FISH HEALTH SECTION
AFS
NEWS LETTER

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FHS ELECTIONS

The Nomination Committee has completed a good slate of candidates:

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Randy MacMillan, Chairman of the Membership and Balloting Committee, has sent ballots to the members of the Section. Please return your choices to him prior to 31 July 1987.

VOTE TODAY!

WESTERN FISH DISEASE CONFERENCE

The 28th Annual Western Fish Disease Conference was hosted by Beth MacConnell and Charlie Smith of the USFWS Fish Technology Center in Bozeman, Montana. The Conference was held June 23-25 and was attended by approximately 150 individuals. Following tradition of this meeting, informal round table discussions produced valuable information and considered problems for which there are currently no solutions. After a keynote address by Rowan Gould from the USFWS offices in Washington, D.C., the conferees heard more than 30 technical papers. Reports on *Renibacterium salmoninarum* and IHNV predominated in the bacteriology and virology sections, respectively. The parasitology section included papers on *Ceratomyxa shasta,* PKX, and *Myxosoma cerebralis.*

Bozeman-style hospitality was enjoyed throughout the meeting, but especially at the pig roast on the grounds of the Fish Technology Center. Beth and Charlie did a great job in organization and in production of the conference.

The site of next year's meeting is planned for Orcas Island, Washington, and will be hosted by Wayne Brunson of the Washington Department of Game.

TESTS INDICATE INABILITY OF *AEROMONAS SALMONICIDA* TO BE TRANSMITTED VERTICALLY

G.L. Bullock
U.S. Fish and Wildlife Service
National Fish Health Research Laboratory, Box 700
Kearneysville, WV 25430

It is widely recognized that *Aeromonas salmonicida* is transmitted horizontally, and there is no direct evidence that the bacterium is transmitted vertically. Nevertheless, concerned trout growers and biologists ask if it is possible. Since the late 1970's experiments have been carried out to determine if *A. salmonicida* could be transmitted vertically. In 1978 fertilized eggs were obtained from rainbow trout that were known to harbor the pathogen. Although *A. salmonicida* was isolated from the sperm from one male, vertical transmission could not be demonstrated in the progeny. In 1982, fertilized eggs were taken from carrier brook trout and again no evidence was found that vertical transmission occurred. In both the 1978 and 1982 trials year-old progeny from carriers were injected with the corticosteroid Kenalog, and then subjected to heat-stress (20 C) — a procedure known to evoke overt disease in latent carriers. None of the injected and heat-stressed trout showed the presence of *A. salmonicida.*

A final test for possible vertical transmission was initiated using brook trout injected with *A. salmonicida.* Three female and three male (3-years-old) brook trout were pair-mated. Four hours before spawning, each female was injected intraperitoneally with 5mL of washed *A. salmonicida* suspension containing 7.4×10^8 cells per mL. Immediately after spawning, ovarian fluids contained 5.2×10^8 to 4×10^8 bacterial cells per mL. Three lots of eggs were fertilized, water-hardened, and divided into six portions. Three portions were disinfected for 15 min in 100 ppm iodine (Betadine) and three portions were not disinfected. All portions were treated with 1:500 formalin every 3 days to control fungus. Only a third of the eggs hatched in four of the test portions; therefore, fry from the disinfected eggs were combined into one lot and fry from disinfected eggs were combined into a second lot. During the ensuing year, trout that died were examined bacteriologically but in no case was *A. salmonicida* isolated. In addition, 70 trout from the disinfected lot and 70 from the nondisinfected lot were injected with Kenalog then heat-stressed. In no case was the presence of *A. salmonicida* demonstrated during the next 2 weeks.

Results from the two tests with naturally infected carriers and from the single trial with artificially infected brook trout strongly reinforce decades of practical observation—namely that furunculosis is not transmitted vertically. However, prudence dictates that eggs from carriers should be disinfected if they are to be moved to locations free of the pathogen.

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BONAMIA OSTREAE IN *OSTREA EDULIS* FROM CALIFORNIA

R.P. Hedrick, J.M. Groff and T. McDowell
Aquaculture and Fisheries Program
Department of Medicine
University of California, Davis, CA 95616

and
J.T. Hollibaugh
Great American Oyster Co. and Tiburon Center
San Francisco State University
San Francisco, CA 94920

Bonamia ostreae is a poorly understood intracellular haplosporidan that has been associated with extensive mortality among European flat oysters (*Ostrea edulis*) both in Europe and as most recently reported by Elston et al. (1986) in Washington, USA. There is considerable speculation regarding the source of the parasite but it was first described by Comps et al. (1980) among oysters in Europe after importation of larvae from hatcheries in California.

