Detroit, Mich.) tubes (incubated at 22°C and 37°C, respectively), and subculture onto blood agar for oxidase testing, allows preliminary identification: *Aeromonas* spp. are ODC negative/Ox^+, *Yersinia* are Ox^- and motile at 22°C but non-motile at 37°C.

Isolation frequency from fecal specimens can be increased using alkaline peptone water[7,8] or gram-negative broth[8] as enrichment. Also, cold enrichment (4°C) in phosphate-buffered saline as recommended for *Yersinia* spp. yields additional isolates (M. Altwegg, unpublished). The isolation procedure used in our laboratory is outlined in figure 2.

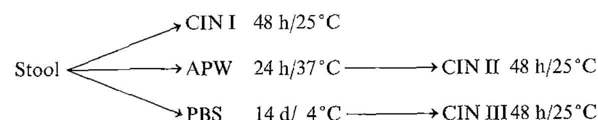
Aeromonas salmonicida

The causative agent of furunculosis in fish (salmonids) is commonly isolated on tryptic soy agar. Atypical isolates that may cause an ulcerative form of this disease in a wide variety of fish

Figure 1. Inhibition of *Aeromonas* spp. on CIN agar at 25°C at different concentrations of Cefsulodin; ○, *A. caviae*; ⊕, *A. hydrophila*; ●, *A. sobria*.

Cefsulodin concentration	Log inhibition < 1	1–< 2	2 < 3	3–< 4	4–< 5	⩾ 5
4 mg/l	○○○○○					
	○○○○○					
	⊕⊕⊕⊕⊕					
	⊕⊕⊕⊕⊕					
	●●●●●					
	●●●●	●				
15 mg/l	○○○○○	○○				
	○○○					
	⊕⊕⊕⊕⊕	⊕	⊕	⊕	⊕⊕	
	●	●	●●		●	●●●●●

Figure 2. Procedure for the simultaneous isolation of *Yersinia* and *Aeromonas* spp. from stool specimens.



species are fastidious, slow growing organisms and require media supplemented with blood or hemin[5]. Media should be incubated at 20°C–25°C, since *A. salmonicida* does not grow at 37°C.

Plesiomonas shigelloides

There are no media available that are especially designed to isolate *Plesiomonas* sp. Due to the low incidence of this organism the use of a special medium for fecal specimens may not be cost-effective. Inositol-bile salts-brilliant green (IBB) agar can be used for both *Aeromonas* and *Plesiomonas*[3,9], but direct oxidase testing may not be done for the latter. In this laboratory a suboptimal method (oxidase test with lactose-negative colonies on enteric media used for the isolation of *Salmonella* and *Shigella*) has yielded several *Plesiomonas* isolates without much effort, but strains that readily ferment lactose are missed by this procedure. Quite often fermentation of sugars by *Plesiomonas* strains is delayed on solid media. The fact that about 50% of *Plesiomonas* strains are susceptible to ampicillin[2] precludes the use of media containing this antibiotic. The use of alkaline peptone water for enrichment is controversial[9].

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Identification of *Aeromonas* in the routine laboratory

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Key words. Aeromonas; identification; taxonomy; routine.

Over the last decade there has been a markedly increased interest in the possible role of *Aeromonas* species as a cause of disease, in particular diarrhoea, in man. Unfortunately this research has often been hampered by the confused state of the taxonomy of the genus. The division of *Aeromonas* into the species *A. hydrophila, A. sobria, A. caviae,* and *A. salmonicida* which appears in Bergey's Manual of Systematic Bacteriology[6] is based on a numerical taxonomic study of 68 strains by Popoff and Véron[8] and a subsequent DNA homology study[7]. Some of Popoff and Véron's[8] phenotypic results do not correlate well with other published data. They found all the strains examined to be lysine decarboxylase negative although many others have found this to be a useful test for differentiating species of *Aeromonas*. Despite their astounding preponderance in the aquatic environment and possible role in human disease, there have been remarkably few taxonomic studies of aeromonads in recent years.

