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Enzymatic Profile and Antibiotic Sensitivity of Some *Vibrio* and *Aeromonas* Strains

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API-ZYM system and antibiotic sensitivity assay were used to characterize twelve strains of *Vibrio* and three strains of *Aeromonas*, each belonging to different species. API-ZYM showed that all tested strains exhibited negative activity for α-galactosidase, β-glucuronidase, α-mannosidase and α-flucosidase. All strains expressed positive activities for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, and naphthol AS-BI phosphohydrolase. β-glucosidase activity was a characteristic of the three *Aeromonas* strains. Also, *Aeromonas* strains as well as *V. damsela* gave very high N-acetyl β-glucosamidase activities. *Aeromonas hydrophila, A. trota* and *V. hollisae* were susceptible to all 9 tested antibiotics, and C10 and C30 were active against all tested strains. API-ZYM system and antibiotic susceptibility profile gave additional information that can help in identifying and separating very close species of *Vibrio* and *Aeromonas*.

Key words: Vibrio, Aeromonas, API-ZYM system, antibiotic susceptibility

Several species of *Vibrio* and *Aeromonas* have been recovered at high rate from seafood samples (Wong et al., 1992). Under the genus *Vibrio*, eleven species are known to be pathogenic or potentially pathogenic to humans, and six of them *(V. choleroa, V. parahaemolyticus, V. vulnificus, V. hollisae and V. furnissii)* are recognized to cause foodborne diseases (Buck, 1998; Hackney & Dicharry, 1988). *Aeromonas* species are ubiquitous in the aquatic environment, and are known as gastrointestinal pathogens (Gelbart *et al.*, 1985).

After primary screening on selective media, further biochemical and serological tests are always sought to differentiate among the members of Vibrio and Aeromonas. Enzymatic profiling of micro-organisms has been an additional and valuable method in microbial classification (Sakai *et al.*, 1993). The API-ZYM is a semi quantitative and rapid method that helps in studying the enzymatic reactions of microorganisms and has been used to study the enzymes of Gram positive and Gram negative bacteria (Velazquez & Feirtage, 1997; Kalogridou-Vassiliadou, 1992; Slade, 1992; Desjardins & Roy, 1990; Corral & Buchanan, 1990; Tzanetakis & Litopoulou-Tzanetaki, 1989; McKellar, 1986), yeast (Schmidt *et al.*, 1979; Schmidt & Lenior, 1980), and mold (Hsiao & Hsiao, 1998; Vagvology *et al.*, 1996; Bridge *et al.*, 1993).

Sensitivity to antibiotics is another tool that may be applied to differentiate among microorganisms. Moreover, certain antibiotics may be incorporated to culture media to make them more selective to resistant strains (Lentsnet *et al.*, 1980; Sakazaki *et al.*, 1986; Sloan *et al.*, 1992). In particular, several studies have focused on the antibiotic sensitivity of Vibrios (Pedersen *et al.*, 1995; Abraham *et al.*, 1997; Khin *et al.*, 1996; Koga & Takumi, 1996; Pradeep & Lakshmanaperumalsamy, 1985; Buogo *et al.*, 1980).

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The aim of this study was to characterize and compare different species of *Vibrio* and *Aeromonas* using their enzymatic profiles and antibiotic sensitivity patterns.

Materials and Methods

Vibrio hollisae ATCC 33563), *Vibrio vulnificus* (ATCC 27562), *Vibrio parahaemolyticus* (ATCC 17802), *Vibrio alginolyticus* (ATCC 17749), *Vibrio fluvialis* (ATCC 33809), *Vibrio mimicus* (ATCC 33653), and *Vibrio furnissi* (ATCC 35016) were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA). *Vibrio anguillarum, Vibrio harveyi, Vibrio cholerae, Vibrio carchariae, Vibrio damsela, Aeromonas hydrophila, Aeromonas media* and *Aeromona trota* were isolated from fish samples and identified with the aid of API 20E Kit (bioMerieux sa, France) and the Biolog Microbial Identification System (GN plates, Biolog, Inc., California, USA). All strains were maintained on tryptic soy agar (TSA) containing 3% NaCl.