Katkansky and Warner (1974) had observed similar parasites, referred to as microcells, as early as 1966 in the hemocytes of *O. edulis* imported to California from Milford, Connecticut in 1963, 1964 and 1965. The poor survival of these oysters over the next three years was believed to be associated with the presence of microcells parasitizing the "leukocytes" of affected animals as determined by scientists at the NMFS Laboratory, Oxford, Maryland and Katkansky and Warner (1974). Oysters planted into three locations, Morro Bay, Elkhorn Slough and Drake's Estero became infected and experienced mortalities as a result of the parasite.

Since the completion of the study of Katkansky and Warner in 1972, observations on the prevalence and occurrence of microcell disease have been lacking although Elston et al. (1986) reported *Bonamia ostreae* among *O. edulis* reared in the state of Washington that had been imported from Elkhorn Slough. They further documented the similarity of the condition to that described as microcell disease by Katkansky and Warner.

In examining the causes of poor survival of *O. edulis* in another oyster-rearing area in California, Tomales Bay, we have detected the presence of microcells that share identical properties to the haplosporidan *B. ostreae*. The oysters were approximately 5.5 cm in shell size and 2 years in age. The mortality in past years had approached 80% prior to harvest. Infected oyster showed no visible external or internal signs other than being thin and watery. Histological exams showed abundant accumulations of hemocytes around the digestive diverticuli and congested in the interstitium. The intracellular parasites were of uniform circular shape (2 um) with an eccentric nucleus and parasitized cells contained up to 10 microcells.

The source of the parasite is unknown but if the original oysters imported to California were free of the disease, some indigenous species may be the reservoir. Studies underway are examining other bivalves as possible sources of the infection. This report documents the first finding of the parasite in oysters from Tomales Bay and the first observations of microcells in California since 1972.

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The health officer of Brewer, ME reported that he had purchased shucked oysters from the Maryland area at a seafood market in Brewer that had orange colored "things" about the size of the end of his thumb in with the oysters. He had also received complaints from the public, wondering if these "things" posed a health hazard. Investigation showed the "things" to be oyster crabs, *Pinnotheres ostreum* that were evidently living in the oysters at the time they were shucked and inadvertently added to the containers of shucked oyster meats sent to seafood dealers in Maine. These oyster crabs, in times past, were themselves considered a gourmet item. Stuart W. Sherburne, Maine Department of Marine Resources, West Boothbay Harbor, ME 04575.

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POTENTIAL IMMERSION VACCINE FOR FURUNCULOSIS

W.T. Cox and R.P. Hedrick
Aquaculture and Fisheries Program
Department of Medicine
University of California, Davis, CA 95616

An unusual isolate of *Aeromonas salmonicida* was recovered from chinook salmon (*Oncorhynchus tshawytscha*) injected with an avirulent strain, 3.101-1 (Cipriano 1982), of the bacteria. The new strain, 3.101-2, differed from the parent strain and from other avirulent strains of this bacteria by possessing autoaggregation qualities in broth culture, a property unique to virulent strains. However, tests have shown strain 3.101-2 to be avirulent by either injection or immersion methods (see Table 1).

Autoaggregation is associated with the possession of an "additional protein" layer, or A-protein (Udey and Fryer, 1978). This protein has been postulated to be of primary importance in the development of furunculosis vaccines. A-protein has been identified from strain 3.101-2 by SDS-PAGE analysis of cell extracts, and appears to be identical to the A-protein of a virulent strain, AS1R. Non-autoaggregating strains (ASIS-2) lack both A-protein and virulence, as determined by SDS-PAGE and virulence testing.

The discovery of strain 3.101-2 offered a unique opportunity to test vaccines possessing A-protein, but usable in an undenatured form. Five bacterins were prepared from saline washed cells suspended to 10 g/l in sterile 0.85% saline: 1) saline washed cells, 2) alkaline hydrolysis (ALKOH) extracted cells plus supernatant (McCarthy et al. 1983), 3) identical to bacterin no. 2 but inactivated with 0.3% formalin, 3) identical to bacterin no. 2 but inactivated with 0.3% formalin, 4) ALKOH cell fraction only, 5) ALKOH soluble fraction only. Bacterin no. 3 was stored at 4° C in pathogen-free well water at the UCD Fish Pathology Lab.

Fish received a 5 min. vaccination in bacterin diluted 1:4 on day 1, followed by a 5 min. immersion in 1:2 diluted bacterin on day 14 (as recommended by Johnson and Amend, 1984). Duplicate groups of 30 fish each were challenged on day 28 by a 20 min. immersion in a suspension of 1.0×10^4 cfu/ml of strain AS1R of *A. salmonicida* (virulent strain). Mortalities were monitored for 28 days, and those due to *A. salmonicida* were confirmed by reisolation of bacteria from the kidneys. Vaccine efficacy was evaluated as recommended by Amend, 1981. Results are shown in Table 2.