For comparison, 48 strains of *Aeromonas* were included in a taxonomic study of *Vibrio fluvialis*[2] but, although groups corresponding to those of Popoff and Véron[8] were found, it was not possible to reliably identify them. Accordingly we embarked on a numerical taxonomic study of *Aeromonas*[3]. The 163 strains studied included a selection of reference strains and isolates from a wide range of environments including human faeces, other clinical material, veterinary specimens, natural waters, seafood, other foods and the domestic and hospital environment. Duplicates of ten strains were included to enable test and operator error to be estimated. Of 195 characters examined for each strain eight were insufficiently reproducible (growth at different pHs,

methylene blue sensitivity, tyrosine degradation, acid from gluconate, growth on D-ribose, arbutin, putrescine and caprate) and 68 were constant for all strains. Resemblance (Euclidean distance) was estimated on the basis of the remaining 119 characters. Most of the strains clustered in phenons corresponding phenotypically to *A.hydrophila, A.sobria, A.caviae* and *A.salmonicida* but the type strain *A.sobria* CIP 7433 did not cluster. Møller's[5] lysine decarboxylase proved to be a useful test.

Using the phenotypic identification scheme derived from the study of Lee and Bryant[3] and a suckling mouse test for enterotoxigenicity it was found that 95% of *A.hydrophila* and 94% of *A.sobria* were enterotoxigenic but only 11% of *A.caviae*[9].

Table 1. Characters useful for identifying the *Aeromonas* phenons defined by Bryant et al.[1]

Phenon	2 *sob*	3 *hyd*	5	6	8 *cav*	45 *salm*	46 *salm*
Number of strains	55	65	9	14	76	15	7
Lysine Møller	**98**	**94**	**67**	**–**	**5**	**93**	**43**
Voges Proskauer	**76**	**+**	**89**	**93**	**–**	**7**	**57**
Gluconate oxidation	**60**	**97**	**44**	**+**	**–**	**7**	**14**
Glucose gas	**84**	**80**	**89**	**79**	**7**	**60**	**–**
Resistance to:							
0/129 150 µg/ml	91	83	89	–	99	80	29
Hydrolysis of:							
Aesculin	**12**	**96**	**+**	**–**	**79**	**64**	**–**
Elastin	**–**	**86**	**–**	**7**	**1**	**87**	**14**
Lecithin	87	89	89	+	30	93	–
Haemolysis human blood	81	98	88	43	6	36	–
Acid from:							
L-arabinose	**7**	**82**	**17**	**43**	**89**	**80**	**50**
Arbutin	**10**	**94**	**50**	**–**	**79**	**+**	**–**
Salicin	27	90	50	–	79	20	17
Sucrose	**98**	**+**	**+**	**–**	**97**	**47**	**+**
Growth on:							
Cellobiose	38	28	89	–	86	–	–
Galactose	82	95	+	93	99	–	–
Melibiose	2	–	+	–	3	–	–
Raffinose	–	–	+	–	8	–	–
Motility	96	97	67	+	82	40	29

Values are the percentage of strains possessing the character and 100% or 0% are printed as + and –, respectively. Characters in bold face are recommended for the routine identification of strains. 0/129, 2–4 diamino-6,7-di-isopropylpteridine phosphate; *sob, A.sobria; hyd, A.hydrophila; cav, A.caviae; salm, A.salmonicida.* Phenon 45 includes only strains of *A.salmonicida* subsp *salmonicida* and 46 contains *A.salmonicida* subspp. *mascoucida* and *achromogenes.* All strains are Kovács oxidase positive, fermentative in the O/F test, Møllers arginine positive, grow in 0% but not 6% NaCl and resistant to 10 µg/ml 0/129. All of the tests should be carried out at 25 to 30°C which gives more consistent results than 37°C. Møller's decarboxylases are read after 1 and 2 days incubation at 30°C and 1 and 3 days at 25°C. 0/129 resistance, haemolysis and motility are read after 1 day and other tests over 5 days.

