

Frequency distributions in rainbow trout populations of absorbance values from an ELISA for *Vibrio anguillarum* antibodies

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ABSTRACT: An enzyme-linked immunosorbent assay (ELISA) was used to measure *Vibrio anguillarum* serotype 1 antibody activity in sera collected from negative reference and from cohort-vaccinated and naturally exposed farmed rainbow trout *Salmo gairdneri* populations. Frequency histograms of each sample's absorbance values (OD) were prepared. Statistical distributions were fit to the data from the reference groups in order to determine seronegative, suspect and seropositive cut-off levels. The OD of reference populations followed either a normal or a log-normal distribution. The frequency histograms for the OD of vaccinated groups had a wide range and clustered around the median. From 57 to 88 % of vaccinated trout were classified as seropositive. The OD of naturally exposed groups were strongly skewed to the right, indicating elevated antibody production in at least part of the population (18 to 36 % suspect or seropositive).

INTRODUCTION

The enzyme-linked immunosorbent assay (ELISA) for circulating specific antibody activity, because of its objective nature and rationalized design, may become a useful tool in seroepidemiologic studies of fish disease. However, high background levels of antibodies in commercially reared and wild fish could limit its applicability (Hamilton et al. 1987). Difficulties in distinguishing between low levels of specific reactivity and non-specific reactivity is a general, and critical, problem in the development of ELISA (Taylor et al. 1978, York et al. 1983). The probability of false positive results due to non-specific reactions can be reduced by using a relatively high positive-negative cut-off level; this will, however, increase the probability of false negative results.

Various methods have been used to determine cut-off levels for absorbance values (OD) to detect fish antibodies by ELISA. Some workers have used arbitrarily determined fixed levels (Bortz et al. 1984, Cossarini-Dunier 1985). It is preferable to base cut-off levels on the frequency distribution of the OD of negative sera (van Loon et al. 1981). Kodoma et al. (1985) and Thorburn (1986) set the minimum positive OD at the mean OD of samples collected from non-exposed populations

plus 2 or 3 standard deviations of the mean. This method, however, is possibly invalid because it assumes the OD in the negative population are normally distributed.

An ELISA to detect *Vibrio anguillarum* serotype 1 antibody in rainbow trout *Salmo gairdneri* has been developed in Sweden, and has been used to investigate the immunogenicity of vaccines (Thuvander et al. 1987) and to follow the immune response of vaccinated and non-vaccinated fish in field trials (Thorburn 1986). The objective of the present study was to determine the frequency distributions of OD provided by this ELISA system in selected populations of rainbow trout. The reason for investigating these distributions was 2-fold. First, examination of the frequency distributions of OD may be quite useful in seroepidemiologic studies for comparison of samples from 2 or more populations (Woodruff et al. 1978). Disparities in the forms and percentile markers of distributions may help elucidate subtle population differences which are not readily apparent from summary statistics. Second, determination of the frequency distribution of OD in known specific-pathogen-free and known exposed or immunized populations is the best way to accurately estimate the true sensitivity and specificity of an ELISA.

MATERIALS AND METHODS

Fish. The sampled rainbow trout populations originated from winter 1986 hatches on 3 commercial freshwater hatcheries (F1, F2, and F3) in southern/central Sweden. Trout from each hatchery were transported to 1 of 3 brackish water (B1 and B3) or saltwater (S2) net-pen farms. All trout designated for B1 and S2 were intra-peritoneally (IP) vaccinated prior to transport in the spring of 1987. The negative reference (non-vaccinated) populations from F1 and F2 (F1REF and F2REF) were full-siblings of their vaccinated cohorts (B1VACC and F/S2VACC, respectively). Trout from F3 were transported to B3 in autumn 1986. Most of these fish were IP-vaccinated on site (B3) the following spring, 1987 (B3VACC); 1 pen of non-IP-vaccinated fish was retained (B3REF). The F3 and B3 populations had been dip-vaccinated at 3 to 5 g during summer 1986, 9 and 12 mo respectively prior to the first sampling occasion. The nature of the relationship between F3REF and the B3 population was not known. They had, however, been hatched and raised, until B3's transport, on the same small hatchery.