The agglutinating antibody response during the course of the study was also followed. Pooled sera samples (5 fish each) from each group were collected and titrated against live cells in sterile 0.85% (Paterson and Fryer, 1974). Reciprocal antibody titers were recorded for each group on days 1, 14, 21, 28, 42 and 49. All groups had titers of zero on day 1, which had risen to 20 (0 to 20) by day 14, at which time the booster vaccination was administered. By day 21 the average titer for all groups was 80 (40 to 160). Unfortunately a water failure resulted in the loss of some fish on day 23 of the study, so serum titers were not recorded for groups treated with bacterins 2, 3, or 4 for the remainder of the experiment. On day 28, fish treated with bacterins 1 and 5 each had titers of 60. On day 42 these titers had declined to 10 and zero, respectively. By day 49 both groups had titers of zero. Sera from control fish was sampled only on days 1 and 14 of the study, and titers were zero on both occasions.

In summary, equal agglutinating antibody titers were observed in fish vaccinated with each of the five different bacterins. This titer, however, was not correlated with protection against challenge with virulent strain AS1R. Similar experiences have been recorded by Michel and Faivre, 1982 and Cipriano, 1983.

Fish vaccinated with bacterins 1 and 3, consisting of cells and cells plus solubilized cell extracts, respectively, had the best survival. Bacterins 4 and 5, consisting of soluble extracts and ALKOH treated cells both failed to provide significant protection. However, the combination of the components in bacterins 4 and 5 into a single bacterin (equal to bacterin 3) gave the best protection. These results indicate that the combination of soluble and particulate antigenic materials provides better protection than either component alone. Also, there seems to be a slight advantage to treating cells by the ALKOH method over using untreated cells (bacterin 3 vs bacterin 1).

Fish vaccinated with bacterin 1 had poorer survival than fish teated with bacterin 3. These bacterins were equal except that bacterin 2 had been treated with 0.3% formalin. These results suggest that undenatured antigenic structures are more effective in producing immunity.

Table 1. Virulence of *Aeromonas salmonicida* strains by injection and immersion to chinook salmon.

Strain	Characteristics	Dose (cfu/ml)	Route	Mean Time to Death	% Cum. Mort
AS1R	A+ virulent	$1x5x10^1$	im	4.8 days	90
AS1S-2	A- avirulent	$2.1x10^4$	im	---	0
3101-2	A+ avirulent	$1.4x10^4$	im	---	0
AS1R	A+ virulent	$1.0x10^4$	immersion	12.6 days	96
3.101-2	A+ avirulent	$5.0x10^8$	immersion	---	0

Table 2. Efficacy of five different immersion bacterins in protecting chinook salmon fingerlings from mortality following bath challenge with *Aeromonas salmonicida.*

Bacterin	Treatment	Cumul. Survival	RPS[1]	Chi Square	Prob.
control	saline, unchallenged	100.0%	---	---	---
control	saline, challenged	4.0	0	---	---
1	washed cells	22.4	19.5	6.0	.025
2	ALKOH, 0.3% formalin	16.0	4.2	2.7	.10
3	ALKOH	28.6	25.7	9.4	.005
4	ALKOH, soluble	4.0	0	0.3	NS[2]
5	ALKOH, cells	10.2	6.4	1.2	NS

[1] Relative Percent Survival
[2] Not significant

PREVALENCE OF IHNV IN ADULTS AT ROUND BUTTE HATCHERY, OREGON

S.E. LaPatra, W.J. Groberg, R.J. Robart, L.J. Freeman, J. Kaufman, J.J. Loomis, D.E. Ratliff
Round Butte Hatchery
Madras, Oregon

During the past five years at Round Butte Hatchery located in Central Oregon, adult salmonids have been monitored for the prevalence of infectious hematopoietic necrosis virus (IHNV). This facility posseses an annually returning stock of summer steelhead trout (*Salmo gairdneri*) which are trapped on the Deschutes River and subsequently transported to the hatchery where they are held until spawning. Since 1976, these adults have had a consistent IHNV carrier rate ranging from 20 to 50%. but in 1986 and 1987 the IHNV carrier rate declined to negligible levels (Table 1). However, during the time when IHNV prevalence was declining at Round Butte Hatchery, in other summer steelhead trout stocks many new isolations of IHNV were being made. This indicates the virus was still prevalent and indeed becoming more widespread.

Evaluation of the situation at Round Butte Hatchery revealed several factors which had changed in 1986 and 1987 from previous years. These changes included: 1) a delay in the transporting and holding of adults at the facility until just prior to spawning; 2) the use of formalin as a fungicide in adult treatments and; 3) a doubling of the flow rate (300 gpm to 600 gpm) through the adult holding ponds. These management strategies were primarily implemented to decrease adult mortality and to meet adult treatment regulations. Interestingly, these changes correlate with the decrease in the IHNV prevalence in the adults.

By changing the long term holding of adults in a high density, low flow situation, to one of a short duration with higher flows, adult mortality was decreased and possibly adult to adult transmission of IHNV was decreased as well. Furthermore, the use of formalin instead of malachite green as a fungicide, could allow better sanitation of the environment within the holding ponds, again decreasing horizontal transmission of virus.

