Immunofluorescence of *Aeromonas hydrophila* as measured by fluorescence photometric microscopy[1]

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Strain-specific fluorescent antibodies were prepared for three isolates of *Aeromonas hydrophila*. These antisera were reacted with 255 other *A. hydrophila* isolates, and their immunofluorescence was measured quantitatively by photometric fluorescence microscopy. Only 27.5% of the isolates reacted with the prepared antisera which indicated that other undetected serogroups are present. Statistical analyses indicated that the *A. hydrophila* isolated from aquatic habitats were distinct from the other isolates as measured by immunofluorescence.

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Des antisérums fluorescents spécifiques de souche ont été préparés contre trois souches d'*Aeromonas hydrophila*. Ces sérums ont été vérifiés contre 255 autres isolements d'*A. hydrophila* et la réaction d'immunofluorescence a été mesurée quantitativement par microscopie en fluorescence photométrique. Seulement 27.5% des souches ont réagi avec les antisérums, ce qui suggère la présence d'autres sérogroupes. L'analyse statistique des mesures faites par immunofluorescence indique que les souches d'*A. hydrophila* recueillies de milieux aquatiques se distinguent des autres souches isolées.

[Traduit par le journal]

Introduction

The use of immunofluorescence has significantly expanded identification, enumeration, and localization of specific microorganisms in both aquatic and terrestrial habitats (Fliermans *et al.* 1974; Fliermans and Schmidt 1975; Schmidt *et al.* 1968; Schmidt 1973). There are two basic approaches to the fluorescent antibody technique: direct and indirect. The direct approach relies on the production of an antibody against a given antigen and the coupling of that antibody with a fluorescent dye forming the fluorescent antibody (FA) complex. The antigen is then directly reacted with the FA under a specific set of conditions and observed qualitatively by fluorescence microscopy. The fluorescing antigen–antibody complex is scored by a subjective measurement of fluorescent intensity in relation to background, and (or) comparing the staining of an unknown antigen to a homologous known system.

The indirect approach is a two-stage system whereby the antigen is first complexed with a homologous antibody. This antigen–antibody complex is then reacted with a fluorescent labeled antiglobulin, homologous to the unlabeled antibody used in the initial reaction. The indirect procedure allows the identification of an unknown antigen as well as the titer of antibodies in an unknown serum. Both approaches require extensive controls to evaluate properly the immunofluorescence reaction (Schmidt 1973).

Immunofluorescence data derived from ecological studies have not been quantified other than by subjectively comparing specimen fluorescence with that of the background and (or) other heterologous specimens (Bohlool and Schmidt 1970; Pugsley and Evison 1974*a*, 1974*b*; Bohlool and Brock 1974). Such measurements of immunofluorescence do not allow the direct assessment of similarity among fluorescing bacteria. Because FA specificity may vary depending on the antigenicity of the microorganism (Fliermans *et al.* 1974; Schmidt 1973), antigenically related microor-

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ganisms would be separated microscopically only when measured quantitatively.

In this paper the preparation of antibodies for various strains of *Aeromonas hydrophila* is described, the strain specificity of the antibodies by direct immunofluorescence is demonstrated, and the utilization of fluorescence photometry to measure differences and similarities among strains isolated from a variety of natural habitats is described.

Materials and methods

Cultures

Stock cultures of *A. hydrophila* 7966, 19570; *A. liquefaciens* 14715; *A. proteolytica* 15338; *A. salmonicida* 14174; *A. punctata* 14486, 15468; *A. formicans* 13137; and *A. anaerogenes* 15467 were originally obtained from the American Type Culture Collection (ATCC). All axenic cultures were routinely grown in nutrient broth shake cultures at 37°C. Samples from aquatic habitats were filtered onto sterile membrane filters (0.45 μm; Millipore Corp., Bedford, MA) and placed on a medium (RS) differential and selective for *A. hydrophila* (Shotts and Rimler 1973; Hazen *et al.* 1978). Samples (e.g., fish or alligator tissue, aquatic plants, and sediments) were plated directly on RS medium and incubated at 37°C for 20 h. Isolates were confirmed as *A. hydrophila* using the profile recognition system for Enterobacteriaceae (API 20E) as described by Smith *et al.* (1972). Over 350 different isolates of *A. hydrophila* from across the United States and from Par Pond (Fliermans *et al.* 1977) have been tested for differences in biochemistry, antibiotic sensitivity, and immunofluorescence relations (Hazen *et al.* 1978).

