Bacteriological Ring Testing – USFWS Fish Health Centers
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Summary:

Six unknown bacterial samples were sent to each of the nine Fish Health Center laboratories of the U.S. Fish and Wildlife Service (USFWS) for laboratory ring testing. The samples consisted of four known bacterial fish pathogens and one Gram positive cocci: *Yersinia ruckeri*, *Aeromonas salmonicida*, *Aeromonas salmonicida* (*subspecies achromogenes*), *Edwardsiella tarda, Aeromonas hydrophila,* and *Staphylococcus aureus*. One additional mixed culture comprised of Y. *ruckeri* and *A. salmonicida* was included. Eight of nine laboratories participated and satisfactorily completed testing. In addition, one laboratory involved all staff members in the exercise to conduct individual proficiency testing for bacteriological testing and identification skills.

Test Principle:

This exercise was designed to test bacteriological pathogen detection capabilities of the USFWS Fish Health Centers, including presumptive identification and confirmatory methodology for the two primary fish pathogens *Yersinia ruckeri* and *Aeromonas salmonicida*. Additional isolates were included in the panel to provide diversity in the possible test results and included the fish pathogen *Edwardsiella tarda*, *Aeromonas hydrophila,* and *Staphylococcus aureus* (a gram positive bacteria),

All labs were provided with identical sets of unknown isolates numbered 1 through 6, in liquid medium, and were instructed to correctly identify the two primary fish pathogens following the Standard Procedures for Aquatic Animal Health Inspections (SPAAHI, 2007 edition).

Methods:

Bacteriological protocols in SPAAHI require initial isolation and determination of primary bacterial characteristics: colony morphology, motility, cytochrome oxidase, and gram stain. Unknown bacterial cultures are struck onto Brain Heart Infusion Agar or Tryptic Soy Agar plates for single colony isolation. Individual colonies are selected for establishment of pure colony isolates to be used for subsequent testing. Based on initial bacterial characteristics, 24 hour cultures are inoculated onto the appropriate biochemical medium; either Triple Sugar Iron (TSI) or Oxidative-Fermentative (OF) tests. Metabolic characteristics lead to presumptive identification of each isolate following the identification flowchart (SPAAHI Appendix 3 A1) for gram-negative bacteria.

Several labs used API 20E biochemical test strips to provide supplemental biochemical tests for isolates, although this step is not required in SPAAHI protocols. Following presumptive identification, the final confirmation methods require serological testing with appropriate antibodies using direct or indirect Fluorescent Antibody Test (FAT).

Ring Testing Results:

Eight labs completed the ring testing exercise, and submitted their test results, in summary or detailed datasheet documents, describing each test method and result. API profiles, when completed, were submitted. Confirmation method, as well as specific reagent manufacturer and working concentration were included in most cases (see Table 2 for detailed test results and methods).

In summary, all eight labs determined the correct presumptive identity of the two primary fish pathogens. Confirmation testing, following the method of Fluorescent Antibody Test (FAT) under SPAAHI protocols, was performed by 7 of 8 labs for *Y. ruckeri* and 7 of 8 labs for *A. salmonicida*. One laboratory used PCR exclusively for confirmation testing of both fish pathogens, which is not an approved confirmation method under SPAAHI protocols. Therefore, the presumptive identification and confirmation scores are not in total agreement.

Difficulties occurred with the mixed culture, sample #6, and not all labs were able to recover both bacteria from the culture broth. Surprisingly, the generally faster growing *Y. ruckeri* was not recovered as frequently as *A. salmonicida* bacteria. Therefore only 6 of 8 labs were able to isolate, and then correctly identify *Y. ruckeri* from the mixed culture, compared to 8 of 8 labs that correctly isolated and identified A. salmonicida.

The additional atypical *A. salmonicida* subspecies *achromogenes* isolate was correctly identified to the subspecies level by five labs. This particular culture (ATCC 36659) performed very poorly (scant growth) at room temperatures of 20-25ºC, which are normally very conducive to good growth for *A. salmonicida.* Several labs also reported production of various shades of brown pigment after prolonged growth, approximately 5-7 days, on BHIA. The poor growth and unexpected pigmentation confounded test results for several labs which resulted in a lower overall presumptive score for this particular bacterial strain.