All vaccinations were done with commercially available formalin-killed bivalent vibriosis bacterins.

Blood samples. Blood samples were collected from the caudal vein, at the times and locations listed in Table 1. After clotting overnight at 4°C, samples were centrifuged at 300 × g for 12 min. The sera were stored at -20°C until tested with the ELISA.

ELISA. Sera from F1, B1, F2 and S2 were assayed in

one run (Run 1) and sera from F3 and B3 in a second (Run 2).

Polystyrene microtitre plates (Linbro, Flow) were coated with *Vibrio anguillarum* serotype 1 (according to the system of Sørensen & Larsen 1986) O-antigen. The plates were incubated overnight at room temperature, then washed 3 times in washing fluid. Subsequently 100 µl of fish serum (diluted 1:100 in PBS with 0.5% Tween 20) was added to each of the wells. Duplicate wells were used for each serum sample. After incubation at 4°C for 20 h, the plates were washed 3 times and rabbit anti-trout immunoglobins (IgM) conjugated to horse radish peroxidase (diluted in PBS containing 0.5% Tween 20 and 1% horse serum) was added to each well. The plates were incubated at 4°C for 15 to 20 h. After washing, trimethylbenzidine (TMB, Sigma) in acetate buffer with H₂O₂ was added to the microtitre wells. The reaction was stopped with H₂SO₄ and the optical density was read spectrophotometrically at 450 nm. This method is described in detail by Thuvander et al. (1987).

Control sera. Control positive and negative sera were included in triplicate on every plate. These pooled sera originated from rainbow trout reared in flowing pathogen-free ground water aquaria; positive trout had been repeatedly injected with a formalin-killed vibriosis bacterin. In addition 2 high OD (B3VACC) sera and 2 low OD (F1REF and F3REF) sera were included, in duplicate, on every plate.

Statistical analysis. The average OD of the 7 negative wells (\overline{OD}_{neg}) on each plate was determined.

Table 1. *Salmo gairdneri*. Summary of sampled populations and sampling scheme for determination of frequency distributions of absorbance values from an ELISA for *Vibrio anguillarum* antibodies in rainbow trout.

Source ^a	Group	IP vaccination date	Site ^a	Sampling procedure			n
				Date	Water temp. (°C)	Fish size (g)	
F1	F1REF	No	F1	Jun 87	12	70	65
	B1VACC	May 87	B1	Jun 87	15	NA	42
F2	F2REF	No	F2	Apr 87	3	200	64
	F2VACC	Nov 86	F2	Apr 87	3	200	32
	S2VACC	Nov 86	S2	Aug 87	15	NA	31
F3	F3REF	No ^b	F3	May 87	7	35	75
	B3REF1	No ^b	B3	Jun 87	7	65	32
	B3REF2	No ^b	B3	Jul 87	17	NA	36
	B3REF3	No ^b	B3	Sep 87	11	NA	33
	B3VACC2	Jun 87 ^b	B3	Jul 87	17	NA	37
	B3VACC3	Jun 87 ^b	B3	Sep 87	11	NA	10

^a F1, F2 and F3 were commercial freshwater hatcheries located in southern/central Sweden. B1, S2 and B3 were commercial brackish/salt water net-pen farms on the south (B1), west (S2) and east (B3) coast of Sweden

^b F3 and B3 groups were dip-vaccinated at an average weight of 3 to 5 g in August (F3) and June (B3) 1986
NA: not available

Within each run, the observed OD from each plate were corrected by subtracting the difference between the plate's \overline{OD}_{neg} and the lowest within-run \overline{OD}_{neg} . For between-run statistical comparisons, the difference between the runs' average OD of the F3REF control serum was subtracted from all OD in the run with the higher average. The averages of the duplicated corrected OD were used in all analyses.

Separate histograms showing relative frequency versus OD range were prepared for each group. Based on visual evaluation of these histograms, likely statistical distributions were selected and fit to the data from each reference group, F1REF, F2REF, F3REF and B3REF1. The Kolmogorov-Smirnov test was used to test the goodness-of-fit of selected distributions. Critical values, determined according to Lilliefors (1967), of >0.20 were considered necessary to conclude that a hypothesized distribution adequately described the observed distribution.