Fluorescent antibody preparations

Cloned stock culture isolates of *A. hydrophila* 7966 and axenic field isolates from various habitats (*A. hydrophila* HDW1: water; *A. hydrophila* B323L: lesion of largemouth bass; *A. hydrophila* GLN1: lung of an alligator; Gorden *et al.* 1979) were grown to a density of about 10^8 cells/mL for the preparation of individual antisera. Cells were harvested by centrifugation, washed three times in physiological saline, and resuspended in phosphate buffer (pH 7.2) to a Klett turbidity of 180 (green filter). The cell suspension was then heated in a boiling water bath for 60 min to inactivate flagellar antigens. Merthiolate (1:100 000 final concentration) was added to the antigen suspension, and the suspension was injected intravenously into three New Zealand rabbits. After injections, test bleedings were made, and antibody levels were determined by tube agglutination. When agglutinating antibody titers for the homologous antigen were greater than 1:2560, antisera were harvested by cardiac puncture, pooled from among the triplicate rabbits, fractionated, and conjugated to fluorescein isothiocyanate isomer I (BBL, Cockeysville, MD). All staining procedures were as described previously by Schmidt *et al.* 1968.

Cross-reactions between the various strains of *A. hydrophila* were measured using immunofluorescence reactions and tube agglutination titers. No absorption techniques (Fliermans *et al.* 1974) were used to increase the strain specificity of any of the *A. hydrophila* antisera. A large number of isolates lacking the colonial morphology and biochemical characteristics of *A. hydrophila* when grown on RS medium were reacted with the prepared FA to determine whether bacteria other than *Aeromonas* spp. demonstrated any cross-reactions. Those reactions were negative.

Photometric fluorescence microscopy

The basic fluorescent microscopy equipment used in these experiments was a Zeiss Photomicroscope III equipped with an epifluorescence condenser III RS with both barrier and exciter filters optimized for the excitation and emission of fluorescein isothiocyanate (FITC). The light path was from the high pressure 200-W mercury arc lamp through a KG1 heat-absorbing filter and a BG-38 red suppressor filter. Two exciter filters, LP455 and KP500, allowed transmission of light from 455 to 500 μm, since the maximum wavelength for absorption of FITC is 490 μm (Nairn 1969). Additionally, the LP510 chromatic filter transmitted light above 510 μm through an LP520 barrier filter to decrease background and enhance the maximum fluorescence of FITC at 520 μm (Nairn 1969). Planachromat oil immersion objectives 40 ×/1.0 and 100×/1.25 with variable numerical apertures were used for viewing specimens. Color photomicrographs were taken with GAF film ASA 200-24 Din with a 30-s exposure. The homologous reactions of the three antisera used in this study are shown in Fig. 1.

Immunofluorescence was measured quantitatively with a Zeiss Photometer-Indicator MPM01 connected to a photomultiplier cell capable of measuring fluorescence from a 0.5-μm-diameter particle. Both background fluorescence and FITC-fluorescing specimens were measured with a 1-μm-diameter probe. Since FITC-fluorescing specimens fade during excitation, only one specimen was measured in each microscopic field. For each determination 50 fields were measured. Photometric indicator settings were as follows: damping control for averaging fluorescence display by digital readout, 0.5 (40 individual readings are averaged during 0.5 s); mode selector was in the percentage of transmission mode; high voltage selector was optimized for sensitivity at a setting of 4 (approximately 850 V); gain control for amplifier sensitivity was set at 100; and the zero suppression and concentration factor dials were in the off position. Individual cells were measured by first measuring the fluorescence intensity of the bacterial cell and dividing it by the background intensity. Simultaneously, cellular intensity was measured by conventional subjective methods (LaBrec *et al.* 1959; Nairn 1969).