In addition to the primary fish pathogens, all labs correctly identified *Aeromonas hydrophila.* The majority of labs (7 of 8) also correctly identified *Edwardsiella tarda* by consulting additional protocols in AFS Blue Book or through their experience working with this regional fish pathogen. The SPAAHI protocols do not list *E. tarda* for routine hatchery inspections, however Blue Book does provide a flowchart and recommends API test strips to confirm an expected biochemical profile for this bacterium. For this exercise, we considered the recommended procedure in Blue Book to constitute a confirmed identification. *Staphylococcus aureus* was also correctly identified to genus, and more frequently to species level, by 7 of 8 laboratories.

Table 1 - Ring Testing Results. Presumptive and confirmatory scores for 8 Fish Health Centers for two primary fish pathogens and additional bacterial isolates.

Isolate ID	Expected Results	FHC Presumptive Score	FHC Confirmation Score
1	***Yersinia ruckeri* (ATCC 29473)**	**8/8**	**7/8**
2	*Aeromonas hydrophila* (ATCC 7965)	8/8	N/A
3	***Aeromonas salmonicidia ssp. achromogenes* (ATCC 33659)**	5/8	4/8
4	*Staphylococcus aureus*	7/8	N/A
5	*Edwardsiella tarda* (ATCC 23659)	7/8	2/8
6A Mixed	***Yersinia ruckeri* (ATCC 29473)**	**6/8**	**5/8**
6B Mixed	***Aeromonas salmonicida ssp. salmonicida* (ATCC 33658)**	**8/8**	**7/8**

Detailed test results by each laboratory's anonymous code are provided in Table 2 including API biochemical profiles (if done) and antibody reagent source and concentrations used for confirmation testing by FAT. Two labs used an alternative test, latex bead agglutination, which could be considered serologically comparable to the standard FAT method. As previously described, one lab used PCR exclusively for confirmation tests, and two labs also used PCR, but in addition to FAT confirmation method for select test samples.