Table 2. Tests useful for differentiating *A.caviae* from *V.fluvialis* and *V.furnissii*

	A.caviae	*V.fluvialis*	*V.furnissii*
Growth on:			
0% NaCl	+	41[a]	37
6% NaCl	–	97	95
Indole	76	48	15
Resistance to:			
0/129 150 µg[b]	99	5	5
Growth on:			
Ethanol	1	97	88
Propanol	–	+	93
Galacturonate	30	95	98

[a] Percentage of strains possessing character; [b] Discs containing 150 µg 2–4 diamino-6,7-di-isopropylpteridine phosphate.

Subsequently a study was initiated with workers in Australia (Lee and Robinson, unpublished results) to test the reliability of the identification methods in different laboratories and to test our ability to predict the suckling mouse results from the same identification tests. Data from 142 strains have been analysed. In our laboratory the probability of a test error was 1.96%. Between the laboratories two tests had poor reproducibility: growth on TCBS and growth at 43°C. With these excluded the probability of a test error between laboratories was 5.6% which is acceptable as is confirmed by our identifications which were in agreement for 97% (138) of the strains. In the UK, on the basis of the identification tests, we predicted the enterotoxigenicity result correctly for 85% (121) of the strains.

Recently data for 1091 strains of Vibrionaceae collected in five separate studies has been merged in a single data matrix and analysed in a taxonomic study[1]. The tests in each study were done using the same techniques. One study originated from the University of Maryland, USA, and the others from one laboratory in England. Included in the data set was information on 256 strains of *Aeromonas* from three separate studies[2,3,10]. A set of 142 characters was compared for cultures of 90 strains included in more than one study to estimate test error and inter and intra study variability. Thirty-one tests gave too poor a reproducibility (variance > 0.1) to be useful for the final analysis including the following which have been recommended at different times for the identification of *Aeromonas:* growth on TCBS, growth at 43°C, acid from cellobiose, sensitivity to methylene blue and growth on salicin, L-arginine and L-histidine. The majority of strains of *Aeromonas* fell into the phenons shown in table 1 which also lists the tests necessary to separate each phenon by at least three characters from each of the other phenons. Phenons 5 and 6 probably represent new species of *Aeromonas.*

The key characters of strains of the genus that are most useful for their identification are that they are Kovâcs oxidase positive, fermentative, Møller's or Thornley's arginine positive, Møller's ornithine decarboxylase negative, grow in 0% but not 6% NaCl and are resistant to the vibriostatic agent 0/129 (2–4 diamino-6,7-di-isopropylpteridine, 10 µg disc). Tests are more reliable when incubated at 25°C to 30°C than at 37°C. It is important to distinguish *Aeromonas* species, in particular *A.caviae,* from *Vibrio fluvialis* and *V.furnissii.* This is best achieved routinely by testing for the ability to grow in tryptone waters containing 0% and 6% NaCl but additional tests selected from table 2 may also be necessary. Nutritional screening of *Aeromonas* strains should be done on the medium described by Lee and Donovan[4]. The other methods used for the tests listed in tables 1 and 2 can be found in several publications[1,2,4,10].

The 'species' *A.hydrophila, A.sobria* and *A.caviae* described by Popoff[6] are each divisible by nucleic acid homology into two or more species that are currently phenotypically indistinguishable[7]. This information is used by some workers to justify lumping all strains of 'motile' *Aeromonas* together as *A.hydrophila* without further qualification. This practice should be strongly discouraged as it will only perpetuate taxonomic ignorance and hinder epidemiological, ecological and other studies. The results discussed here indicate that, although the speciation within *Aeromonas* requires further clarification, a selection of tests from table 1 can provide a reliable means of phenotypically identifying the majority of strains isolated in the routine clinical laboratory and indicating their enterotoxigenicity, as determined in suckling mice, without having to use mice.

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Serology of mesophilic *Aeromonas* spp. and *Plesiomonas shigelloides*

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Key words. O-antigen; H-antigen; mesophilic *Aeromonas; Plesiomonas shigelloides;* antigenic relationship.