Results

Specificity of prepared antibodies

A series of experiments were conducted to determine the relationship between subjective fluorescence and photometric fluorescence measurements. The data are shown in Table 1. Fluorescent antibodies prepared for various species of bacteria with a broad range of specificity were reacted with a number of homologous and heterologous bacteria to obtain immunofluorescent reactions of varying intensities. The intensity of the fluorescence was measured both subjectively and quantitatively (photometric microscopy). The various antigen–antibody complexes demonstrated various degrees of fluorescence, and the distinction among the weak fluorescence (± to 2+) was clear photometrically but not always subjectively. Cells which reacted well with the FA (3+ and 4+) were readily distinguished from the weaker reactions and from each other. An additionally brighter level of fluorescence (5+) was seen only photometrically with some of the cells. Although the number of determinations of the brighter fluorescence were fewer ($n = 41$), the photometric readings demonstrated good separation from the weaker fluorescence.

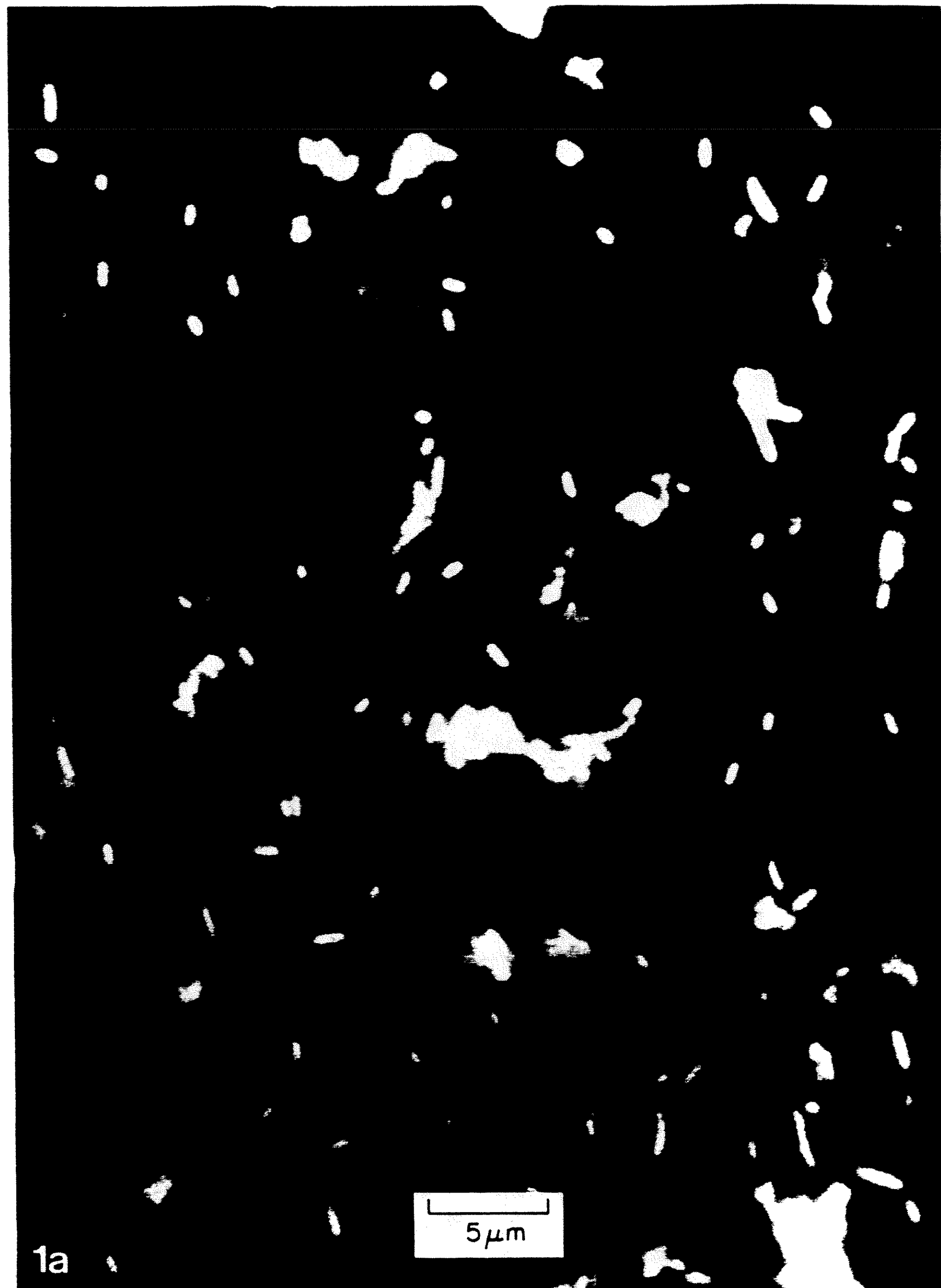

FIG. 1*a*. Strain-specific *A. hydrophila* HDW1 fluorescent antibody staining the homologous system.