Table 2 - Detailed Ring Testing Results for Each Laboratory

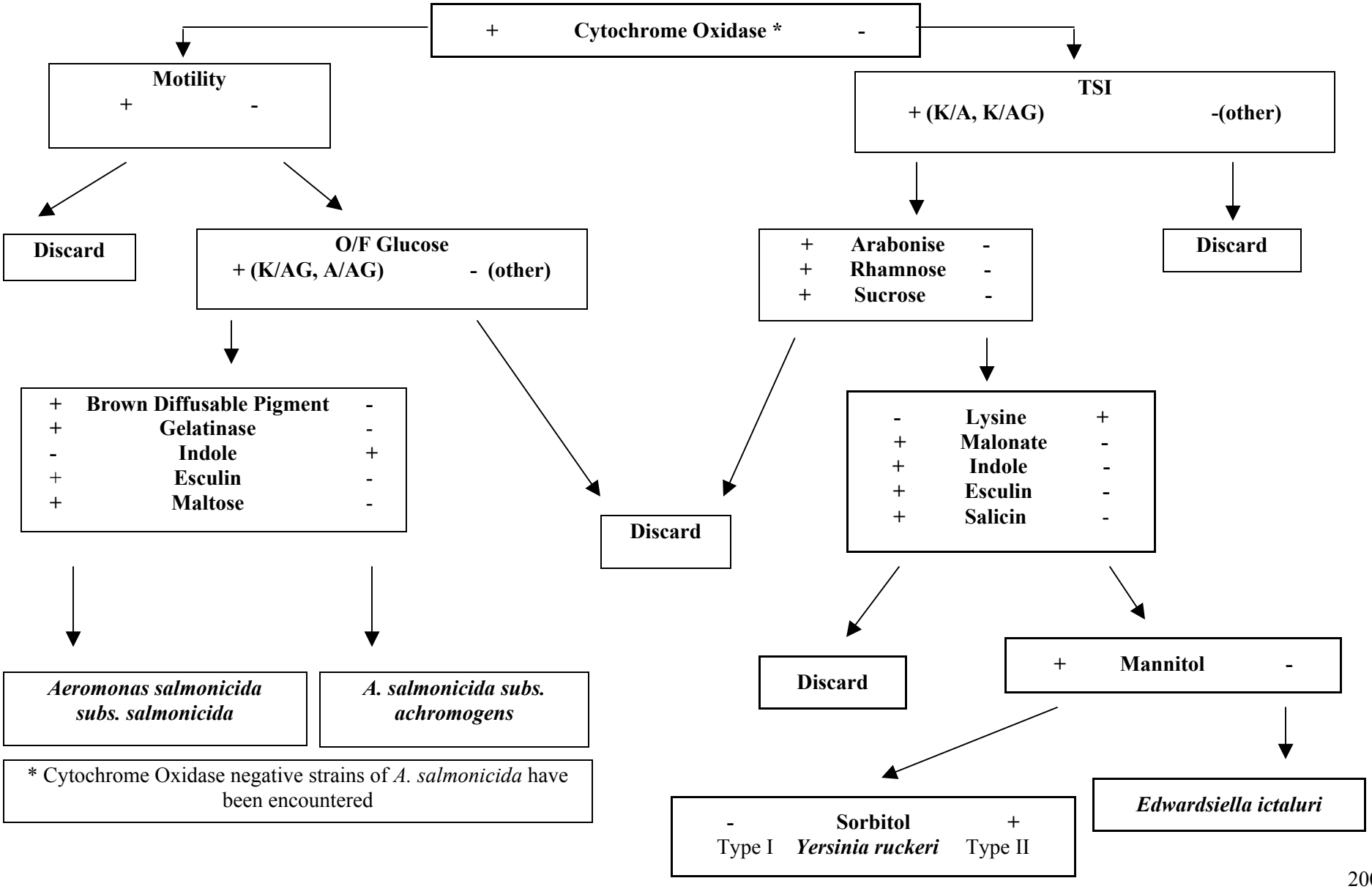
	Culture 1	Culture 2	Culture 3	Culture 4	Culture 5	Culture 6
ID	***Yersinia ruckeri***	***Aeromonas hydrophila***	***Aeromonas salmonicida ssp. achromogenes***	***Staphyloccus aureus***	***Edwardsiella tarda***	**Mixed culture** ***A. salmonicida ssp. salmonicida*** **and *Y .ruckeri***
LAB A Results	***Yersinia ruckeri*** **Type 1**	*Aeromonas hydrophila*	*Vibrio alginolyticus*	*GPC "Staphyloccus or Micrococcus sp"*	*Edwardsiella tarda*	***Aeromonas salmonicida ssp. salmonicida***
API Profile[1]	5104100	7247527	0045124	ND	4744000	6006104
Confirmation Method / (Reagents[2])	**FAT (Microtek 1:50)**					**FAT (Microtek 1:50)**
Lab B Results	***Yersinia ruckeri***	*Aeromonas hydrophila*	*Pasteurella multocida*	*GPC "Staphylococcus or Micrococcus sp"*	*Edwardsiella tarda*	**A= *A . salmonicida*** **B= *Y. ruckeri***
API Profile	5104100	7007527	0040124	2012000	4744000	A-0006104 B-1104100
Confirmation Method / (Reagents)	**FAT and Bead Agglutination (reagent and dilution not given)**					**A-FAT B-Bead Agglutination**
Lab C Results	***Yersinia ruckeri***	*Aeromonas hydrophila*	***Aeromonas salmonicida***		*Edwardsiella tarda*	**A= *Y. ruckeri*** **B= *A. salmonicida***
API Profile	ND	7577755	5046644		4544000	A-ND B-5046644
Confirmation Method / (Reagents)	**PCR**		**PCR**		**PCR**	**A- PCR B- PCR**