Significant ($p < 0.05$) differences in the median values of groups were tested with the Mann-Whitney test. Significant ($p < 0.05$) differences between distributions were tested by the 2-sample Kolmogorov-Smirnov test. The above procedures were performed using Statgraphics (Statistical Graphics Corporation, 1986, STSC Inc.).

The OD corresponding to the 95th percentile of the fitted distribution, OD_{95} , was determined for each reference sample. By setting the positive-negative cut-off limits at OD_{95} , 95 % of the fish in each negative sample should be correctly identified, yielding a test specificity (probability of correctly identifying a negative fish) of 0.95. However, since these cut-off limits were calculated on the basis of one sampling from each population, they differ, most likely, from the true population cut-off limits by some unknown amount. To incorporate this uncertainty, 90 % confidence intervals (CIs) were constructed around OD_{95} (Elveback & Taylor 1969).

When using sample-based cut-off limits in field studies, fish with OD falling within the CI boundaries might best be considered questionable. Those with OD below the lower boundary of the CI (L_{CI}) would be classified as seronegative and those with OD above the upper boundary of the CI (U_{CI}) as seropositive.

The cut-off limits (L_{CI} and U_{CI}) from each reference group were used to classify the cohort IP-vaccinated and exposed fish as seropositive, suspect or seronegative. The proportion of correctly classified (as seropositive) fish in B1VACC, B/F2VACC and B3VACC were used to estimate the sensitivity (probability of correctly identifying a positive fish) of the ELISA. The 95th percentiles for the exposed groups, B3REF2 and 3, were determined as described by Mainland (1971).

RESULTS

A vibriosis outbreak, due to *Vibrio anguillarum* serotype 1, began in B3REF in mid-August 1987, after samples B3REF1 and B3REF2 were taken, but prior to sample B3REF3. The fish were treated orally with 75 mg oxytetracycline (kg fish) $^{-1}$ d $^{-1}$ for 10 d. Mortalities totalled 6.5 %. *V. anguillarum* serotype 1 was also isolated from S2VACC fish, and sporadic mortalities (totaling less than 8 %) occurred throughout summer 1987.

Control sera

The plate average OD of the positive control serum ranged from 0.81 to 1.17 in Run 1 and from 0.83 to 1.05 in Run 2. The \overline{OD}_{neg} ranged from 0.05 to 0.11 in Run 1 and from 0.10 to 0.19 in Run 2. The average OD of the control F3REF serum was 0.05 in Run 1 and 0.08 in Run 2.

The results shown in Tables 2 and 3 and Fig. 1, 2 and

Table 2. *Salmo gairdneri*. Description of the frequency distributions of *Vibrio anguillarum* absorbance values in reference rainbow trout populations

Group ^a	Fitted distribution	Mean OD (SD)	Median OD	OD_{95}	L_{CI}	U_{CI}
F1REF	Log-normal	0.18 (0.06)	0.17	0.29	0.27	0.32
F2REF	Normal	0.14 (0.06)	0.14	0.24	0.23	0.26
F3REF	Normal	0.25 (0.11)	0.25	ND	ND	ND
B3REF1	Log-normal	0.21 (0.09)	0.19	0.37	0.33	0.40
B3REF2	ND	ND	0.28	0.59	ND	ND
B3REF3	ND	ND	0.20	0.55	ND	ND

^a Groups are described in Table 1

OD_{95} = 95th percentile of the OD distribution

L_{CI} , U_{CI} : lower and upper limits of the 90 % confidence interval for OD_{95}

ND: not done

3 are based on the corrected OD, as described earlier. For instance, OD from the plate with the highest \overline{OD}_{neg} in Run 1 were corrected by subtracting 0.06 ($0.11 - 0.05$) prior to inclusion in any analysis or graphics.

Reference populations

Table 2 lists the summary statistics and cut-off levels for F1REF, F2REF, F3REF and B3REF1. The observed and fitted distributions of OD in F1REF, F2REF and B3REF1 are shown in Fig. 1.