Experiments are planned at Round Butte Hatchery to evaluate the effects of density and flows on the prevalence of IHNV in the adults.

Table 1.

Year	Total Adults	Mortality	Number Spawned	% IHNV (+)
1983	733	86	200	48
1984	1061	496	505	44
1985	1351	115	699	39
1986	1147	59	570	4
1987	710	33	508	5

* * * * * *

The first record of a neoplasms (germinoma) in Maine ocean quahogs *Mercenaria mercenaria* was in a 67 mm specimen submitted in April, 1987 in a sample of 50 from a commercial aquaculture operation in Maquoit Bay, Brunswick, ME. This specimen had, upon consultation with Austin Farley of the NMFS pathology lab at Oxford, ME, the apparent initial stages of ovarian cancer. The aquaculturist started losing quahogs in the fall of 1986 but did not submit specimens for examination until April 24, 1987, after he had experienced 60% mortalities. We are doing follow-up work on this to determine if other quahogs in his operation have neoplasms and, if so, whether the neoplasms are in Maine native stock or in cultured stock that the aquaculturist previously imported from Massachusetts. Stuart W. Sherburne, Maine Department of Marine Resources, Fisheries Research Station, West Boothbay Harbor, ME 04575.

IHN VIRUS IN WASHINGTON CHUM SALMON

Kathy Hopper
Washington Dept. of Fisheries
115 General Administration Bldg.
Olympia, WA 98504

Infectious hematopoietic necrosis virus was isolated from wild chum salmon adults trapped in tributaries to the Stillaguamish River (North Puget Sound) during December 1986. This is the first known isolation of IHNV from chum salmon in Washington State or from fish in the Stillaguamish watershed.

Ovarian fluid samples from the fish were diluted 1:2 with standard antibiotic incubation mix (gentamicin and antimycotic-antibiotic in HBS). After inoculation of preformed monolayers of EPC cells, the samples were routinely saved until proven negative (often 4 weeks). When typical CPE appeared in cells inoculated with the ovarian fluid samples, we recultured the original sample and found that the titers remained the same or increased during storage at 4°C. In this case, we obtained infectious virus until we ran out of the diluted ovarian fluid at 6 weeks after the original inoculation.

CAPACITY OF FORMALIN TO INACTIVATE IHN VIRUS

S.E. LaPatra and J.S. Rohovec
Department of Microbiology
Oregon State University
Corvallis, OR 97331

Traditionally malachite green has been used effectively to combat fungus infections of adult salmon and their eggs. In compliance with FDA regulations, a substitute chemotheraputent, formalin, has been used more or less effectively. A potential benefit of using formalin instead of malachite green is its virucidal activity against enveloped viruses, such as infections hematopoietic necrosis virus (IHNV). In-vitro comparison of IHN virus inactivation by these two compounds, at concentrations commonly used to treat adult salmonids, showed that formalin was more effective against IHNV than malachite green (Table 1). Furthermore, the higher concentrations of formalin used to treat eggs exhibited an even greater potential for virus disinfection (Table 2). Although these test systems did not account for organic matter which could decrease the virucidal capacity of formalin, they did show a potential of limiting horizontal or fish-to-fish transmission of IHNV between adults and of further external virus disinfection of eggs during incubation.

Table 1. IHNV Inactivation by Two Compounds

Compound	Concentration (ppm)	% Virus Reduction After 1 Hr.
Malachite green	1	20
Formalin	200	60

Table 2. Formalin Inactivation of IHNV

Concentration (ppm)	% Virus Reduction After 1 Hr.
100	53
200	71
1000	92
2000	98

ORGAN COMPRESSION AND ATROPHY IN HOST FISH DUE TO PLEROCERCOIDS OF *LIGULA* SP.

Richard A. Heckmann
and
Sidney N. Beers
Department of Zoology
Brigham Young University
Provo, UT 84601

There are many species of fish in North America that are hosts for the plerocercoid stage of cestodes (Hoffman, G.L., 1967. Parasites of North American Freshwater Fishes. University of California Press, Berkeley). The pathogenic effects of the cestode larvae includes: change in blood chemistry and formed elements, mechanical compression of organs, inability to maintain a normal position in the water, release of toxic materials, decrease in reproductive capacity, parasitic castration, disruption in carbohydrate and fat metabolism, underdevelopment of gonadal tissue and death of host fish (Dubinina, M.N. 1966. Tapeworms (Cestoda, Ligulidae) of the Fauna of the USSR. Nauka Publishers, Moscow. English Translation 1980 NMFS, NOA, U.S. Dept. of Commerce, NSF, by Amerind Publishing Co., New Delhi, India).

To gain additional data on organ compression and atrophy two projects were completed at the Brigham Young University Fish Disease Laboratory.

