Asian Fisheries Science 6(1993): 11-22.
Asian Fisheries Society, Manila, Philippines

https://doi.org/10.33997/j.afs.1993.6.1.002

Numerical Taxonomy and Virulence Screening of *Aeromonas* spp. Isolated from Healthy and Epizootic Ulcerative Syndrome-Positive Fishes

J.L. TORRES
Division of Biological Sciences
College of Arts and Sciences
University of the Philippines in the Visayas
Iloilo City, Philippines

K. TAJIMA
Laboratory of Microbiology
Faculty of Fisheries
Hokkaido University
Hakodate 041, Hokkaido, Japan

M. SHARIFF
Faculty of Fisheries and Marine Science
Universiti Pertanian Malaysia
43400 Serdang, Selangor, Malaysia

Abstract

Numerical taxonomic analysis was done on 54 previously identified motile *Aeromonas* spp., including 6 reference strains. At 85% similarity value, 34 of the 54 strains were classified into 8 phenons. When the strains were screened for virulence, phenon I proved to be the most virulent.

The majority of isolates in phenon I (highly pathogenic and serologically homogenous *A. hydrophila*) came from fish exhibiting epizootic ulcerative syndrome (EUS), while those from phenon II (nonpathogenic and serologically heterogenous *A. hydrophila*) came from healthy fish. Two host species, *Channa striata* and *Clarias* sp., from which the greatest diversity of motile aeromonads was recovered, were heavily affected by EUS.

Introduction

Epizootic ulcerative syndrome (EUS), characterized by severe ulcerations of the skin and musculature, is a serious disease of freshwater fishes in Asia (Anon. 1986). It affects bottom-dwelling fishes in paddy fields, lakes and rivers, like snakeheads (*Channa*

spp.), catfishes (*Clarias* spp.), cyprinids (*Puntius* spp.) and gouramies (*Trichogaster* spp.). EUS was first observed in Indonesia in the early 1980s (Anon. 1986) and was soon reported in Malaysia (Shariff et al. 1988), the Philippines (Llobrera and Gacutan 1987), Thailand, Bangladesh, Cambodia, Laos, Burma, Papua New Guinea (Anon. 1986) and Sri Lanka (Anon. 1988) and threatened the livelihood of small-scale fishfarmers.

Aeromonas hydrophila was the organism most frequently isolated from fish exhibiting EUS (Anon. 1986). However, identification of *A. hydrophila* is not yet resolved because of wide variations in phenotypic, serological and genotypic descriptions of the organism (MacInnes et al. 1979; LeBlanc et al. 1981; Allen et al. 1983; Popoff 1984; Torres et al. 1989). *A. hydrophila* was found to be pathogenic to fish while the other two recognized motile aeromonads, *A. caviae* and *A. sobria*, were not (LeBlanc et al. 1981; Popoff 1984). Recent studies show that not all *A. hydrophila* strains are pathogenic (de Figueiredo and Plumb 1977; Torres et al. 1989), although it is generally accepted that the nonpathogenic strains are opportunistic or are secondary invaders.

Isolates (total, 54; 48 from healthy and EUS-positive fish, 6 reference) used in the study had been previously identified and screened for virulence (Torres et al. 1989). Numerical taxonomic analysis was done to determine relationships among these isolates. Results were then correlated with the results of the virulence screening.

Methods

Isolates used consisted of 41 from Malaysia, 7 from the Philippines, and 6 reference strains. Two of the six reference strains came from the American Type Culture Collection (ATCC), one from the Southeast Asian Fisheries Development Center Aquaculture Department (SEAFDEC-AQD), and three from the Laboratory of Microbiology, Faculty of Fisheries, Hokkaido University. The isolates were maintained at -86°C.

All 54 isolates were identified as *Aeromonas* spp. according to the description by Shotts and Bullock (1975). These were Gram-negative short rods, motile, oxidase positive, fermentative on O/F (glucose) medium and yellow on Rimler-Shotts medium. Species identification were confirmed using Popoff's (1984) description in

Bergey's Manual of Systematic Bacteriology (see Torres et al. 1989).

For each isolate, 102 characters were examined (Table 1), including the basic characters of motile *Aeromonas* spp. Tryptic Soy Agar (TSA) was the basal medium. Incubation was at 25°C for 48 hours except for decarboxylases and Voges-Proskauer (VP) tests which were incubated for four days. An uninoculated control medium was also tested.