The frequency distributions of F3REF and B3REF1 differed significantly. Since the nature of the relationship between F3REF and B3REF1 was unknown, we decided to use B3REF1 to determine the cut-off limits for classification of the subsequent samplings of B3REF and for B3VACC. The median OD of B3REF1 was significantly lower than that of F3REF, suggesting that no serological response to environmental levels of *Vibrio anguillarum* had occurred during the fish's first 7 mo in brackish water. In the period between transport and the first sampling, B3 water temperatures had never exceeded 7°C and no sign of *V. anguillarum* infection had been noted.

Neither the median OD nor the distributions of F1REF and B3REF1 differed significantly from one another. The medians and distributions of both groups, however, differed significantly from F2REF.

Vaccinated populations

Fig. 2 presents the observed distributions of ODs from the IP-vaccinated groups. The OD in all groups showed a broad distribution. Some fish in each group had OD below or in the region of 'suspect' classification, the majority had OD near their group's median OD, and some had extremely elevated values.

Table 3 lists the median OD and the sero-classification percentages of each vaccinated group. The estimated test sensitivities were 0.81 in B1VACC, 0.84 in F2/S2VACC (determined by dividing the total number of seropositives by the total number sampled in both groups) and 0.60 for B3VACC2/3. The OD distributions of F2VACC and S2VACC did not differ significantly from one another.

Exposed population

The distributions of the ODs of B3REF2 and B3REF3 are shown in Fig. 3. The distributions were skewed to the right, indicating that a small proportion of fish had substantially higher ODs than did most of the fish.

Table 2 lists of the 95th percentiles for B3REF2 and

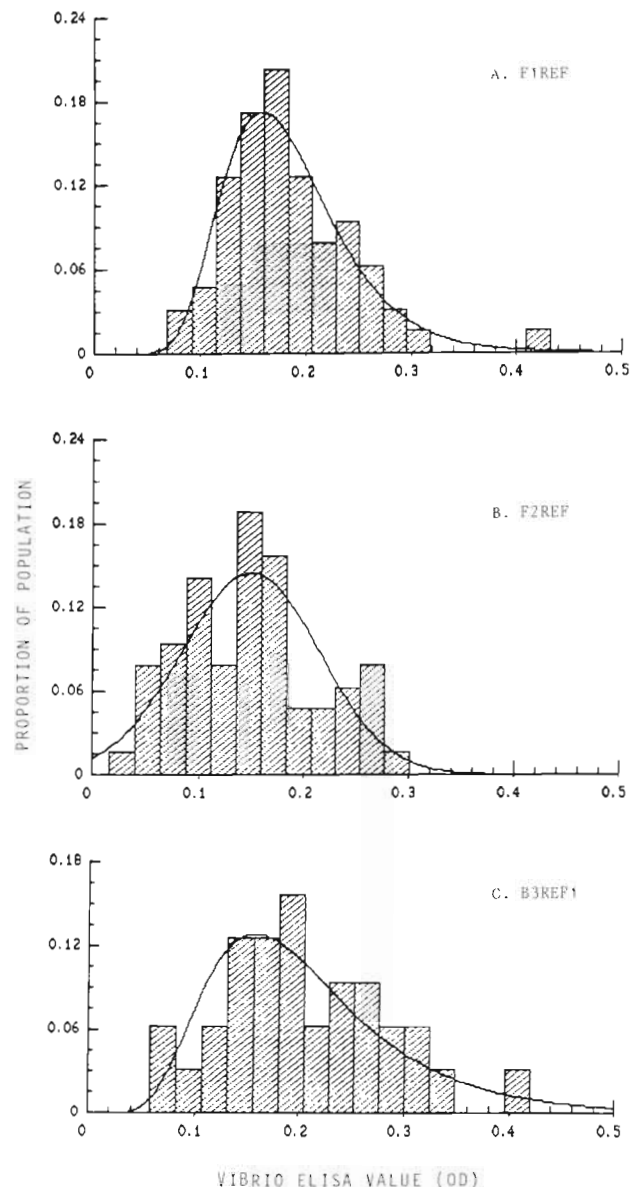


Fig. 1. *Salmo gairdneri*. Observed and fitted distributions of vibrio ELISA values among reference populations of rainbow trout. (A) Group F1REF. (B) Group F2REF. (C) Group B3REF1. Groups F1REF and F2REF had no previous exposure to the bacterium. Group B3REF1 was dip-vaccinated 1 yr prior to sampling, at an average weight of 3 to 5 g