The Utah Division of Wildlife Resources (Fisheries) trapped 200 year-old *Perca flavescens* from Yuba Reservoir, approximately 50 miles south of Provo, for part of this study. Each fish was weighed and measured, then examined for plerocercoids. The plerocercoids of *Ligula* sp. from the infected fish were counted, measured and weighed. Infected fish were weighed before and after the plerocercoids were removed. The volume of the infected fishes abdominal cavity was estimated by measuring length, width and height of the area. This measurement would not account for a distended body cavity. An estimate of volume was also calculated for the plerocercoids removed from the fish.

Sixty-eight percent of the yellow perch examined were infected with an average of 2.8 worms (maximum, 9 per one fish). The average total length of the uninfected fish was 78.1 mm while the infected fish measured 76.7 mm. The average weight of the infected fish with worms was 3.34 grams and those without plerocercoids weighed 3.74 grams. The infected fish had an average weight of 2.90 grams after the plerocercoids were removed. Thus the average percent weight of worms from infected fish was 13.2% with one fish containing 43.8% body weight in worms. The average volume for a single plerocercoid was 421.1 mm^3 and for infected *P. flavescens* the average abdominal cavity volume was 1260 mm^3 (this is not the volume of a distended abdomen). Fish infected with 3 plerocercoids would have 100% of their **normal** body cavity filled with worms since on an average one plerocercoid of *Ligula* sp. in *P. flavescens* occupies 33.4% of the body cavity. These figures account for the grossly distended fish which have multiple worm infections. Also, excessive number of plerocercoids could strangulate visceral organs and rupture the body wall with subsequent host death. The data also corroborate accounts of organ atrophy and compression due to plerocercoids. State Fisheries managers for Yuba Reservoir have noted a decrease in yellow perch and walleye populations in the Reservoir.

A similar study was completed at Bottlehollow Reservoir, Utah which contained fathead minnows (*Pimephales promelas*) and salmonids (*Salmo trutta* and *S. gairdneri*). *Pimephales* was infected with *Ligula* plerocercoids. The maximum number of worms per fish was three with all posted fish infected with at least one plerocercoid. Using a similar technique to measure volume, it was estimated that 19% of the body cavity per infected fish was occupied with one plerocercoid.

As indicated in most articles pertaining to fish containing plerocercoids of cestodes it is important to "manage" this parasitic problem for optimum fish growth and production.

CHARACTERISTIC PISCINE ERYTHROCYTIC NECROSIS (PEN) FROM NATURAL POPULATIONS OF FRESHWATER FISH FROM FOUR MAINE LAKES

Stuart W. Sherburne
and
Laurie L. Bean
State of Maine, Department of Marine Resources
Fisheries Research Station
West Boothbay Harbor, ME 04575

Shortly after the 1975 discovery of piscine erythrocytic necrosis (PEN) in anadromous alewives, *Alosa pseudoharengus*, fish were sampled in lakes used as spawning areas by alewives to determine if freshwater species might show evidence of this disease. The first instance of cytoplasmic inclusions, characteristic of PEN, in red blood cells of a freshwater species was from a 13.3 cm T.L. large-mouth bass, *Micropterus salmoides*, caught at Sherman Lake, North Edgecomb, on July 15, 1976.

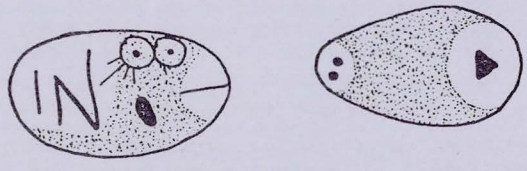
From July 1976 to September 1981 we took blood samples from 968 freshwater fish, consisting of 13 species, from seven alewife spawning areas and six non-alewife areas. Fish with characteristic PEN were found only in alewife spawning areas — Damariscotta Lake, Damariscotta; Nequasset Lake, Woolwich; Sheepscot Pond, Palermo and Sherman Lake. Of 652 fish sampled from alewife spawning areas, 8.4% (55/652) had cellular pathology characteristic of PEN.

PEN was evident in four freshwater species — yellow perch, *Perca flavescens*, pumpkinseed sunfish, *Lepomis gibbosus*, chain pickerel, *Esox niger*, and large-mouth bass. Individual infections were light, with only one to about 20 infected cells on a slide, which has been characteristic in some of our marine species. The red acidophilic cytoplasmic inclusions varied from small to large, up to 2.0 microns. Inclusions were associated with nuclear degeneration in some instances.

As only 316 fish were sampled from non-alewife areas, we do not want to infer that there is a relation between alewives and characteristic PEN in freshwater species. More fish need to be sampled.

We have found PEN in juvenile alewives that have never been to sea. As juvenile alewives are consumed by freshwater species, there could be a possible association between alewives and characteristic PEN in freshwater species. The question remains as to whether the characteristic PEN in these freshwater species represents PEN (VEN) as we know it in marine species, i.e., if electron microscopy were performed on these cells, would a virus be found such as has been evident in certain marine species?

