Effects of incorporating ampicillin, bile salts and carbohydrates in media on the recognition and selection of *Aeromonas* spp. from faeces

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Summary. The effects of incorporating ampicillin, some bile salts and sugars into media on the primary recognition and selection of aeromonads from faeces were examined. Most (88%) of the 101 *Aeromonas* strains examined had an ampicillin MIC of ≥ 40 mg/L, and would be isolated on blood agar containing ampicillin 30 mg/L. The strains with an ampicillin MIC of < 40 mg/L were all of human origin and predominantly *A. caviae*. Although ampicillin at 10, 20 or 30 mg/L in blood agar inhibited faecal bacteria, the ability to detect *Aeromonas* strains with a high ampicillin MIC was less when the lower concentrations of ampicillin were used, without any improvement in the isolation of those strains with a low ampicillin MIC. Thirty-seven strains were tested for sensitivity to several different bile salts and found to be at least as resistant to them as *Escherichia coli* NCTC 10418. Bile salt sensitivity was not related to the species or source of a strain. There were minor differences in sensitivity to bile salts between some strains which related to whether strains had been isolated originally in the presence of bile salt or not. The effects of the presence of *E. coli*, *Klebsiella* spp. and *Enterococcus faecalis* on the growth of *Aeromonas* strains in mixed culture on media with and without carbohydrate were examined. The colony size of some *Aeromonas* strains was reduced in mixed culture but colony counts were not affected with any *Aeromonas* strains. The effect of carbohydrate in the medium on *Aeromonas* colonies was apparent in the presence of other bacteria that fermented carbohydrate, when the non-fermenting *Aeromonas* colonies were often indistinguishable from those of the fermenters. When xylose was added to the medium the recognition of colonies of the non-fermenting aeromonads among those of other fermenting bacteria was difficult because their colonies were often of the same colour. This may explain the difficulty of isolating aeromonads from mixed cultures on some carbohydrate-containing media. It is recommended that if aeromonads are to be recognised in selective culture from faeces by their failure to ferment carbohydrate, a medium be used which inhibits the growth of non-aeromonad bacteria as much as possible.

Introduction

On MacConkey agar, or other media designed for the isolation of salmonellae and shigellae from faeces, *Aeromonas* colonies cannot always be distinguished easily from those of members of the Enterobacteriaceae. Therefore, several special selective or indicator media have been devised to facilitate their recognition and isolation. Most of these media contain one or more of ampicillin, bile salts and xylose.^{1,2,3} The first two are used as selective agents and the third with an indicator, to

distinguish xylose non-fermenting *Aeromonas* colonies from the xylose-fermenting organisms of the Enterobacteriaceae. However, some strains of *Aeromonas* may be inhibited by the ampicillin or bile salts. Strains vary in their resistance to ampicillin⁴ and some may be isolated only on media without bile salts.⁵ When carbohydrates are present, the recognition of aeromonads in mixed culture is much reduced. This has been attributed to the effects of carbohydrate metabolism by other genera.⁶ Therefore, we have evaluated the effects of incorporating these agents in media on the recognition and selection of aeromonads from faeces.

Materials and methods

Bacterial strains

Aeromonas strains from faeces and water samples were isolated on xylose desoxycholate citrate agar or brilliant green bile salts agar as described previously.^{6,7} Strains from foods and other human sources were isolated on horse blood agar. Strains were identified to species level as described before.⁸

Media

Horse-blood agar was prepared by supplementing Columbia Agar Base (Oxoid, CM331) with sterile defibrinated horse blood (Tissue Culture Services) 5%. Nutrient Broth was Oxoid (CM67) and Nutrient Agar was Oxoid (CM3). Media were sterilised by autoclaving at 121°C for 15 min.