B3REF3 and Table 3 presents the median OD and sero-classification percentages. The median of B3REF2 was significantly higher than that of B3REF1 and B3REF3.

DISCUSSION

In order to fully exploit the potential of ELISA in fish disease research and control, meaningful methods of expressing the output data, OD, must be developed. A

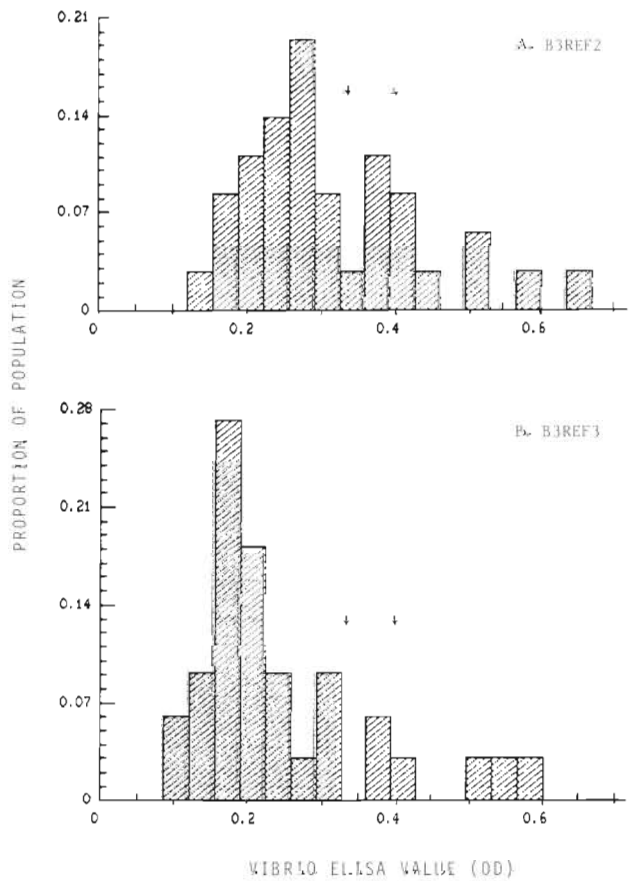
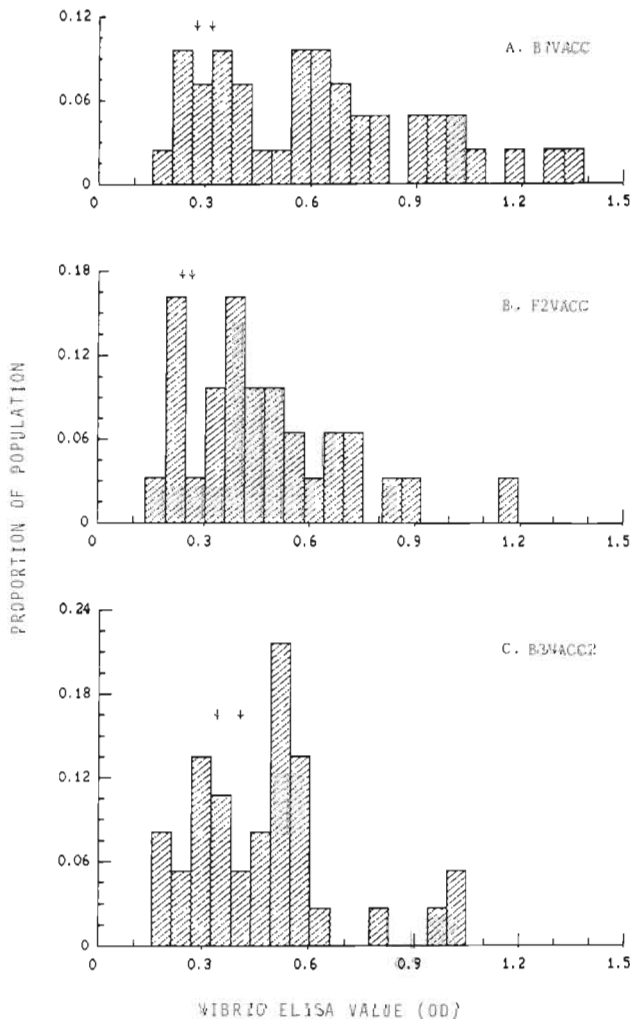