The data in Table 2 indicate that initial tests with the unconjugated antisera from the various strains of *A. hydrophila* gave good agglutination titers and each conjugated antiserum demonstrated excellent immunofluorescence staining reaction with its homologous antigen. However, antisera prepared for *A. hydrophila* 7966, the neotype strain from ATCC, demonstrated cross-reactivity against the tested heterologous antigens both for tube agglutination and immunofluorescence, and did not react with most isolates from water; redsore infected largemouth bass; nor tissue from necropsied alligators (Fliermans, unpublished results). Thus, three additional isolates of *A. hydrophila* (HDW1, B323L and GLN1, respectively) were selected as representatives of the *A. hydrophila* in the studied

FIG. 1*b*. Strain-specific *A. hydrophila* B323L fluorescent antibody staining the homologous system.

habitats. Fluorescent antibodies were prepared against each of these isolates and demonstrated a high degree of specificity to the homologous system with minimal cross-reactivity.

Numerous *A. hydrophila* isolates (255) were tested against the three prepared antibodies, and the serological relationships were compared by photometric microscopy (Table 3). The individual samples and immunofluorescence reactions for each antigen–antibody complex have been condensed, so that only the data for the number of isolates tested, the mean, range, and standard error

FIG. 1c. Strain-specific *A. hydrophila* GLN1 fluorescent antibody staining the homologous system.

of the reaction, are reported for each group of isolates. Analysis of variance and Student–Newman–Keul multiple-range tests indicated that the mean of the immunofluorescence reaction for *A. hydrophila* from freshwater habitats stained with HDW1-FA was significantly different (0.05 level) from all other isolates. Additionally, all other groups of isolates did not react significantly with FA prepared against B323L of GLN1. The data in Table 3 further indicate that in each group of isolates, a wide range of immunofluorescent reactions was observed. While 20.0% (51/255) of the isolates

TABLE 1. Measurements of immunofluorescence using photometric microscopy

Subjective fluorescence reading	No. of determinations	Quantitative photometer reading
±	52	1.41±0.04[a]
1+	53	1.99±0.05
2+	57	2.86±0.06
3+	53	5.03±0.10
4+	58	9.82±0.13
5+	41	20.1±0.44

[a]One standard error.

were highly reactive with a single FA, only H2ML3, K1W4 (water isolates), and B603L (bass intestinal isolate) reacted strongly with all three prepared fluorescent antibodies. Seventy-three percent (185/255) of the isolates did not react strongly with any of the prepared antisera.

Discussion

To determine if a relationship existed between subjective and objective fluorescence measurements, immunofluorescence reactions were quantified for 314 fluorescing antigen–antibody complexes using a variety of conjugated sera. These sera were prepared in rabbits from seven different species of bacteria. The specificity of the sera differ in both their heterologous and homologous staining characteristics (Table 1). The immunofluorescence reactions were grouped initially by subjective observations and then by photometric readings. The antigen–antibody complexes were placed in one of six reactive groups and such groupings were confirmed by quantitative photometry. An additional group, 5+, was observed only by photometry and did not appear brighter than the 4+ grouping using subjective measurements. Standard errors among the groups were low and allowed distinctions between microorganisms having the various degrees of fluorescence.