Determination of ampicillin MIC for *Aeromonas* strains

Ampicillin Adatabs® (Mast Laboratories) were dissolved in water and then diluted in Iso-Sensitest Broth (Oxoid, CM471) to make two-fold dilutions of ampicillin from 160 to 0.1 mg/L; 0.1-ml volumes of the dilutions of ampicillin (or Iso-sensitest broth alone as a control) were dispensed into wells of a microtitration plate. Bacterial inocula were made by adding to 10 ml of Iso-sensitest broth 0.1 ml of a suspension prepared by emulsifying four colonies from a blood-agar plate in 3 ml of saline; a 0.1-ml volume of the inoculum (*c.* 10⁵ cfu/ml) was added to each well of the microtitration plate. *Escherichia coli* NCTC 10418 and the Oxford strain of *Staphylococcus aureus*, NCTC 6571, were used as control strains. Plates were incubated overnight at 37°C and then inspected for bacterial growth by eye. Each MIC determination was made twice.

Effect of different concentrations of ampicillin on the isolation of aeromonads from faeces artificially inoculated with aeromonads

Six strains of *Aeromonas*, representing those with a wide range of sensitivities to ampicillin, were incubated overnight at 37°C in nutrient broth and then serially diluted in 10-fold steps in normal saline; 0.1-ml volumes of each dilution were added to 1 ml of emulsified faeces (10% w/v in normal saline). *Aeromonas*-inoculated faecal samples, and uninoculated faecal samples as controls, were mixed well and 0.1-ml volumes were subcultured on blood-agar plates containing ampicillin 10, 20 or 30 mg/L and on antibiotic-free medium. Inocula were spread in a standard way; there were two sets of outbreaks from the well each of four streaks, and a final single streak. The loop was flamed after each set of streaks. After incubation overnight at 37°C, plates were flooded with oxidase reagent (tetramethyl-*p*-phenylene-

diamine dihydrochloride 1% w/v in sodium thiosulphate 0.025% w/v) and the relative number of oxidase-positive and -negative colonies was estimated. Numbers of colonies were scored on a semi-quantitative scale: 1 = growth only in the "well" of the plate; 2 = growth only in the first group of outbreaks; and so on. Oxidase-positive colonies were immediately subcultured on blood agar, and their identity as aeromonads was confirmed with Hugh and Leifson's O/F test and API 20E tests (API System S.A., La balme les Grottes, 38390 Montalieu Vercieu, France).

Effect of bile salts on the growth of aeromonads

Two groups of *Aeromonas* strains were examined; 23 strains whose primary isolation had been made on media containing bile salts, and 14 strains whose primary isolation was made on blood agar. *E. coli* NCTC 10418 was used as a control. Nutrient-agar plates were made containing a range of concentrations of different bile salts from 10 to 0.1 g/L. The bile salts used were: Oxoid L55 and Difco; bile salts no. 3 (Oxoid L56 and Difco); sodium desoxycholate (Oxoid L57 and Difco); and sodium taurocholate (Difco). Nutrient-agar plates without bile salts were used as controls. A 0.1-ml inoculum from suspensions of 10⁷, 10⁵ or 10² cfu/ml of each strain was spread as a lawn over the plates.

Effect of carbohydrate on the isolation of aeromonads from mixed cultures

Four media were prepared; MacConkey Agar (Oxoid, CM7), and three media of similar formulation comprising (/L) peptone (Oxoid, L37) 20 g, bile salts (Oxoid, L55) 5 g, sodium chloride 5 g, neutral red 0.075 g, agar (Oxoid, L11) 12 g but in which lactose was either omitted or replaced by xylose (Oxoid, SR 22) 10 g or inositol (Sigma) 10 g. Four strains of *A. sobria*, four of *A. hydrophila* and four of *A. caviae*, each species group having one strain found to ferment lactose on primary isolation, were selected. These strains and fresh isolates of *E. coli*, *Klebsiella* sp. and *Enterococcus faecalis* from faeces were incubated overnight at 37°C in nutrient broth. Viable counts of the pure cultures were made by the Miles and Misra technique⁹ on blood agar, MacConkey agar and bile salt medium without carbohydrate. Mixed cultures were prepared by mixing equal volumes of each pure *Aeromonas* culture with a culture of *E. coli* or *Klebsiella* or *Ent. faecalis* and viable counts were made on MacConkey agar and bile salt medium without carbohydrate. Viable counts of the same *Aeromonas* cultures, both before and after mixing with equal volumes of the *E. coli* broth culture, were also determined on bile salt medium containing xylose or inositol, or no carbohydrate, by spreading 0.1 ml of the culture as a lawn over the whole of the plate. All plates were incubated overnight at 37°C.