	Culture 1	**Culture 2**	**Culture 3**	**Culture 4**	**Culture 5**	**Culture 6**
	Yersinia ruckeri	***Aeromonas hydrophila***	***Aeromonas salmonicida ssp. achromogenes***	***Staphylococcus aureus***	***Edwardsiella tarda***	**Mixed culture** ***A. salmonicida ssp. salmonicida*** **and *Y. ruckeri***
Lab D Results	***Yersinia ruckeri*** **Type 1**	*Aeromonas hydrophila*	***Aeromonas salmonicida ssp. achromogenes***	*GPC*	*Edwardsiella tarda*	**A- *Y. ruckeri* (Type 1)** **B- *A. salmonicida***
API Profile	ND	ND	ND	ND	ND	ND
Confirmation Method / (Reagents)	**FAT (reagents and dilution not given) and PCR**		**FAT (Reagents not given) and PCR**			**FAT (reagents and dilution not given) and PCR**
Lab E Results	***Yersinia ruckeri***	*Aeromonas hydrophila*	***Aeromonas salmonicida ssp. achromogenes***	*Staphylococcus sp.*	*Edwardsiella tarda*	**A= *A. salmonicida*** **B= *Y. ruckeri***
API Profile	110410057 (48hr)	104752447 (48hr)	‘3A’=3000004 ‘3B”= 3000205	6732153	474400017 (48hr)	A=1414325 B=110410057 (48hr)
Confirmation Method / (Reagents)	**FAT (Microtek IFAT – 1:40) and PCR**		**FAT (Diagxotics 1:100) and PCR**			**FAT and PCR**

	Culture 1	Culture 2	Culture 3	Culture 4	Culture 5	Culture 6
	Yersinia ruckeri	***Aeromonas hydrophila***	***Aeromonas salmonicida ssp. achromogenes***	***Staphylococcus aureus***	***Edwardsiella tarda***	**Mixed culture** ***A. salmonicida ssp. salmonicida*** **and *Y.ruckeri***
Lab F Results	***Yersinia ruckeri***	*Aeromonas hydrophila*	***Vibrio alginolyticus***	*'Gram positive isolate'*	*Edwardsiella tarda*	**A=*A. salmonicida***
API Profile	5104100	3047527	0045124		4744000	
Confirmation Method / (Reagents)	**IFAT (reagents and dilution not given)**					**Bead Agglutination (Microtek – dilution not given)**
Lab G Results	***Yersinia ruckeri*** **Type 11.1**	*Aeromonas hydrophila*	***Aeromonas salmonicida ssp. salmonicida***	*Staphylococcus sp*	*Edwardsiella tarda*	**A=*A. salmonicida*** **ssp. *salmonicida*** **B=*Y. ruckeri* 11.4**
API Profile	Biochems by std tube methods	Biochems by std tube methods	Biochems by std tube methods	Biochems by std tube methods	Biochems by std tube methods	Biochems by std tube methods
Confirmation Method / (Reagents)	**FAT (Leetown FITC 1:40)**		**FAT (Microtek 1:40)**		**FAT**	**A-FAT** **B-FAT**
Lab H Results	***Yersinia ruckeri*** **Type 11.1**	*Aeromonas hydrophila*	***Aeromonas salmonicida ssp. salmonicida***	*Staphylococcus sp*	*Edwardsiella tarda*	**A=A. *salmonicida*** **ssp. *salmonicida*** **B=*Y. ruckeri* 11.4**
API Profile	5104100	4046627 6047526			6744000 1042000	
Confirmation Method / (Reagents)	**FAT (Microtek IFAT 1:40)**		**FAT (Diagxotics 1:100)**		**FAT**	**A=FAT**

[1] API 20E Profile (24 hr test unless otherwise noted)
[2] Reagents used for confirmation testing and working dilution (if given)

3.A1 Laboratory Reference Flow Chart for Identification of Gram-Negative Bacterial Pathogens Which Grow on TSA or BHIA and are Targeted for Detection to Complete Fish Health Inspection Requirements



* Cytochrome Oxidase negative strains of *A. salmonicida* have been encountered

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Ring Testing Discussion:

Six unknown bacterial samples were sent to each of the nine Fish Health Center laboratories of the U.S. Fish and Wildlife Service (USFWS) for laboratory ring testing. The samples consisted of four known bacterial fish pathogens, one Gram positive cocci, and a mixed culture. Eight of nine laboratories participated and completed testing in a satisfactory manner, with 8/8 (100%) of laboratories correctly identifying and confirming *Yersinia ruckeri*. For *Aeromonas salmonicida* subspecies (ssp.) *salmonicida*, the success rate was lower at 75% due to problems recovering this isolate from a mixed culture. This occurred for the submitting laboratory as well, but most likely the mixed culture was not a fair test of laboratory proficiency due to competition of the two bacteria. For *Aeromonas salmonicida* subspecies *achromogenes*, the success rate was lower at 62% but this appears to be due to problems with the viability of the atypical strain used, as well as the contradictory characteristics regarding pigmentation. Nearly all laboratories successfully identified the optional isolates of *E. tarda, A. hydrophila* and *S. aureus* to genus level, and most labs identified them to species level even though this was not required.