Fig. 2. *Salmo gairdneri*. Observed distributions of vibrio ELISA values among populations of rainbow trout following IP-vaccination. (A) Group B1VACC. (B) Group F2VACC. (C) Group B3VACC2. Group B3VACC2 was also dip-vaccinated, more than 1 yr prior to sampling. Arrows indicate L_{CI} and U_{CI} , as defined in Table 2, determined from the OD distributions of F1REF (A), F2REF (B) and B3REF1 (C)

Fig. 3. *Salmo gairdneri*. Observed distributions of vibrio ELISA values among a population of naturally exposed rainbow trout. (A) Group B3REF2. (B) Group B3REF3. This population had been dip-vaccinated more than 1 yr prior to sampling. Vibriosis mortalities occurred in the population after sample B3REF2, but before sample B3REF3 was taken. Arrows indicate L_{CI} and U_{CI} , as defined in Table 2, determined from the OD distribution of B3REF1

Table 3. *Salmo gairdneri*. Median absorbance values (OD) and percentages of sampled rainbow trout classified as *Vibrio anguillarum* seronegative, suspect and seropositive in vaccinated and exposed populations

Group ^a	Median OD	% neg	% suspect	% pos
B1VACC	0.60	12	7	81
F2VACC	0.42	16	3	81
S2VACC	0.43	6	6	88
B3VACC2	0.53	27	16	57
B3VACC3	0.49	10	20	70
B3REF2	0.28	64	17	19
B3REF3	0.20	82	9	9

^a Groups are described in Table 1

The following reference populations were used: F1REF for B1VACC, F2REF for F2VACC and S2VACC, B3REF1 for B3VACC2, B3VACC3, B3REF2 and B3REF3

critical initial step in this process is to investigate the variation of OD in negative populations and in diseased and immunized populations. Workers in human and veterinary medicine have found the distribution of ELISA values in non-diseased populations to follow a normal pattern (van Loon et al. 1981) or to have a positive skew (de Savigny et al. 1979, Gillis et al. 1984). Quantitative information on the distribution in diseased populations is less available.

Studies of the distribution of measured antibodies in fish populations are scarce. Robohm et al. (1979) examined the distribution of \log_{10} values of agglutination titres to various bacterial antigens in flounder and weakfish. This information was then used to classify sampled populations of fish either having had no recent exposure to a specific antigen (Poisson distribution), or as having had a recent exposure (normal or 'mixed' distribution). Dunier (1983) estimated the variances of agglutination titres obtained in rainbow trout injected with dinitrophenol bound to the carrier, keyhole limpet haemocyanin, and found them to be considerably higher than variances found in positive mammalian populations. Although the continuous nature of ELISA data lends itself readily to distribution-fitting, comparable studies on the variation of ELISA values in fish populations have not been published.

The OD of the reference populations in this study followed either a normal or a log-normal distribution. Group F2REF had a normal distribution and a significantly lower median OD than the other reference groups. The blood samples from this group were collected at a lower water temperature and earlier in the year than were the other reference samples. These factors may have contributed to a lower background level of cross-reactive antibodies. Differences in the background readings of negative serological samples have also been attributed to husbandry (Taylor et al. 1978) and environmental (Grab & Pull 1977) effects.