Mesophilic *Aeromonas* spp. and *Plesiomonas shigelloides* have been noticed as possible etiological agents of diarrheal diseases in recent years. There have been few studies on the serology of these organisms. Ewing et al.[2] distinguished 12 O- and 9 H-antigens of *A. hydrophila,* but their serotyping system is no longer available at the present time. Recently, Leblanc et al.[5] studied O-antigens of *A. hydrophila* isolates from fish and divided them into 12 O-groups with no designation of antigenic symbols. In the serology of *P. shigelloides*, on the other hand, Quincke[6] demonstrated 16 O-groups, but his study has not yielded an applicable serogrouping system for *P. shigelloides* because his reference strains are not available. Based on 307 strains, Sakazaki and Shimada[7] (and unpublished data) recently established an antigenic scheme of mesophilic *Aeromonas* spp. Also, Shimada and Sakazaki[8,9] defined 50 O- and 17 H- antigens of *P. shigelloides* within 194 strains. In this workshop, the serology of *Aeromonas* and *Plesiomonas* is presented on the basis of our studies.

O- and H-antigens

There are two classes of antigens, O- or somatic and H- or flagellar antigens, that are important in the serotyping of *Aeromonas* and *Plesiomonas* strains. Agglutination of many strains of these organisms in O-antisera is inhibited in the living state. The inhibitory effect is inactivated by heating cell suspensions at 100°C or treating them with 50% ethanol or 1 N HCl. These findings may suggest the presence of some masking antigens. However, those masking antigens have not been taken into consideration in our serotyping system.

O- antigens for antiserum production and agglutination tests are prepared in the same manner as those used for *Salmonella* and *Escherichia coli.* It was recognized, however, that O-antisera contained varying amounts of R antibodies, which cause confusions in O-agglutination even if they are produced with complete S-form cultures. It should be emphasized, therefore, that all O-antisera of *Aeromonas* and *Plesiomonas* should be absorbed before use with the R-form culture of the same species. Slide agglutination is the method of choice.

It was demonstrated that polymeric flagellin extracted with 0.05 N HCl using the method of Fey and Suter[4] was sufficient to produce high-titered H-antisera for *Aeromonas* and *Plesiomonas.* Although *Aeromonas* and *Plesiomonas* are defined as polarly flagellated rods, the majority of strains produce lateral or peritrichous flagella in young agar cultures. It appears, however, that no difference of antigenicity is present between polar and lateral flagella. For the determination of H-antigens of *Aeromonas* and *Plesiomonas* an overnight culture of actively motile organisms in brain heart infusion is prepared, to which one adds an equal volume of PBS containing 0.1% sodium azide. The H-antigen determination is best performed by a quali-

Table 1. O-antigenic relationships between *Plesiomonas shigelloides* and *Shigella* spp.

P. shigelloides	*Shigella* spp.
11	-*S. dysenteriae* 8
17	= *S. sonnei*
22	-*S. dysenteriae* 7
23	-*S. boydii* 13

=, identical relationship; -, a, b-a, c type relationship.

Table 2. Extrageneric relationship of O-antigens of mesophilic *Aeromonas* species

Aeromonas O-antigen	O-antigen of allied species
3	-*V. cholerae* 51
4	-*V. cholerae* 59
11	-*V. cholerae* 19
13	= *P. shigelloides* 5
17	-*V. cholerae* 2
	-*V. cholerae* 9
19	= *P. shigelloides* 15
23	= *V. cholerae* 39
	= *V. fluvialis* 5
28	-*P. shigelloides* 22
29	-*P. shigelloides* 14
38	-*V. cholerae* 62

=, identical relationship; -, a, b-a, c type relationship.

Table 3. Relationships of O-antigen groups of *Plesiomonas shigelloides* between serogrouping systems of Aldova and Shimada-Sakazaki

Aldova	Shimada and Sakazaki	Aldova	Shimada and Sakazaki
1	6	16	39
2	(R)	17	17
3	27	18	12
4	(R)	19	45
5	32	20	47
6	2	21	46
7	33	22	40
8	34	23	41
9	26	24	42
10	25	25	19
11	35	26	43
12	(R)	27	44
13	36	28	7
14	37	29	4
15	38	30	18