The *Aeromonas* colonies in mixed culture on plates with and without carbohydrate were recognised by flooding the plate with oxidase reagent. Their number in

mixed cultures was compared with that from the pure cultures. If there was no inhibition, it would be expected that the counts of aeromonads in mixed cultures would approximate to half that of the pure culture on the same medium. Examination was also made for the effect of the presence of non-aeromonad bacteria on the size and colour of the *Aeromonas* colonies on the different media.

Results

Determination of the MIC of ampicillin for the Aeromonas isolates

A total of 101 *Aeromonas* strains comprising 68 from faeces, four from other human sources and 29 from food and water samples was tested. These included 24 strains of *A. sobria* of which 18 were from faeces, one from a blood culture, and five from water samples. There were also 18 strains of *A. hydrophila*, seven from faeces, two from other human sources, and nine from water samples, and 54 strains of *A. caviae*, 40 from faeces, one from a perineal swab, one from food and 12 from water samples. Five strains, three from faeces, and two from water could not be assigned to any of these species.

The MIC values of ampicillin for most (88%) of the *Aeromonas* isolates were ≥ 40 mg/L (table I). However, nine strains (9%), of which seven were *A. caviae*, had MIC values of ≤ 10 mg/L. All strains with ampicillin MIC values of < 40 mg/L were of human origin.

Effect of different concentrations of ampicillin on the isolation of aeromonads from faeces artificially inoculated with aeromonads

All the six *Aeromonas* strains examined were isolated from human faeces. They included a strain of *A. sobria* with an ampicillin MIC of ≥ 80 mg/L,

two strains of *A. hydrophila* with ampicillin MICs of ≥ 80 and 40 mg/L, and three strains of *A. caviae* with ampicillin MICs of 10, 5 and 5 mg/L respectively.

All the concentrations of ampicillin used caused some reduction in the growth of the non-aeromonad bacteria. Plates without ampicillin had a score of 4, those with ampicillin 10 and 20 mg/L scores of 3, and those with ampicillin 30 mg/L gave scores of 2. On plates without ampicillin and those with ampicillin 10 mg/L, oxidase-positive colonies of the three strains with MIC values ≥ 40 mg/L could be detected at ratios of *Aeromonas*: non-*Aeromonas* bacteria of between 10:1 and 1:100. However, they were present only in the well of each plate. For the faecal samples inoculated with the strains of *A. caviae* with ampicillin MICs of either 10 or 5 mg/L, no oxidase-positive colonies were detected on any plate containing ampicillin. This remained true even for samples containing 10 times more viable aeromonads than faecal bacteria. Ampicillin at 20 and 30 mg/L improved the isolation of the two strains with ampicillin MIC values ≥ 80 mg/L, but only marginally improved the isolation of the strain with an ampicillin MIC of 40 mg/L (table II).

Effect of bile salts on aeromonads

The 37 strains examined included five strains of *A. sobria*, nine of *A. hydrophila* and 23 of *A. caviae*; 13 strains were from human faeces, three from foods and 21 from water samples. With an inoculum of 10^2 cfu/ml neither the control *E. coli* strain nor most of the *Aeromonas* strains grew on any medium containing a bile salt at any concentration. Four *Aeromonas* strains (three of which were isolated originally on media containing bile salt) grew on media containing bile salts no. 3 (Difco) at a concentration of ≤ 2.5 g/L. Minor differences only were observed in the results with inocula of 10^5 or 10^7 cfu/ml and the latter was used routinely. The *E. coli* control strain was inhibited by bile salts (Difco) and sodium taurocholate (Difco) at concentrations of 10 g/L and by all the other bile salt formulations tested at 2.5 g/L.