Similarity values between isolates were computed using the formula (Sneath 1957):

$$S_{value}\ (\%) = N_s/(N_s + N_d) \times 100$$

where N_s was the number of similarities between isolates and N_d, the number of dissimilar characters between the isolates. A similarity matrix was made and rearranged until fixed in descending order.

Forty-eight original isolates (excluding reference strains) were screened for virulence by injecting fingerling grass carps (*Ctenopharyngodon idella*) weighing 10-20 g with 6.4×10^4 cells. Ten fingerlings per isolate were used in two trials. Prior to intraperitoneal injection, the isolates were passaged twice in fish and reisolated. Fresh isolates were then stored at -86°C until used. Only one subculture from the stock was done just prior to injection.

After seven days, the virulence of the isolates was categorized as follows: a) highly virulent, 90-100% mortality; b) weakly virulent, 50-89% mortality; and c) avirulent, 0-49% mortality. Freshly dead fish were examined for motile *Aeromonas* to satisfy Koch's postulates.

Results

Thirty-four (59%) of the 54 isolates were classified into 8 phenons with mean similarities of more than 85% (Fig. 1). This is based on 34 of the 102 characters studied (Table 1) which were variable among the isolates and thus used in computing similarity values.

Phenons I and II were identified as *A. hydrophila* with a perfect fit according to Popoff's (1984) description given in Bergey's Manual. Phenons V and VI were identified as *A. hydrophila*-like, i.e., with one or two characters differing from those of *A.*

Table 1. Biochemical tests used in characterization of motile *Aeromonas* spp. Thirty-four tests (with asterisks) gave variable results and were used in the computation of similarity values using Sneath's (1957) formula.

Colony characters:
- *Cream-white on TSA
- *Cream on TSA
- *Cream on TSA with diffused brown pigment
- Yellow on Rimler-Shotts Agar

Micromorphology:
- Gram reaction
- Rods
- Motility

Biochemical tests:
- Oxidase
- Catalase
- Oxidation/Fermentation (open tube)
- Oxidation/Fermentation (closed tube)
- Arginine dihydrolase
- *Lysine decarboxylase
- Ornithine decarboxylase
- *Alkaline slant on TSI
- *Acid butt on TSI
- Hydrogen sulfide on TSI
- *Gas on TSI
- Indole production
- *Methyl Red
- Voges-Proskauer
- Nitrate
- *Gas from glucose
- *Gas from glycerol
- *Hydrogen sulfide from cysteine

Growth on NB at:
- pH 3
- pH 4
- pH 5
- pH 6
- pH 7
- pH 8
- pH 9

Growth on NB at:
- 0.0% NaCl
- 1.0% NaCl
- 2.0% NaCl
- 3.0% NaCl
- 4.0% NaCl
- *5.0% NaCl

Growth on:
- MaConkey agar
- Cetrinamide agar
- *1% Peptone water

Degradation of:
- Urea
- Starch
- Gelatin
- *Aesculin
- *Arabinose
- Tween 20
- Tween 40
- Tween 80
- Casein
- 4% bovine blood

Utilization of:
- *Citrate
- *Potassium cyanide
- Arabinose
- *Tyrosine
- *Cysteine
- *Glycine
- *Phenylalanine
- Glutamine
- Lysine
- *Glutamic acid
- *Ornithine
- Arginine

Alkalinization of Amino Acid medium:
- *Tyrosine
- Cysteine
- *Glycine
- Phenylalanine
- Glutamine
- Lysine
- *Glutamic acid
- Ornithine
- Arginine

Sensitivity to:
- Cephaloridine (5 μg)
- Fusidic acid (10 μg)
- Novobiocin (2 μg)
- Cloxacillin (5 μg)
- Lincomycin (2 μg)
- Penicillin G (1.5 iu)
- Ampicillin (2 μg)
- *Tetracycline (10 μg)
- *Vibriostat 0/129 (10 μg)
- *Vibriostat 0/129 (150 μg)

Fermentation of:
- Maltose
- *Trehalose
- Lactose
- Sorbiton
- Dulciton
- *Rhamnose
- Inositol
- Xylose
- Adonitol
- Glycerol
- *Aesculin
- *Arabinose
- Melibiose
- Glucose
- *Salicin
- Sucrose
- Mannitol