A. salmonicida subs. *achromogenes* appears to be biochemically different than what is stated in the SPAAHI manual. Further investigation was done regarding the SPAAHI protocols and the characteristics described by ATCC for this atypical isolate. It was determined that there is significant strain variation within this subspecies that can give alternative tests results for gelatinase, as well as the ability to produce brown pigmentation. It appears that there are various strains within the subsp. *salmonicidia* and *achromogenes* and current gelatinase and pigmentation characteristics may not adequately differentiate *A. salmonicida* strains or subspecies.

An additional error in the SPAAHI flowchart occurs for possible test results for OF Glucose. The possible test results for OF Glucose are currently described as

+ (K/AG, A/AG) *(and conversely)* - (other)

A test result of K is not appropriate for OF Glucose test. The correct reactions for an OF Glucose test result can be listed in terms of gas production (A, or AG) in but more appropriately should refer to the metabolic pathway and read as:

+ Oxidative/Fermentative *(or)* - Fermentative (only)

A further look into the biochemical variability for *A. salmonicida* subs. *achromogenes* should be evaluated by the SPAAHI manual revision committee and a clarification made to the flowchart for the OF Glucose test results. The OF test results can be reported in terms of acid production (A, AG), however the results in terms of metabolic pathway might be clearer, or the results could be reported with both sets of terminology.

In terms of administering the ring testing program, two additional suggestions are offered that relate to test sample preparation and testing time period. Several labs reported problems related to leaking sample containers during initial shipment. It is recommended that cultures used in future testing be provided on BHIA slants, versus the liquid medium used here, to prevent leakage during shipment. Laboratories receiving suspect or cross-contaminated samples should be encouraged to notify the submitting lab to request a new set of isolates to complete the ring testing exercise.

Participation overall was very good, with eight of nine laboratories reporting results. However, the time period from start to finish for this exercise exceeded two months. This was the first formal ring testing for the Fish Health Centers and many questions occurred regarding process and reporting expectations. For future testing, concise written instructions should accompany the test samples. It is also recommended that

the testing be completed within 2-3 weeks to simulate the standard time frame that Fish Health Centers normally conduct inspections for hatchery and aquaculture facilities.

Standard bacteriological protocols in SPAAHI do not require the use of additional tests such as API 20E biochemical test strips. Several laboratories do use this additional tool for presumptive identification of common fish pathogens, and therefore these results should be discussed. Table 3 lists detailed results for the API profiles obtained by the submitting lab prior to testing, and the profiles generated by labs during this exercise. The information is derived from the APIWeb website (http://industry.biomerieux-usa.com/industry/cosmetic/api/apiweb.htm#) which provides some notations, in addition to identity, including the percentage of significant taxa associated with a particular profile score.

Because API test strips were primarily designed for human and animal clinical microbiology, the protocols require incubation of test strips at 37ºC. API test strips have been used for fish pathogens for over 20 years by modifying the incubation protocol to room temperatures (20-25ºC). This is required to culture bacterial pathogens isolated from cool and cold water fish species. Profiles obtained at the modified temperatures have been validated through years of testing known bacterial isolates and control standards. Several common API profiles are listed for *Y. ruckeri* and *A. salmonicida* in the SPAAHI manual (Appendix 3.A2 – Profiles Obtained with API-20E for Known Fish Pathogens).