Microorganisms were grown in tryptic soy broth (TSB) containing 3% NaCl for 18 h at 30°C for *V. hollisae* and *V. vulnificus* and at 36°C for the rest of the microorganisms. Each culture was then lawned onto the surface of TSA plates supplemented with 3% NaCl. After 24 h incubation period, cells were suspended in physiological saline (0.85% NaCl). The turbidity was adjusted to correspond to MacFarland No.5 standard. The API-ZYM strip (bioMerieux sa, France) contains 19 microtubes of dehydrated chromogenic enzyme substrates and one microtube of buffer as a control. Each microtube was inoculated with two drops of the bacterial suspension, and was then incubated within its humidified chamber for 4 h at 37°C. After incubation, one drop of ZYM A and one drop of ZYM B reagents were added to each well. Color reactions were allowed to develop for 5 min, after which the enzyme activity level was determined in accordance with the colour chart supplied by the manufacturer. A value in the range between 0-5 was assigned to the developed color, where zero corresponds to a negative reaction, 1, for the liberation of 5 nanomoles, 2, for 10 nanomoles, 3 for 20 nanomoles, 4 to 30 nanomoles and 5 to 40 nanomoles of reaction product. Each API-ZYM result (of one strip) was scored as described by Sakai *et al.* (1993). The 19 enzymes were divided into 7 groups, each one composed of 3 enzymes, except the last group that contained only one enzyme (a-flucosidase). When the activity of the first enzyme in each group is positive, the score is 1. When the activity of the second enzyme

Table 1. Antibiotics used, concentration per disc, symbols and interpretative creiteria of inhibition zones

Antibiotic	Concentration or µg/disc	Symbol	Inhibition zone* (diameter mm)		
			Resistant	Intermediate	Susceptible
Chloramphenicol	10	C10	-	-	-
Chloramphenicol	30	C30	≤12	13-17	≥18
Amoxycillin	25	AML25	-	-	-
Nalidixic acid	30	NA30	13	14-18	19
Doxycyclin-HC1	30	DO30	16	17-18	19
Sulfamethoxazole	25	SXT25	10	11-15	16
Polymyxin B	(300)	PB300	8	9-11	12
Colistin sulfate	25	CT25	-	-	-
Tetracycline	30	TE30	14	15-18	19
Neomycin	30	N30	12	13-16	17

*Cited from Pradeep and Lakshmanperumalaswamy, 1985

is positive, the score is 2. When the activity of the third enzyme is positive, the score is 4. No score was given for negative reaction. The scores of each group are totalled and expressed as a seven-digit number.

For antibiotic sensitivity assay, all bacterial strains were activated (24 h) in TSB supplemented with 3% NaCl, and then inoculated onto the surface of Muller Hinton agar using sterile cotton swabs. The inoculum was allowed to dry for about 15 min and ten different antibiotic discs (Table 1) were aseptically placed on the surface of the inoculated agar (3-4 discs/plate). The plates were then incubated for 24 h at 30°C for *V. hollisae* and *V. vulnificus* and 37°C for the rest of tested microorganisms. The diameter of inhibition zone of each antibiotic was measured (mm) and recorded based on the sensitivity level (resistant, intermediate and susceptible).

Results and Discussion

Data (Table 2) indicate the absence of α-galactosidase, β-glucuronidase, α-mannosidase, and α-fucosidase in all tested *Vibrio* and *Aeromonas* strains. Besides other enzymes, these four enzymes had negative activity when tested in *A. sobria* and *A. hydrophila* (Gelbart *et al.*, 1985; Waltman II *et al.*, 1982) and in *V. vulnificus* (Biosca & Amaro, 1996). Similar results had been obtained by Sakai *et al.* (1993) except that some strains of *V. anguillarum* and *A. hydrophila* showed positive reactions with β-glucuronidase. All tested strains showed positive activities in respect of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Sakai *et al.* (1993) reported similar results, but there was one strain (out of 11) of *V. anguillarum* that showed negative reaction for acid phosphatase. Similar results were also obtained with *A. hydrophila* from different sources (Waltman *et al.*, 1982) and with *V. vulnificus* (Biosca & Amaro, 1996). Fous *et al.* (1993) reported similar enzymatic activity for *V. damsela* except for cystine arylamidase that gave weak (<5 nanomoles) reaction. The results also revealed that *A. media*, *A. hydrophila*, *A. trota* and *V. damsela* had very high (40 nanomoles) activity for n-acetyl-β-glucosamidase. Also, the results indicated that the three *Aeromonas* strains were the only ones among the tested strains showing positive β-glucosidase activity. Therefore, this enzyme can be used to distinguish between the selected strains of the two genera. *V. damsela* can be separated from the other Vibrio strains by its high activity of n-acetyl-β glucosamidase.