Growth on NB at:
- 25°C
- 30°C
- 37°C

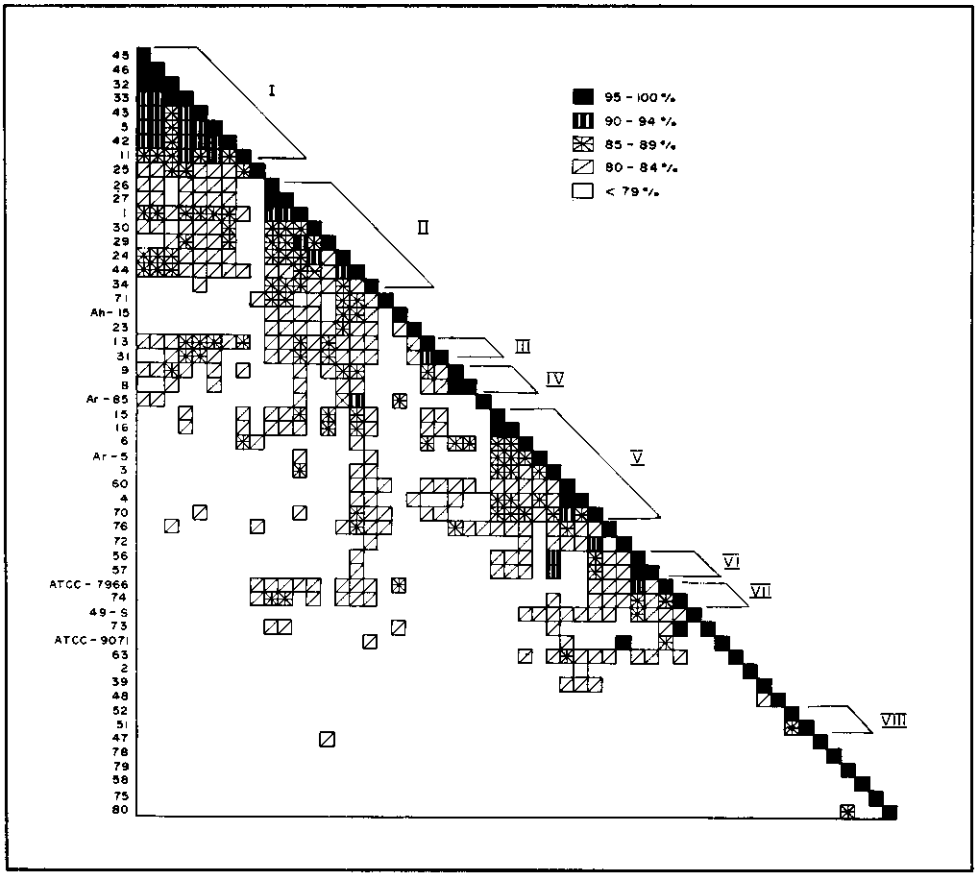

Fig. 1. Diagrammatic representation of the relationships of the 54 motile *Aeromonas* spp. based on S-values. Squares are shaded to represent similarity values such that areas with the highest S-values received the densest shading. Clusters of isolates are represented by triangular areas with darker shading.

hydrophila (except the reference isolate Ar-5, having three differing characters and which was therefore identified as unclassified *Aeromonas* sp.). Phenons III, IV and VII were identified as either *A. hydrophila* or *A. hydrophila*-like. Phenon VIII proved to be unclassified *Aeromonas* sp.

Twenty-two isolates, including the two reference strains *A. hydrophila* (ATCC 7966) and *A. sobria* (ATCC 9071), did not belong to any cluster (Fig. 2). However, the identification of reference isolates (Torres et al. 1989) sufficiently agreed with the description found in the 1984 edition of Bergey's Manual (Popoff 1984). Five of the unclustered isolates were identified as *A. hydrophila* and another five as *A. hydrophila*-like. Three isolates were identified as *A.*

sobria and four as *A. sobria*-like. One was *A. caviae* and two unclassified *Aeromonas* spp.

Five of the seven isolates recovered from Philippine fish were identified as *A. hydrophila* (isolates 42-46). Almost all were highly virulent except isolate no. 44 (avirulent) and isolate no. 43 (weakly virulent) (Fig. 2). All of these isolates came from EUS cases in Laguna de Bay. The other two, *A. caviae* and *A. sobria*-like (isolates 47 and 48, respectively), were avirulent. Isolate no. 47 came from Laguna de Bay while no. 48 was isolated from healthy *Oreochromis* sp. from the ponds of the Brackishwater Aquaculture Center, University of the Philippines in the Visayas, Iloilo.