Groups F1REF and B3REF followed log-normal distributions. Characteristics of the log-normal distribution include a concentration of individuals near the low end of the range and a moderate positive skew. The right-hand tail incorporated, most likely, a small proportion of individuals with some non-specific reactivity. In the B3 reference group, it may, however, also have reflected fish with residual specific antibody activity. F3REF and B3REF1 had been dip-vaccinated during summer 1986. We believed, when planning this study, that antibodies elicited by these vaccinations should have declined to background levels prior to our sampling in 1987. However, elevated vibrio titres have been detected by ELISA in aquaria-maintained rainbow trout for up to 46 wk after dip-vaccination at 12 g (Thuvander et al. 1987). Although the duration of elevated titres decreases when fish are reared in the field,

as opposed to the laboratory, and are vaccinated at smaller sizes (M. A. Thorburn unpubl.), some of the sampled F3 and B3 reference fish may have had residual specific antibody activity. The presence of specific reactivity was more probable in F3REF, which had a significantly higher median OD than B3REF1, and a different (normal) distribution. F3REF was vaccinated later and sampled earlier than B3REF1. Since there is variation among individual fish in the rate at which they lose specific antibody activity, OD frequency distributions will be time-dependent in vaccinated populations.

The frequency histograms of the IP-vaccinated groups revealed quite similar profiles. An initial peak was consistently observed near the cut-off region, indicating that a small proportion of fish did not respond to IP-vaccination with significantly elevated antibody production. This finding confirms the phenomenon of individual antibody non- (or poor-) responders in fish populations (M. Tatner pers. comm. 1987).

The statistical comparison of samples F2VACC and S2VACC showed no change in the OD distribution following the occurrence of vibriosis in S2VACC. While secondary exposure may have helped maintain antibody levels, it did not appear to cause an increase in the titres of individual fish, or to stimulate antibody production among the non-responder proportion of the population.

At a given cut-off level, test sensitivity is a function of both the quality (accuracy and precision) of the ELISA and of the frequency distribution of the true OD in the positive populations. Because of the occurrence of non-responders, any test which requires the presence of circulating antibody for identification of positive fish cannot approach 100% sensitivity. The sensitivity of the ELISA in B1VACC and F2/S2VACC was, however, quite reasonable. It was not as good in the B3 vaccines. The relatively higher mean OD and larger standard deviation in B3REF1 resulted in a higher positive cut-off level for the classification of B3VACC.

The OD of B3REF1 were significantly higher than those from the same population sampled 7 wk earlier (B3REF1). Apparently, environmental levels of *Vibrio anguillarum* became, at some point, high enough to stimulate humoral immune responses in at least part of the B3REF population. That the first vibriosis mortalities occurred ca 3 wk after sampling B3REF2 suggests that: (1) when initiated by low environmental pathogen concentrations, vibriosis may have a long incubation period, or (2) the mere presence of *V. anguillarum*, at levels which can elicit antibody production in some fish, is not sufficient cause for a disease outbreak. Thorburn (1986) has previously demonstrated the dose-dependency of the incubation period for vibriosis. Rosenkvist-Jensen (1983) and Thorburn (1986) have

observed vibrio antibody production in field-tested rainbow trout populations which remained disease-free.

The occurrence of elevated ELISA values in the B3REF population was fairly transitory. By the final sampling (B3REF3), most OD were similar to those observed in the first sampling. However, relatively more fish were classified as suspect or seropositive in B3REF3 (18%, as opposed to 3% in B3REF1). Also, B3REF3 had a much higher 95th percentile (0.55) than did B3REF1 (0.37).

In conclusion, we suggest that no single method for interpretation of ELISA data will suffice for all aspects of fish disease work. Determination and comparison of median OD values may be adequate to assess humoral responses to immunization, but may not be sensitive enough to ascertain natural exposure in seroepidemiologic surveys. In the latter case, examination of the form and percentile markers of the OD frequency distributions in representative samples should provide helpful complementary information. Cut-off levels, which allow estimation of percent sero-positive, are valuable if they are based on the OD frequency distribution of comparable negative reference populations (Mainland 1971). Further research is necessary to determine which specific fish and environmental characteristics must be similar to achieve adequate comparability.

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