However, nearly all the 37 *Aeromonas* strains tested were more resistant to bile than the *E. coli* control irrespective of the type of bile salt used (table III). Sensitivity to bile salts did not appear to be influenced by the species or source of the strain and only minor differences in sensitivity to bile salts, which appeared to relate to the medium of original isolation of the strain, were observed. All the *Aeromonas* strains were inhibited by each bile salt tested at 10 g/L but sodium taurocholate, which

Table I. MICs of ampicillin for 101 *Aeromonas* strains

Ampicillin MIC (mg/L)	Number of strains of				Total (%)
	<i>A. sobria</i>	<i>A. hydrophila</i>	<i>A. caviae</i>	Other*	
≥ 80	21	16	42	3	82 (81)
40	2	2	3	0	7 (7)
20	0	0	2	1	3 (3)
10	1	0	4	1	6 (6)
5	0	0	1	0	1 (1)
2.5	0	0	2	0	2 (2)

**Aeromonas* strains not belonging to any recognised species.

Table II. Recovery of *Aeromonas* strains from faeces artificially inoculated with aeromonads on blood agar containing different concentrations of ampicillin

Concentration of ampicillin (mg/L) in blood agar	Log ₁₀ of the ratio of the number of cfu/ml of <i>Aeromonas</i> : faecal bacteria									
	-1-<0		0-<1		1-<2		2-<3		3-<4	
	A	B	A	B	A	B	A	B	A	B
0	-	NT	-	-	-	-	-	-	NT	-
10	-	NT	-	-	-	-	-	-	NT	-
20	+	NT	+	+	-	+	-	+	NT	(+)
30	+	NT	-	+	-	+	-	+	NT	+

+ Aeromonads isolated in all tests; (+), aeromonads isolated in some tests; -, aeromonads not isolated in any test; NT, not tested.

A, results for a strain with an ampicillin MIC of 40 mg/L; B, results for two strains with ampicillin MICs of ≥ 80 mg/L.

was the least inhibitory of the bile salts, did not inhibit any strain at ≤ 5 g/L. However, Difco and Oxoid L55 bile salts inhibited nearly all strains at this concentration. All but five strains grew on media with all the bile salts at ≤ 2.5 g/L. These latter strains failed to grow in the presence of Difco bile salts at this concentration.

Effect of carbohydrate on the detection of aeromonads in mixed culture

The presence of *Aeromonas* strains had no effect

Table III. Inhibition of growth of *Aeromonas* strains by different bile salts in nutrient agar

Bile salt	Cumulative percentage of strains inhibited by bile salts at					
	2.5 g/L		5 g/L		10 g/L	
	B	N	B	N	B	N
Oxoid L55	0	0	83	86	100	100
Difco	4	29	95	100	100	100
Difco no. 3	0	0	9	50	100	100
Oxoid no. 3 L56	0	0	9	50	100	100
Sodium desoxycholate (Difco)	0	0	9	7	100	100
Sodium desoxycholate (Oxoid L57)	0	0	4	14	100	100
Sodium taurocholate (Difco)	0	0	0	0	100	100

B, strains isolated on primary isolation media with bile salts; numbers are percentages of a total of 23.

N, strains isolated on primary isolation media without bile salts; numbers are percentages of a total of 14.

on the count, size or colour of the colonies of *E. coli*, *Klebsiella* spp. or *Ent. faecalis* strains on any medium.

Mixed cultures of Aeromonas strains on MacConkey agar. Colony counts of 10 of the 12 *Aeromonas* strains in mixed culture with *E. coli* were within 10^{-1} of the expected number. Two strains each had counts of 10^{-1} – 10^{-2} of the expected number. In mixed culture experiments with *Klebsiella* sp., viable counts of eight of the *Aeromonas* strains were within 10^{-1} of the expected number. Colony counts of the remaining four strains were difficult because the presence of colonies was obscured by capsular material produced by the *Klebsiella* sp. on all plates. In mixed culture experiments with *Ent. faecalis*, counts of all 12 *Aeromonas* strains were within 10^{-1} of the expected number. Two of the *Aeromonas* strains with counts 10^{-1} of the expected number produced smaller colonies in mixed culture with *E. coli* on MacConkey agar and medium without carbohydrate. One of these *Aeromonas* strains also behaved similarly in the presence of *Klebsiella* sp. and *Ent. faecalis*. On MacConkey agar, the colonies of two non-lactose fermenting strains of *Aeromonas* in mixed culture with *E. coli* or *Klebsiella* sp. were indistinguishable from lactose fermenters when no more than 0.5 mm from the periphery of a fermenting colony. More distant *Aeromonas* colonies were clearly non-lactose fermenting.