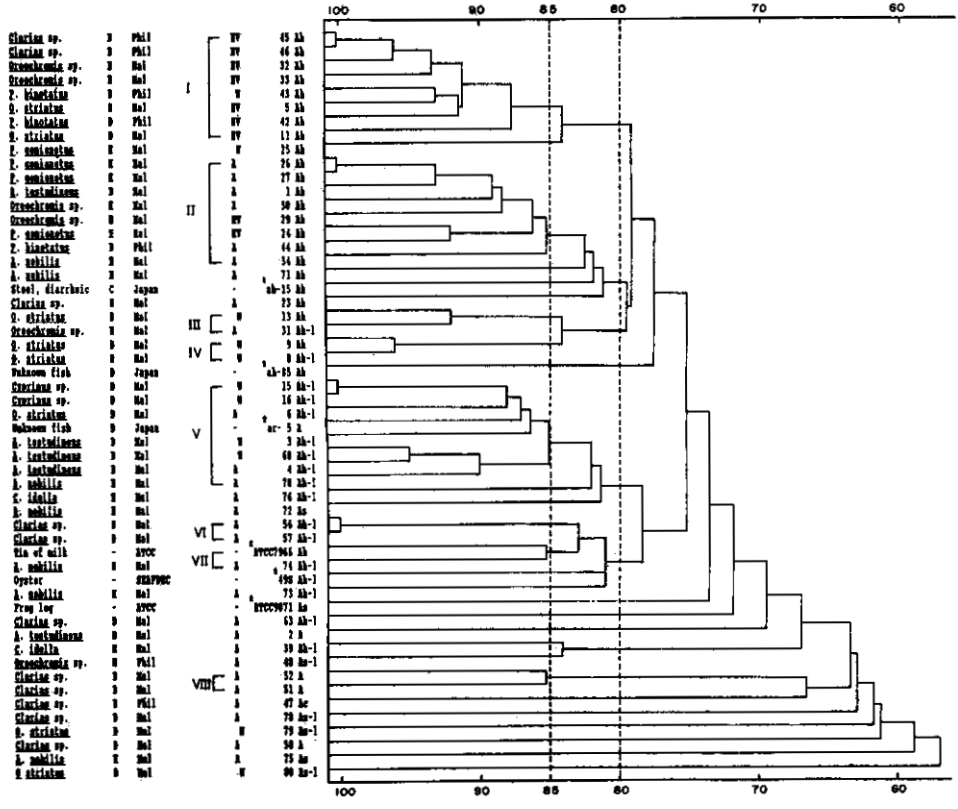

Fig. 2. Dendrogram based on average linkage of 54 strains studied. Phenons are evident above 85% phenon line. If phenon line is at 80%, more isolates will comprise a phenon. (I-VIII, phenons; Ah, *A. hydrophila*; Ah-l, *A. hydrophila*-like; As, *A. sobria*; As-l, *A. sobria*-like; Ac, *A. caviae*; D, EUS-positive fish; H, healthy fish; C, clinical isolate; ATCC, American Type Culture Collection; SEAFDEC, Southeast Asian Fisheries Development Center; Phil, Philippines; Mal, Malaysia; HV, highly virulent; W, weakly virulent; A, Avirulent.

Six of the 41 isolates from Malaysia were found to be highly virulent. Two came from EUS-positive *Channa striata* (isolates 5 and 11), two from apparently healthy imported *Oreochromis* sp. (isolates 32 and 33) and one from locally cultured apparently healthy *Oreochromis* sp. (isolate 29) and one from still apparently healthy *Puntius gonionotus* (isolate 24).

Based on virulence screening (Fig. 2), Phenon I was highly virulent with no. 43 as the only weakly virulent isolate included. Isolates belonging to phenon II are avirulent except nos. 24 and 29 (highly virulent). All the other phenons were characterized as either weakly virulent or avirulent.

Biochemically (Table 2), two isolates under phenons IV and VIII were sensitive to 150 µg of 2,4-diamino-6,7-di-isopropylpteridine (vibriostatic agent 0/129). Non-alkalinization of glutamic acid separates phenon IV from the rest of the phenons. Similarly, colony color of phenon I (cream-white) separates it from all other phenons (cream). Negative aesculin hydrolysis differentiates phenon V from the rest of the group.