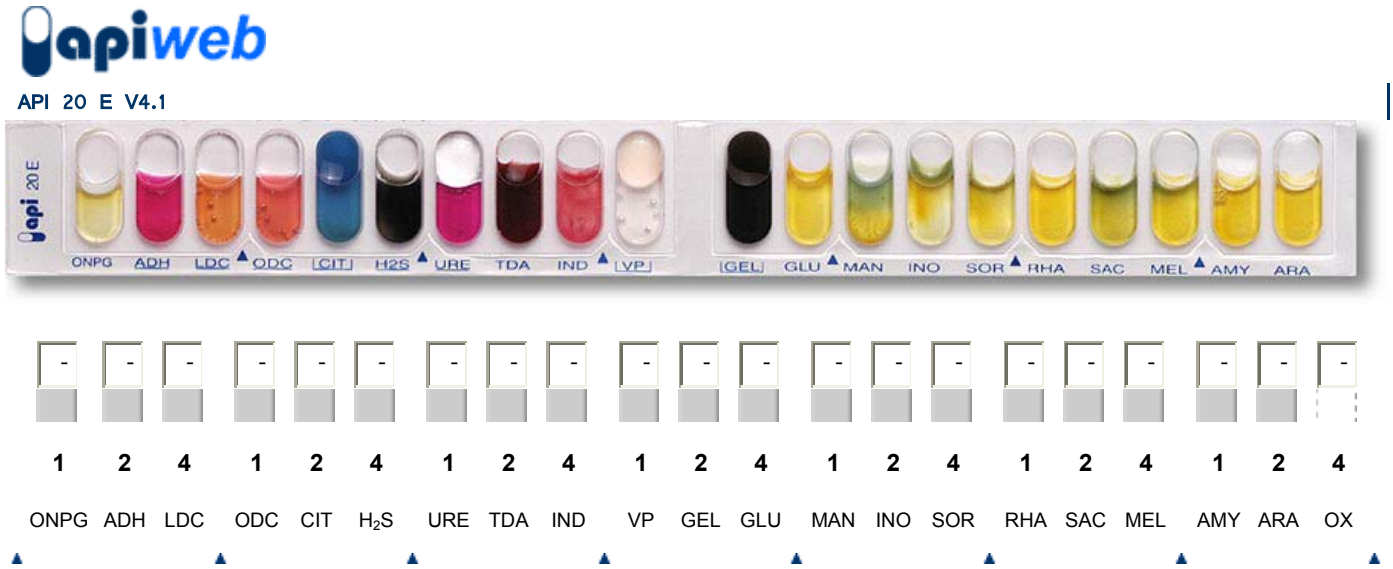
In this exercise, the API profile for *Y. ruckeri* consistently returned *Hafnia alvei*, with a notation of the possibility of *Yersinia ruckeri*. This is the most common identity produced by API for *Y.ruckeri* (Type 1) isolates.

The profile variability for *A. hydrophila* is fairly large. Despite this, the identifications are accurate with the exception of 2 unacceptable profiles generated by 2 labs. The unacceptable profile test results may reflect inexperience in using and interpreting the test strips, being as both of the labs did correctly identify the isolate despite the error with the API test.

The profiles for *A. salmonicida*, and particularly the subspecies *achromogenes*, are more problematic. The results are highly variable and most often produced identifications including *Vibrio alginolyticus, Pasteurella multocida* 1 or 2, *Pasteurella pneumotropica, or Vibrio fluvialis.* The most common profile given for *A. salmonicida* in the known profiles listed in SPAAHI is 0006104 which is identified as *Pseudomonas psuedomalia*. Other common variations of a 24-hour profile are 2006140, 4006104, and 6006104, all of which correctly identify as *A. salmonicida*. It is clear that much of the variation occurs in the first digit of the profile, generated by the ONPG, ADH, and LCD biochemical tests. Subjective and inaccurate interpretation of these specific test results can cause variation in the profile generated.

The profiles generated for *E. tarda* are also quite consistent in correctly identifying this bacterium, although some variability in specific biochemical tests does occur. The variability occurs in the ADH test, and is largely a result of interpreting subtle color reactions between yellowish-orange reactions (yellow is negative) and clearly orange to red (positive) reactions. The growth rate of an isolate, as well as the somewhat subjective color range of the test result can produce scores of 4 or 6 for the first profile digit. While the API instructions and positive color guide (shown below) make a clear distinction for this test, it is our lab's experience that the color produced is often intermediate at 24 hours.

The image below shows colors for a positive test result for all API 20E 24-hour capules, and the corresponding values assigned to each test that makes up the profile value. It is recommended that the guide be posted in the laboratory setting, and be used as a standard reference for interpreting color reactions.



Summary of Ring Testing Objectives

In summary, bacteriological ring testing conducted by the USFWS Fish Health Centers served several purposes, including:

- Demonstrated detection capabilities of eight Fish Health Centers, including presumptive identification and confirmatory methodology for the two primary fish pathogens *Yersinia ruckeri* and *Aeromonas salmonicida*.