The code number (Table 2) given to each strain based on the enzymatic profile can be easily used to separate and identify the microorganisms to the genus level. However, at the species level, most strains were well separated except in 2 cases. *V. parahaemolyticus*, *V. alginolyticus* and *V. damsela* had the same code (7623020), but can be easily separated based on the differences in their enzymatic activities, where esterase lipase and trypsin showed higher values in *V. parahaemolyticus* compared to *V. alginolyticus*, which in turn, gave higher activity of n-acetyl-β glucosamidase. On the other hand, *V. damsela* is characterized by very high (40 nanomoles), n-acetyl-β glucosamidase activity and the absence of trypsin. Similarly, *V. anguillarum* and *V. furnissii* had the same code number (7613020). *V. anguillarum*, however, exhibited stronger reaction for esterase lipase (C8) and n-acetyl-β glucosamidase, and weaker reaction for acid phosphatase compared to *V. furnissii*. The results of this study and those reported by Sakai *et al.* (1993) prove the usefulness of the API-ZYM system and the derived strain-code number in identifying closely related species. This code was only based on positive or negative results of the reaction, ignoring the level of enzyme activity. It is suggested, therefore, that identification using such procedure should take an extra step, taking into account the level of enzyme activity. As a result, different codes will be obtained for very close strains. More tests

Table 2. Enzymatic activities of *vibrios* and *Aeromonas* strains by the API-ZYM method[1]

Microorganism	Enzyme*																				Identification code
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
V. hollisae	0	5	2	4.5	0	4.5	0	0.5	0	0	0.5	2	0	0	0	2	0	0	0	0	7213400
V. vulnificus	0	5	2	3	0.5	5	1.5	1.5	0	0	2	2	0	0	0	0	0	0.5	0	0	7713020
V. parahaemolyticus	0	5	2	4	0	4	0.5	0	5	0	5	2	0	0	0	0	0	0.5	0	0	7623020
V. alginolyticus	0	5	2	2.5	0	4.5	1	0	3.5	0	5	2	0	0	0	0	0	2	0	0	7623020
V. anguillarum	0	5	1	2	0	5	0.5	2	0	0	0.5	2	0	0	0	0	0	4	0	0	7613020
V. harveyi	0	5	2	3.5	0.5	4.5	1	0.5	3.5	4	5	2	0	0	0	0	0	0.5	0	0	7773020
V. cholerae	0	5	1.5	2.5	0	5	0.75	1.5	0	0	2	2	0	0.5	0	0	0	1.5	0	0	7613520
V. carchairae	0	5	2	3	0	4	0.5	1	4	3	5	2	0	0	0	0	0	2	0	0	7673020
V. fluvialis	0	5	1.5	2.5	0.75	5	1	0.5	0	0	5	2	0	0.75	0	0	0	2	0	0	7713120
V. mimicus	0	5	3	3.5	0	5	1	3	0	0	5	2	0	1.5	0	0	0	2	0	0	7613120
V. furnissii	0	5	0.5	0.75	0	5	1	2.5	0	0	2	2	0	0	0	0	0	1.5	0	0	7613020
V. damsela	0	5	2	4.5	0	4	0	0.5	0	0	2	2	0	0	0	0	0	5	0	0	7623020
A. media	0	3	2	4.5	0.5	4	0	0.75	0.5	0	2	2	0	3	0	0	1	5	0	0	7333130
A. hydrophila	0	5	2	4	1.5	2.5	0	0.5	1	0	1.5	2	0	0	0	0.5	4.5	5	0	0	7333430
A. trota	0	3	3	5	1	3	0	0	0.5	0.5	2	2	0	3.5	0	0	2	5	0	0	7323130

[1]Enzyme activity mark 0=0 nanmole, 0.5=<5 nanmoles; 1=5 nanmoles; 2=10 nanmoles; 3=20 nanmoles, 4=30 nanomoles and 5=≥40 nanomoles.

*Enzymes 1 = control, 2 = phosphatase alkaline, 3=esterase(C4), 4=esterase lipase (C8), 5=lipase(C14), 6=leucine arylamidase,
7=valine arylamidase, 8=cystine arlamidase, 9=trypsin, 10=chemotrypsin, 11=phosphatase acid, 12=naphthol-AS-phosphohydrolase,
13=α galactosidase; 14=β galactosidase; 15=β glucuronidase; 16=β glucosidase; 17=β glucosidase; 18=N-acetyl-b glucosamidase;
19=α mannosidase; 20=α fucosidase

Data are average of two experiments

Table 3. Antibiotic susceptibility of selected *Vibro* and *Aeromona* strain by disc diffusion method