Among the fish sampled, *C. striata* had the greatest diversity of *Aeromonas* isolates with four phenons and two isolates not found in the clusters (Table 3). Isolates from *Clarias* sp. belonged to three phenons with five isolates not found in the clusters. *C. striata* and *Clarias* sp. were the most heavily affected among the fish examined.

Discussion

The results demonstrate that numerical taxonomy analysis is effective in recording phenotypic variations so that isolate relationships can be studied in detail. This analysis is necessary because motile aeromonad taxonomy is ambiguous (Holder-Franklin et al. 1981; Allen et al. 1983; Popoff 1984). The results of the study have made it possible to select objectively strains for EUS-related studies, for testing antimicrobial compounds, and for vaccine production.

At 85% similarity value, motile aeromonads isolated from healthy and EUS-positive fish could be grouped into eight phenons. Goodfellow (1977) described phenons as groups of organisms that share a high degree of similarity, which means that no single feature is either essential to group membership or is sufficient to make an organism a member of the group or phenon. It is significant that

Table 2. Diagnostic characteristics of phenons I-VIII.

Characteristics	Phenons/number of isolates							
	I 8	II 8	III 2	IV 2	V 8	VI 2	VII 2	VIII 2
Lysine decarboxylase	*5/8	+	-	-	2/8	+	+	-
Simmon's citrate	-	-	+	-	3/8	-	-	1/2
Growth in peptone water	+	+	1/2	+	+	+	+	+
Nutrient broth with 5% NaCl**	-	-	-	-	-	+	1/2	-
Aesculin hydrolysis	+	+	+	+	-	+	+	+
Fermentation of salicin**	+	+	+	-	2/8	-	1/2	-
Fermentation of trehalose	+	+	+	+	+	+	1/2	+
Fermentation of aesculin	+	+	+	+	-	-	-	+
Fermentation of arabinose	-	4/8	-	-	-	-	+	-
Tetracycline sensitivity	+	5/8	+	+	+	+	1/2	+
0/129 vibriostat (150 μg)	-	-	-	1/2	-	-	-	1/2
Alkalinization of tyrosine	5/8	4/8	1/2	-	-	-	-	-
Alkalinization of glycine	5/8	4/8	+	+	-	-	1/2	-
Alkalinization of glutamic acid	+	4/8	+	-	+	+	+	+
Cream-white colony color	+	-	-	-	-	-	-	-
Cream colony color	-	+	+	+	+	+	+	+
Methyl red	-	4/8	-	+	4/8	-	-	+

+, 85-100% positive reaction; -, 85-100% negative reaction.
*No. of isolates with positive results/total no. of isolates tested.
**Excluded in the consideration for identification of *A. hydrophila*.

Table 3. Number of isolates according to conditions of fish species.

Fish species (number of isolates)	Phenons/number of isolates								
	I 8	II 8	III 2	IV 2	V 8	VI 2	VII 2	VIII 2	Others 22
EUS-positive									
Anabas testudineus (5)	0	1	0	0	3	0	0	0	1
Channa striata (8)	2	0	1	2	1	0	0	0	2
Cyprinus sp. (2)	0	0	0	0	2	0	0	0	0
Puntius binotatus (3)	2	1	0	0	0	0	0	0	0
Clarias sp. (11)	2	0	0	0	0	2	0	2	5
Healthy									
Puntius binotatus (4)	0	3	0	0	0	0	0	0	1
Oreochromis sp. (6)	2	2	1	0	0	0	0	0	1
Aristichthys nobilis (7)	0	1	0	0	1	0	1	0	4
Ctenopharyngodon idella (2)	0	0	0	0	0	0	0	0	2
Others									
Reference strains (6)	0	0	0	0	1	0	1	0	4

almost all of the *A. hydrophila* isolates were grouped in the higher hierarchy, followed by the middle *A. hydrophila*-like group, and that the *A. sobria* complex (with *A. caviae*) grouped in the lower hierarchy. While this finding is generally consistent with the present recognition of three-species taxonomy of motile *Aeromonas,* the positions of *A. hydrophila*-like, *A. sobria*-like and *A. caviae* remain unclear. The problem is aggravated because the characteristics of the isolates were distinct from the type strains. It is possible that some of these represent new species. On the other hand, unclustered isolates were the sole representatives of their respective groups/phenons. However, if overall similarity level is decreased to 80%, reference isolates would clearly belong to a specific phenon and a greater number of strains would comprise a phenon.