A major problem in characterizing a large number of isolates as in Table 3 is the relationship of those isolates to the habitat from which they were obtained. We arbitrarily designated HDW1 as a serotype for a water isolate, B323L as a serotype from lesions of a largemouth bass, and GLN1 as the serotype for *A. hydrophila* associated with the American alligator. The chosen *A. hydrophila* may be transient in the habitat and may have been expelled (e.g., from a bass or alligator) before its isolation. Therefore, the three isolates chosen as

TABLE 2. Tube agglutination titers and quantitative fluorescent antibody test reactions of selected strains of *Aeromonas hydrophila*

Antigen	Antiserum	Agglutination titer	Immunofluorescence: Subjective reaction	Immunofluorescence: Quantitative reaction[a]
A. hydrophila 7966	7966	>2560	4+	9.80
	HDW1	1280	2–3+	4.40
	B323L	160	±	1.00
	GLN1	640	2+	3.10
	Normal sera	<20	neg	1.00
A. hydrophila HDW1	7966	320	1+	1.80
	HDW1	>2560	4+	10.30
	B323L	80	Neg.	1.00
	GLN1	160	+	1.20
	Normal sera	<20	Neg.	1.00
A. hydrophila B323L	7966	80	±	1.10
	HDW1	80	Neg.	1.00
	B323L	>2560	4+	8.90
	GLN1	320	1+	2.00
	Normal sera	<20	Neg.	1.00
A. hydrophila GLN1	7966	320	1+	1.10
	HDW1	160	±	1.30
	B323L	320	1+	2.00
	GLN1	>2560	4+	10.70
	Normal sera	<20	Neg.	1.00

[a]Ratio of cell fluorescence to background fluorescence.

TABLE 3. Composite of *A. hydrophila* isolates reacted with three separate specific fluorescent antibodies

Isolates		Antisera		
Source	No. tested	HDW1	B323L	GLN1
		Photometric reading		
ATCC	9	2.11 ± 0.75[a] (1.11–5.00)[b]	1.77 ± 0.24 (1.00–3.20)	1.99 ± 0.40 (1.00–4.35)
Surface slime largemouth bass	33	1.96 ± 0.14 (1.05–5.44)	2.72 ± 0.51 (1.00–13.70)	1.81 ± 0.24 (1.00–6.00)
Intestine largemouth bass	49	2.91 ± 0.40 (1.07–19.00)	2.58 ± 0.31 (1.00–10.43)	2.61 ± 0.42 (1.00–13.00)
Lesion on largemouth bass	35	2.27 ± 0.39 (1.09–11.50)	2.27 ± 0.25 (1.00–6.63)	2.34 ± 0.31 (1.00–8.75)
American alligator	21	2.20 ± 0.50 (1.00–11.40)	3.13 ± 0.56 (1.05–10.31)	3.86 ± 0.92 (1.02–18.00)
Freshwater	108	5.31 ± 0.41[c] (1.03–24.29)	2.62 ± 0.23 (1.00–17.18)	2.19 ± 0.22 (1.00–14.20)

[a]Mean ± standard error.
[b]Range of fluorescence measured.
[c]Antisera significantly different from isolates at 0.05 level using Student–Newman–Keul test.

reference samples from a given habitat may not be, in fact, an integral part of that habitat. This is further supported by the data in Table 3 which demonstrate that each group of isolates has a wide range of fluorescence for a given FA, suggesting that the antigenic makeup within the group is quite diverse in its relationship with the designated serotype. All three serotype isolates demonstrated similar biochemical properties as measured by API-20 (Anatabs, Inc.) and similar antibiotic sensitivity. Single linkage cluster analyses of the biochemical and antibiotic characteristics demonstrated a 90% similarity for all the isolates tested (T. C. Hazen. 1979. Ph.D. Thesis, Wake Forest University, Winston-Salem, NC).

Previous studies by DeFigueiredo and Plumb (1977) on the virulence of *A. hydrophila* strains to catfish (*Ictalurus punctatus*) suggested that a variety of strains may exist in nature. Although only nine different strains of *A. hydrophila* were tested, those isolated from water were significantly less virulent than strains from diseased fish, even though all nine isolates were biochemically identical.

Our data expand those of DeFigueiredo and Plumb (1977) and demonstrate that at least three serological groups of *A. hydrophila* do occur and appear simultaneously in natural habitats. Additionally these serogroups can be differentiated quantitatively by immunofluorescence even when biochemical or antibiotic sensitivity is the same. Because 72.5% of the *A. hydrophila* isolates did not react with any of the prepared antisera, serogroups other than those described are probably present in the natural habitats. The data further indicate that the large number of water isolates are significantly different in their antigenic properties from the isolates taken from bass or the American alligator. The utilization of fluorescence photometric microscopy allows the quantitative screening of isolates to determine similarity in antigenic composition with respect to a given FA.

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