Editor's Note: Although piscine erythrocytic necrosis (PEN) was first used to describe this disease, viral erythrocytic necrosis (VEN) is now commonly accepted and is the term adopted by the Fish Health Section.



Due to increased demand for fishery products and higher prices paid Macrozoarces to the fishermen, underutilized species such as the ocean pout, *americanus*, are being processed for market. Fillets of ocean pout, rejected by the federal inspector at a Rockland fish processing plant were found to be severely infected by *Loma (Plistophora)* cysts. This microsporidian was responsible, in part, for curtailment of a developing fishery for ocean pout during World War II because so many fillets contained these cysts. Stuart W. Sherburne, Maine Department of Marine Resources, Fisheries Research Station, West Boothbay Harbor, ME 04575.

DETECTIONS OF FISH PATHOGENS USING DNA HYBRIDIZATION

W.B. Schill
U.S. Fish and Wildlife Service
National Fish Health Research Laboratory, Box 700
Kearneysville, WV 25430

Detection of pathogenic microbes is often accomplished using various antibody based techniques. In some cases antibodies produced by the infected organism are measured by their interaction with a test antigen. A positive response in this sort of test is not a clear indication of active disease, however, because vaccinated and recovering animals will also be positive. Other antibody tests attempt to identify the microbe directly by using polyclonal or monoclonal antisera to detect the corporeal antigens or metabolites of the pathogen. Polyclonal antisera are often nonspecific, and monoclonal antibodies are difficult to produce and may be so specific that minor variation in the microbe may render the pathogen undetectable.

DNA hybridization is an alternate method for detection of pathogens. This technique has the advantage of not being sensitive to changes in the "antigenic coat" displayed by the pathogen. For example, it has been shown that a DNA probe correctly identified adenovirus type 34 in lung tissue even though the probe was homologous to type 2 adenovirus. Immunological detection would have required using the correct one of 37 antisera.

We have cloned small (50-2000 base pair) fragments of chromosomal and plasmid DNA from *Yersinia ruckeri* and *Aeromonas salmonicida* into M13mp8 and have used both radioactive labeling and nonradioactive (biotin-avidin) labeling of these cloned fragments to produce hybridization probes capable of detecting the homologous pathogen. Twenty-five probes were generated specific for the large plasmid found in Serotype 1 *Y. ruckeri*, and an additional twenty-five probes were produced that were specific for the chromosomal DNA of this organism. Fifty probes were produced specific for *A. salmonicida* total DNA.

Bacterial colonies grown on agar plates using standard sampling methodology were "lifted" to filters of low lignin (Whatman 541) cellulose or nitrocellulose by allowing the filter medium to contact the surface of the agar plates for 5-10 minutes. The bacteria were then fixed to the filters by treatment with NaOH-NaCl solution followed by neutralization with pH 7.0 Tris-HCL + NaCl solution. The Whatman 541 filters were steamed for 3-5 minutes between the NaOH and neutralization steps.

The Whatman 541 filters had the advantage of being very easily handled and required no further treatment if radiolabeled probes were to be used. Unfortunately, 541 lifts prepared by this method showed high nonspecific binding of avidin to the cell debris and were unsuitable to be used with biotin labeled probes.

Nitrocellulose colony lifts had to be exhaustively treated to preclude nonspecific binding of avidin. Filters were treated with lysozyme, proteinase K, phenol-chloroform-isoamyl alcohol, and chloroform to remove non-nucleic acid components. The filters were then dried, rehydrated, and "prehybridized". Finally, the filters were hybridized, washed, exposed to avidin-alkaline phosphatase, washed again and developed with chromogenic substrate solution.

Radiolabeled probes (P^{32}) were always the most sensitive and specific, but were of course difficult to work with and of very limited shelf life (< one month). Biotin labeled probe systems often showed nonspecificity due to binding of avidin to cell wall components of the bacteria. This necessitated a rigorous treatment of the filters that partly compromised the convenience of using a nonradioactive probe. Biotin labeled probes would be usable however under field conditions. Alternately, Whatman 541 lifts are easily prepared with no equipment and once dried are stable indefinitely. Lifts prepared by field personnel could be mailed or transported to a central laboratory for hybridization with radiolabeled probes.

Although the absolute detection limit for biotin labeled DNA probes was less than 1 pg of target sequence, it must be understood that the target sequence may be only a small percentage of the entire genome. Therefore a probe specific for a chormosomal sequence will require the presence of a larger number of cells (number of target copies) to yield a positive result than will a probe specific for a plasmic or ribosomal RNA sequence that may be present in thousands of copies per cell. We observed detection limits of 1×10^5 to 1×10^6 cells for chromosomal sequence probes and 1×10^4 cells for plasmid sequence probes.