The ATCC 7966 (*A. hydrophila*) may not be an ideal choice for a reference strain. ATCC 7966, a member of phenon VII, reacted atypically in two biochemical characters (fermentation of salicin and growth in nutrient broth with 5% NaCl), diagnostic for *A. hydrophila* (see Torres et al. 1989). This strain was isolated from a tin of milk and not in fish/aquatic environments which is their natural habitat (see Popoff 1984).

Phenon I (highly virulent with cream-white colored colonies) differed from phenon II (avirulent with cream colored colonies) in virulence and colony color (Table 2). This is the first report suggesting correlation between virulence and colony color among *A. hydrophila* isolates. This finding must be viewed with caution, however, because color determination of colonies on TSA is subjective. It is likely that phenon I shares a common protein, detectable as cream-white colony color in TSA, not found in other strains.

Serological analysis among the isolates (Torres et al. 1992) indicated that phenon I is serologically homogenous and phenon II is not. Thus, phenon I is referred to as *A. hydrophila* serotype I and phenon II, *A. hydrophila* serotype II. This finding is significant in the selection of isolates for use in vaccine production.

Previous reports correlating biochemical reactions, i.e., VP, gas production, and fermentation of arabinose (Cumberbatch et al. 1979; Kaper et al. 1981; Burke et al. 1984) with virulence, are uncertain in light of Popoff's (1984) new classification of *A. hydrophila* where all of the above mentioned tests were positive. The lysine decarboxylase (LDC) test has also been correlated with virulence (Burke et al. 1984). However, this study found no correlation between LDC and virulence.

Two isolates under phenons IV and VIII were observed sensitive to vibriostatic agent 0/129. Although this vibriostatic agent is used to separate *Vibrio* and *Aeromonas* (Popoff 1984), Takahashi et al. (1986) reported isolation of vibriostatic agent-sensitive *A. hydrophila* from diseased ayu (*Plecoglossus altivelis*).

The majority of isolates in phenon I (pathogenic and serologically homogenous *A. hydrophila*) came from EUS-positive fish while those from phenon II (nonpathogenic and serologically heterogenous *A. hydrophila*) came from healthy fish. The results of this study agree with previous reports of differences in virulence of *A. hydrophila* isolates (de Figueiredo and Plumb 1977; Mittal et al. 1980; Hsu et al. 1981).

The results of this study confirm that some strains of *A. hydrophila* are pathogenic and others are not. Furthermore, in consonance with the results of Lallier et al. (1980) using rainbow trout (*Oncorhynchus mykiss*), *A. hydrophila* strains were found to be more virulent than *A. sobria* and *A. caviae* to grass carp. Additionally, seven phenons were found in EUS-positive fish and five phenons in healthy fish. It is apparent that fish most affected by EUS carry a variety of motile *Aeromonas* spp. strains.

Phenon V isolates came from diverse sources; all but one came from EUS cases. This phenon cannot be identified up to species level of motile aeromonads using the criteria of Popoff (1984) found in Bergey's Manual. Nevertheless, this phenon clearly belongs to the motile aeromonads using the same criteria of Popoff (1984). Because of its distinct phenotypic characters, this phenon is a candidate for a new species of motile *Aeromonas*. It is differentiated from the rest of the phenons by its inability to hydrolyze aesculin, and from *A. hydrophila* by its inability to hydrolyze aesculin and ferment arabinose. Four strains under this phenon displayed virulence.

Other phenons have limited representatives. Thus, identification/classification is difficult to ascertain, although they clearly belong to motile *Aeromonas* spp.

Additional biochemical tests not routinely done should be added to strengthen further the characterization of *A. hydrophila, A. sobria* and *A. caviae*. Genotypic studies must be done to permit establishment of new species, particularly for those isolates displaying virulence. These need urgent attention as the results may have important implications in the study of EUS.

Acknowledgements

We thank the International Development Research Centre (IDRC) of Canada for research funding; and Prof. A.T. Law and Prof. T. Kimura for valuable discussions.

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Manuscript received 21 November 1989; revised ms received 31 July 1992; accepted 12 November 1992.