Mixed cultures of Aeromonas strains with E. coli on bile salt media with xylose or inositol. Counts of all 12 *Aeromonas* strains in mixed culture with *E. coli* were within 10^{-1} of the expected number on inositol- and on xylose-containing media. However, six of the 12 strains produced smaller colonies on media with inositol, xylose or no carbohydrate

when in mixed culture. On inositol-containing media, all bacteria appeared to be non-fermenters but *Aeromonas* colonies were easily recognised after plates were flooded with oxidase reagent. With xylose-containing media, it was difficult or impossible to distinguish the non-xylose fermenting aeromonads from the xylose fermenting *E. coli* without using the oxidase reagent. Although the *Aeromonas* strains were clearly non-xylose fermenting in pure culture, they appeared to be fermenters in mixed culture.

Discussion

In primary media for the isolation of *Aeromonas* from faeces, the concentration of ampicillin used has usually been 10–30 mg/L.^{1,2,3,5} Most (88%) of the 101 strains of *Aeromonas* we have tested had ampicillin MIC values of ≥ 40 mg/L, and should be detected in the presence of 30 mg/L, but 12% of the strains would have failed to grow. Even on media containing ampicillin 10 mg/L, 9% of the strains would not have grown. However, the incorporation of ampicillin into media was useful in suppressing the growth of non-aeromonad faecal bacteria. Any β -lactamase production by these bacteria did not appear to change significantly the MIC of ampicillin for aeromonads in mixed culture.

The selectivity of media containing ampicillin 10 mg/L for aeromonads was little better than that of blood agar without ampicillin. If ampicillin is used as a selective agent, we recommend that it be incorporated into media at a concentration of 30 mg/L. Such a concentration may prevent the growth of some *A. caviae* strains but this might not be important because the pathogenicity of *A. caviae* is uncertain.¹⁰ It is of interest that all strains with an ampicillin MIC of < 40 mg/L were of human

origin. However, many more strains from water should be tested to assess the significance of this finding.

All the 37 *Aeromonas* strains tested by us were inhibited by bile salt at concentrations equal to or greater than those required to inhibit the control *E. coli* NCTC 10418. The bile salt concentrations required for inhibition of *Aeromonas* strains at the higher inocula were between five and 10 times the concentrations normally used. The reported failure of some *Aeromonas* strains to grow on bile salts⁵ may be related to the incorporation of carbohydrates in the media used.

Although the presence of other bacteria reduced the colony size of some *Aeromonas* strains when growing in mixed culture, this inhibition was independent of the presence of carbohydrate in the medium. The apparent failure of some *Aeromonas* strains to grow in the presence of a *Klebsiella* sp. was the outcome of *klebsiella* capsular material obscuring underlying *Aeromonas* colonies.

Whenever large numbers of fermenting bacteria were present in mixed cultures on media containing carbohydrates, the nearby colonies of *Aeromonas* often also appeared to be fermenters. The effect was more obvious when colony counts were performed by spreading the inoculum over the whole plate. Moreover, if sufficient acid is produced in the medium the oxidase test may be falsely negative.¹¹ If carbohydrates are to be used in a primary isolation medium to reveal non-fermenting aeromonads, our results suggest that it is desirable to suppress the growth of fermenting bacteria as much as possible. This may be the reason why xylose desoxycholate citrate (XDCA) agar is successful in aeromonad isolation, whereas less inhibitory media are effective only when there is either no carbohydrate present, or the contaminating flora does not ferment that present in the medium.^{2,6,12}

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