Newer techniques promise to increase the sensitivity and specificity of nonradiolabeled hybridization probes. Hybridization to ribosomal RNA sequences has been mentioned above. Other methods employ amplification of the target sequence or very efficient detection systems to lower detection limits, assay times, and false positive results. One of these techniques uses priming sequences flanking the target sequence to initiate transcription of the target in the presence of DNA polymerase or reverse transcriptase. In subsequent reactions the transcripts from the previous round become templates. The result is an exponential increase in the number of target sequences, and because each round of amplification requires only about seven minutes to perform, this is equivalent to reducing the generation time of the microbe to this amount of time. Using a model system, we have achieved 200,000 fold amplification in about two hours.

More recently a "strand displacement" technique has been described that measures the amount of labeled signal sequence displaced from a performed complex when analyte DNA or RNA is introduced. This method is reported to yield detection limits in the attomole (10^{-18}) range with enzyme detection and has a theoretical ction limit of 10^{-19} to 10^{-20} moles with bioluminescent detection. A small disadvantage with the more advanced techniques described is that sequence information on the pathogen of interest is required and thus application of these methods to fisheries diagnostics will be delayed until this information is available.

Hematopoietic and/or gonadal neoplasms in soft-shell clams, *Mya arenaria*, known to exist at only three sites along the Maine coast prior to 1983, have been evident in 35 locations from Kittery to Perry, ME. This is based on examination of histological sections prepared from 3,565 clams, collected in 108 samples from 42 locations from August, 1982 to May, 1987. Two areas have been sampled on a monthly basis since January, 1986 to determine if the incidence of this disease fluctuates from month to month. Clams from several other areas have been collected on a seasonal basis In addition, clam tissues and sediments from various areas along the coast are being analyzed at the State Public Health Lab for evidence of environmental contaminants that may be associated with these neoplasms. Stuart W. Sherburne, Laurie L. Bean, Rodman G. Getchell, State of Maine, Department of Marine Resources, Fisheries Research Station, West Boothbay, ME 04575.

Italian authorities (as of March, 1987) are now requiring a bloodworm dealer in Wiscasset, ME to provide a health certificate with each shipment of worms going to Mestre, Italy. This dealer had shipped millions of bloodworms to Italy during the past eight years without any health certificate being required. The State of Maine is only required to provide a statement that the worms appear healthy at the time of shipment, that the worms are not for human consumption and that they are to be used for sport fishing only. No histopathological workups are required. Stuart W. Sherburne, Maine Department of Marine Resources, Fisheries Research Station, West Boothbay Harbor, ME 04575.

LARGE PLASMID OF *YERSINIA RUCKERI* HAS NO HOMOLOGY TO THE VIRULENCE PLASMID OF *YERSINIA ENTEROCOLITICA*

W.B. Schill
U.S. Fish and Wildlife Service
National Fish Health Research Laboratory, Box 700
Kearneysville, West Virginia 25430

Sorbitol negative strains of *Yersinia ruckeri* are known to carry a high molecular mass plasmid, and other *Yersinia* species have been shown to sometimes harbor large plasmids that contain genetic sequences coding for virulence determinants. Also, it has been shown that these virulence determinants are universally present in pathogenic strains of *Y. pestis, Y. enterocolitica,* and *Y. pseudotuberculosis.* Because of the long held belief that sorbitol negative *Y. ruckeri* are particularly virulent fish pathogens, some have suggested that the large plasmid carried by this group of organisms is similar, if not identical, to the large plasmids found in the other virulent members of this genus. However, no direct tests for virulence determinants on the *Y. ruckeri* plasmid have heretofore been performed despite the availability of DNA hybridization probes for the known *Yersinia* virulence determinants.

A P^{32}-labeled hybridization probe specific for the virulence determinants discussed above was obtained as a gracious gift from Dr. Walter Hill of the United States Food and Drug Administration. Fifteen sorbitol negative and fifteen sorbitol positive *Y. ruckeri* isolates maintained in the National Fish Health Research Laboratory collection were grown on brain-heart infusion agar plates in a 6x5 grid arrangement. Two virulent, plasmid carrying *Y. enterocolitica* colonies were grown outside the grid and asymmetrically on the plate to serve as controls and orientation markers. The colonies were lifted onto Whatman 541 paper disks and denatured by placing the filters colony side up onto a blotter (in a glass dish) that was saturated with 0.5N NaOH, 1.5M NaCl. The filters, still on the saturated blotter, were placed in a "bamboo steamer" and steamed for 3-5 minutes. The filters were then immersed in 1M Tris, 2M NaCl, pH 7.0 for 4 minutes and then air dried. Colony lifts are stable indefinitely if kept dry, and no "prehybridization" is needed.

Hybridization was performed by placing the filters in "seal-a-meal" bags containing 2 ml hybridization cocktail per filter at 55° C overnight. The hybridization cocktail was 0.6M NaCl, 2mM EDTA, 10X Denhardt's solution, 0.2% SDS, 0.1mg/ml boiled and sonicated salmon sperm DNA, 10mM HEPES, pH 7.0 plus 106 cpm/filter hybridization probe.