- Identified two areas that require clarification in the current SPAAHI bacteriological flowchart regarding *A. salmonicida* subspecies *achromogenes* and Oxidative-Fermentative test results.

- Reinforced the need to follow currently approved SPAAHI protocols (FAT) for bacteriological confirmation testing.

- Identified the limited source of manufacturer's producing much needed serological reagents that are required for bacteriological confirmatory testing.

- Identified a need to review, or provide training in, interpretation of API 20E biochemical test strips (and recommended referencing a positive control chart to provide clarity and consistency in reporting positive color reactions).

Table 3 - API 20E Biochemical Profiles Generated During Laboratory Ring Testing

	Culture 1	Culture 2	Culture 3	Culture 4	Culture 5	Culture 6A	Culture 6B
Ring Testing Culture ID	***Y. ruckeri*** **ATCC 29473**	***A. hydrophila*** **ATCC 7965**	***A. salmonicida ssp. achromogenes*** **ATCC 33659**	***S. aureus***	***E. tarda*** **ATCC 23659**	**Mixed Culture** ***Y. ruckeri*** **ATCC 29473**	**Mixed Culture** ***A. salmonicida ssp. salmonicida*** **ATCC 33658**
Incubation Temp[1]	20-25ºC	20-25ºC	20-25ºC	37ºC	37ºC	20-25ºC	20-25ºC
API 20E 24 hr Profiles		**4046627** *Aeromonas hydrophila* (98%)	**0046124** *Vibrio alginolyticus* (92.9%)		**6744000**[1] *Edwardsiella tarda* (99.4%)		**6006104** ***A. salmonicida ssp. salmonicida*** **(99.5%)**
Lab Results	**5104100** **5104100** **5104100** **5104100** *Hafnia alvei* (99%) Possibility of *Y. ruckeri* **1104100-57** (48hr) *E. coli* (78%) *H. alvei* (9%)	**3047527** ***A. hydrophila*** (96%) **4046627** ***A. hydrophila*** (98%) **6047526** ***A. hydrophila*** (74%) **7247527** *A. hydrophila* (85%) **7007527** *A. hydrophila* (66%) Possibility of *V.fluvialis* **7577755** Unacceptable profile **1047524-47** (48hr) Unacceptable profile	**0045124** **0045124** *V. alginolyticus*(67%) *Pastuerella pneumotropica* (24%) **0040124** *Pasteurella multocida* 1 (51%) *Pasteurella multocida* 2 (42%) **5046644** Possibility of *V. fluvialis* **3000004** *A. salmonicida ssp. salmonicida* **3000205** Unacceptable profile	**2012000** *Pseudomonas aeruginosa* (90%) **6732153** Unacceptable ID	**4544000** *E. tarda* (100%) **4744000** **4744000** **4744000** *E. tarda* (99%) **4744000-17** (48hr) *E. tarda* (99%) **4754000** *E. tarda* (89%) **6744000** *E. tarda* (99%)	**1104100** *Hafnia alvei 2* (57%) Possibility of *Y. ruckeri* (confirm by serological tests) **1104100-57** (48hr) Identification not valid (Possibility of Erwinia sp., Possibility of *Y. ruckeri*) **5046644** *P. pneumotropica* Possibility of *V. fluvialis*	**6006104** (99%) **0006104** *A. salmonicida ssp. salmonicida* (99%) **1414325** Unacceptable profile
Note	***Hafnia alvei*** **is the most common identification produced for known Y.ruckeri standards when testing is done at** 20-25ºC		***A.salmonicida ssp. achromogenes*** **produced variable profiles with identifications of Vibrio or Pasteurella ssp. or unacceptable profiles/ID**		**[1]Profile provided in AFS Blue Book (2007 Edition)**	**Same *Y. ruckeri* isolate as Culture1 (variability may be do to incomplete isolation from mixed culture)**	

[1] API 20E biochemical test strips are designed to be incubated at 37 ºC for testing human and animal diagnostics. For cool and coldwater fish species, modification of the standard temperature protocol is required and test strips are incubated at 20-25 ºC. API 20E profiles for known fish pathogens have been generated to provide alignment with anticipated profiles produced at the recommended incubation temperatures (SPAAHI, Appendix 3.a.2).