication were also negative in this study. Furthermore, bacterial suspensions showed reduced haemolytic activity after treatment with polymyxin B. We consider that the haemolysin produced is so dilute that it could not be detected in the supernates after the destruction of the bacteria or that the intact cell was required to produce the haemolytic activity. Although the haemolysin has been reported to be thermostable and extractable by boiling in saline,⁵ no haemolytic activity was detected for these strains after boiling the bacterial suspensions at 100°C for 30 min.

With the microplate technique described by

Dominguez-Rodriguez *et al.*¹¹ for *Listeria monocytogenes*, we were able to identify and quantitate the haemolytic activity in 94% of the campylobacters tested. This method is simple, distinguishes reliably between haemolytic and non-haemolytic isolates and should be the method of choice, since the agarose technique demonstrated haemolytic activity in only 56% of the isolates. In this study, the haemolytic activity of campylobacters as determined in the microplate assay was low in titre, ranging from 1 to 64.

The detection of haemolytic activity in the majority of campylobacters examined suggests that this cell-associated activity could be a potential virulence factor contributing to campylobacter gastroenteritis. The production of haemolysin is a mechanism for microbes to obtain iron for bacterial growth and the expression of virulence factors.¹⁴ Iron overloading has been shown to increase the virulence of campylobacters,^{15,16} which also provides evidence for the importance of iron sequestration in campylobacter infections.

It is interesting to note that *Shigella* spp.—invasive enteropathogens—are classified as non-haemolytic, but were shown to lyse sheep erythrocytes when in close contact with them.^{17,18} The contact haemolytic activity of *Shigella* spp. was reported to play a role in the lysis of the phagocytic vacuole for the release of virulent shigellae into the host cytoplasm, where replication occurs. The invasive phenotype of shigellae is reported not to be differentiated through this haemolytic activity.^{19,20} All invasive *Campylobacter* isolates except four (three invasive, one non-invasive) detected in the gentamicin HEp-2 cell invasion assay were haemolytic (Tay *et al.*, unpublished observations). Whether the same invasive mechanism plays a role in the campylobacters as in *Shigella* spp. needs further investigation.

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