Following hybridization, the filters were washed twice in 0.3M NaCL, 0.03M sodium citrate, 0.2% SDS at 50° C. for 15 minutes each, and once in the same solution at room temperature for 30 minutes. Filters were then air dried and placed in contact with Kodak XAR X-ray film, and exposed at room temperature for 14 hours. Films were developed according to the package directions.

Results were unequivocal. No hybridization was observed to any *Y. ruckeri* colonies, and very strong signals were observed at the position of the *Y. enterocolitica* control colonies. Thus the plasmids carried by these two pathogens, although similar in molecular mass, are not homologous. The function(s) of the *Y. ruckeri* remain cryptic, and analogies drawn by some to the virulence mechanisms in other *Yersinia* species are without foundation.

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Yellowtail founder, *Limanda ferruginea,* fillets from the Grand Banks, Nfld, packed by a fish processor in Rockland, ME and rejected by the U.S. Army because of small (pin-head size), white, hard nodules distributed throughout the muscle tissue, were submitted for examination in April, 1987. Histological sections showed that inside each nodule was the cystacanth of an *Acanthocephala* (spiny-headed worm). Stuart W. Sherburne, Maine Dept. of Marine Resources, West Boothbay Harbor, ME 04575.

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THE EFFICACY OF PRAZIQUANTEL (DRONCIT) AND IVERMECTIN IN COMBINATION AS A HELMINTHICIDE FOR FISH PARASITES

Richard Heckmann
and
Reid W. Litchfield
Department of Zoology
Brigham Young University
Provo, UT 84601

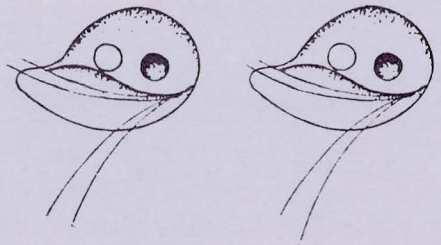
In previous issues of the FHS Newsletter we have reported on results of using Ivermectin (0.05% per ml active ingredient) and Praziquantel (56.8 mg per ml active ingredient) separately as potential pharmaceuticals for fish parasites. During the last six months we have been testing the efficacy of a combination of Ivermectin and Praziquantel against helminths and other parasites of *Cottus bairdi,* the mottled sculpin.

Sculpin were collected with a seine and checked for fish parasites. Parasites common to *Cottus bairdi* included: *Diplostomum spathaceum, Rhabdochona cotti, Dactylogrus* sp., *Gyrodactylus elegans, Myxidium* sp., *Myxobolus* sp., *Apiosoma* sp., *Trichodina* sp., *Epistylus* sp. *Loma (Plistophora)* sp. *Ichthyophthirius multifiliis,* and *Ichthyobodo (Costia) necatrix.*

A series of 7 drug trials were conducted whereby efficacy for a Praziquantel-Ivermectin combination was determined. For each series, 6-8 sculpin were placed in each of 3, 20-gallon aquaria. Varying doses of the two drugs were added to the aquaria with one tank being a control. During one series, two extra aquaria were included with the ideal level of a single doze of Praziquantel or Ivermectin added for comparison with the combination drug.

The drugs, either as a single dose or as a combination, were ineffective against alleviating the protozoan parasite burdens (*Myxidium, Myxobolus, Apiosoma, Trichodina, Epistylus, Ichthyophthirius, Plistophora, Costia).* The fish were stressed and died if the Ivermectin injectible solution was greater than 0.5 ml per 20 gallons. For Praziquantel, we added 3 ml injectable per 20 gallons with no outward stress manifestations from the sculpin. Sculpin are much more sensitive (LD_{50}) to Ivermectin levels than Praziquantel. The ideal combination of drugs that we determined after 7 series of experiments was 1 ml Praziquantel (56.8 mg per ml active ingredient) and 0.1 ml Ivermectin (0.05% active ingredient per ml) for a 20-gallon aquarium. The 7 series required 160 sculpin.

For each experiment, fish were collected, placed in aquaria for 24 hours, drug or drugs added and then one fish was killed 1, 2, 4, 6, 8, 10 days after the pharmaceutical was added. Each time a treated fish was killed one sculpin from the control aquarium was also killed to determine parasite burden. Each fish was carefully dissected for parasite determination both type and number. After 24 hours post treatment the trematodes were dead but the nematodes were still active. From 4 to 5 days post treatment all helminths were dead. Praziquantel-Ivermectin in combination is efficacious against helminths of *Cottus bairdi.*



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Editors:

James R. Winton
National Fisheries Research Center
Building 204
Naval Support Activity
Seattle, WA 98115
206-526-6282

John S. Rohovec
Department of Microbiology
Oregon State University
Corvallis, OR 97331
503-754-4441

FHS NEWSLETTER
Department of Microbiology
Oregon State University
Corvallis, Oregon 97331

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JAMES W WARREN
USFWS, 9317 HWY 99, STE I
VANCOUVER WA 98665