Microorganism	Inhibition zone (mm) and status*										
	C10	C30	AML25	NA30	DO30	SXT25	PB300	CT25	TE30	N30	Gr.#**
V. hollisae	32 S	38 S	36	42 S	36 S	22 S	26 S	24	36 S	18 S	1
V. vulnificus	32 S	38 S	26	38 S	38 S	14 I	0 R	0	40 S	16 I	5
V. parahemolyticus	20 S	22 S	0	20 S	20 S	12 I	0 R	0	22 S	12 R	7
V. alginolyticus	20 S	24 S	0	18 I	22 S	14 I	10 I	10	22 S	12 R	6
V. anguillarum	24 S	28 S	16	26 S	24 S	20 S	14 S	16	30 S	12 R	3
V. harveyi	22 S	28 S	0	26 S	28 S	20 S	0 R	0	28 S	14 I	4
V. cholerae	26 S	32 S	24	24 S	28 S	22 S	10 I	8	30 S	16 I	2
V. carchairae	28 S	32 S	14	30 S	28 S	20 S	0 R	0	32 S	14 I	4
V. fluvialis	24 S	26 S	16	30 S	20 S	20 S	12 S	14	24 S	12 R	3
V. mimicus	20 S	26 S	16	26 S	26 S	18 S	14 S	12	26 S	12 R	3
V. furnissii	20 S	24 S	14	16 I	18 I	16 S	12 S	12	18 I	12 R	6
V. damsela	24 S	28 S	10	28 S	26 S	20 S	8 R	8	26 S	14 I	4
A. media	38 S	44 S	26	46 S	40 S	22 S	26 S	26	46 S	14 I	2
A. hydrophila	38 S	44 S	20	46 S	38 S	30 S	28 S	28	48 S	26 S	1
A. trota	36 S	42 S	14	42 S	36 S	22 S	18 S	22	40 S	24 S	1

*S = susceptible, I = intermediate; R = resistant

** Microorganism group, where # 1 is most susceptible and # 7 is most resistant

such as gram strain, catalase and oxidase activities can be included in the identification code as well.

Table 1 shows the antibiotics used, its concentration, and the interpretative criteria of inhibition zones (Pradeep & Lakshmanperumalsamy, 1985). There were no published data nor any provided by the manufacturer on the interpretative criteria for chloramphenicol (C10), amoxycillin (AML25), and colistin sulfate (CT25). However, the interpretative criteria for C30 were used for C10 since the differences in the inhibition zones between the two concentrations were very small and the results of C10 exceeded the susceptible limit ((18 mm) for the C30 interpretative criteria. Excluding the results of AML25 and CT25, it was noticed that *A. hydrophila, A. trota,* and *V. hollisae* were susceptible to all (eight) antibiotics (Table 3). Also, C10 and C30 were very effective against all tested organisms. On the other hand, neomycin (N30) was the least effective of the tested antibiotic where 20% of the cultures were susceptible. Although, doxycyclin-HCL (D030) and tetracycline (TE30) were very effective against most of the cultures, *V. furnissii* was the only species showing intermediate resistance against these two antibiotics.

Based on the inhibitory pattern of the different tested antibiotics, the tested microorganisms can be ascendingly arranged into seven groups according to their antibiotic susceptibility: 1) *A. hydrophila, A. trota* and *V. hollisae;* 2) *A. media* and *V. cholera;* 3) *V. anguillarum, V. fluvialis* and *V. mimicus;* 4) *V. carchariae, V. damsela* and *V. harveyi;* 5) *V. vulnificus;* 6) *V. alginolyticus* and *V. furnissii;* and 7) *V. parahaemolyticus.* This grouping may change when interpretative criteria for CT25 and AML25 are determined. More antibiotics and higher number of isolates should be investigated to get a more clear picture on using this assay to separate *Vibrio* species. It appears that polymyxin B, colistin sulfate, neomycin and amoxycillin can be easily used for such purpose.

It is evident from this study that both API-ZYM system and antibiotic susceptibility assay can serve as source of valuable additional information useful in identifying and characterizing members of Vibrio and Aeromonas. A modification in the API-ZYM system data interpretation is suggested to aid in separating very close species such as: *V. parahaemolyticus, V. alginolyticus* and *V. damsela.* Antibiotic susceptibility data indicated the possible use of some antibiotics such as polymixin B, colistin sulfate, neomycin and amoxycillin in developing selective media for isolation and identification of *Vibrio* species resisting these antibiotics.

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