Development of antimicrobial susceptibility testing methods for *Flavobacterium columnare* and *F. psychrophilum*

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WHY?

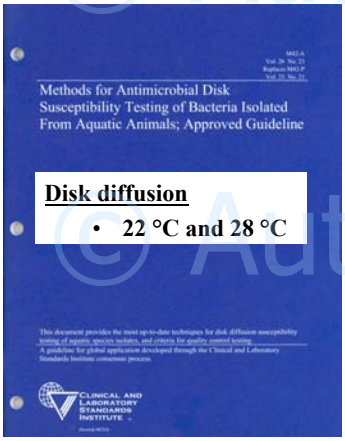
Recent Drugs Approvals (U.S.)

Product	Species	Indication
AquaFlor® (Florfenicol) Intervet Schering-Plough	Catfish	Control of mortality due to columnaris disease associated with ***F. columnare*** (conditional approval) & Control of mortality due to coldwater disease associated with ***F. psychrophilum***
	Freshwater-reared Salmonids	
Terramycin® 200 (Oxytetracycline dihydrate) Philbro Animal Health	Freshwater-reared Salmonids	Control of mortality due to coldwater disease caused by ***F. psychrophilum*** & Control of mortality due to columnaris disease (***F. columnare***)
	All freshwater-reared Oncorhynchus mykiss	

Need standard methods to test drug sensitivity

Method development

M42-A

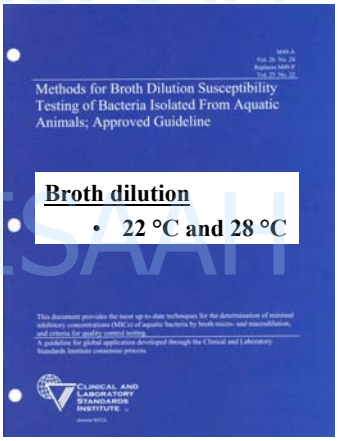


Disk diffusion

- **22 °C and 28 °C**

Methods & QC parameters

M49-A



Broth dilution

- **22 °C and 28 °C**

Aeromonas salmonicida subsp. *salmonicida*
ATCC 33658

Escherichia coli
ATCC 25922

Flavobacteria

- Gram-negative aerobic rods
- 2-5 um long, 0.3-0.5 um wide, rounded or tapered ends
- Gliding motility
- Fastidious – low nutrient requirements
- yellow (cream to orange) colonies on agar

F. columnare

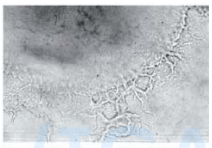


Figure 3. Light microscopy (40×) of *Flavobacterium columnare* colony onto Anacker and Ordal medium.

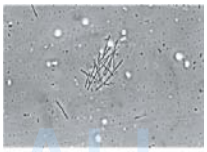
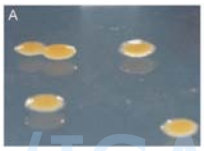
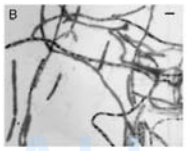


Figure 2. Wet mount strain (100×) of long bacilli *Flavobacterium columnare* isolates.

Pilarski et al. 2008

F. psychrophilum





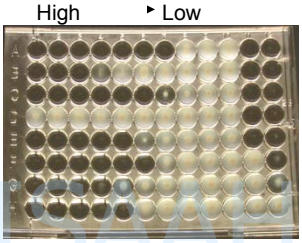
Elizabeth Crump

Antimicrobial Susceptibility Testing

Broth dilution

High ▸ Low

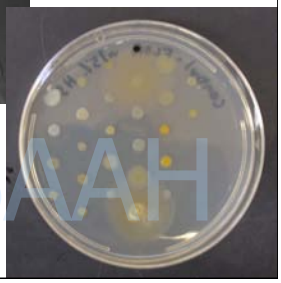
Disk Diffusion



Agar dilution

2- fold dilutions

◂ ◂ ◂ ◂



stock ▾ ▾ ▾ ▾

High conc. ▸ Low conc.

Organism	Medium	Incubation
Group 1:Non fastidious bacteria		
Enterobacteriacae *Aeromonas salmonicida* (nonpsychrophilic strains) *Aeromonas hydrophila* and other mesophilic Aeromonads *Pseudomonas* spp. *Plesiomonas shigelloides* *Shewanella* spp. Vibrionaceae and related bacteria (non obligate halophilic strains)	CAMHB	22°C for 24-28 hours and/or 44 -48 hours, or 28ºC for 24-28 hours
Group 2: Vibrionaceae and Photobacteriaceae (obligate halophilic strains)		
Group 3: Gliding bacteria		
Flavobacterium columnare	Diluted CAMHB (1:7)	28°C (24-28 hours and/or 44 -48 hours
Flavobacterium psychrophilum *Flavbacterium branchiophilum*	Diluted CAMHB (1:7)	15°C (44-48 hours and/or 68 -72 hours
	Recommended supplementation cannot be made but may include cations, NaCl, or horse or fetal calf serum.	

Group 4: Streptococci
Lactococcus spp., *Vagococcus salmoninarum*, *Streptococcus* spp., *Carnobacterium maltaromaticum*, and other streptococci

Group 5: Other fastidious bacteria
Psychrophilic *Aeromonas salmonicida* strains, *Vibrio salmonicida*, *Mortella viscosa*, *Tenacibaculum maritimum*, *Renibacterium salmoninarum*, *Mycobacterium* spp. and *Nocardia seriolae*.

Darwish et al., 2008 – broth microdilution of *F. columnare* with 1:5 CAMHB (4g/L)

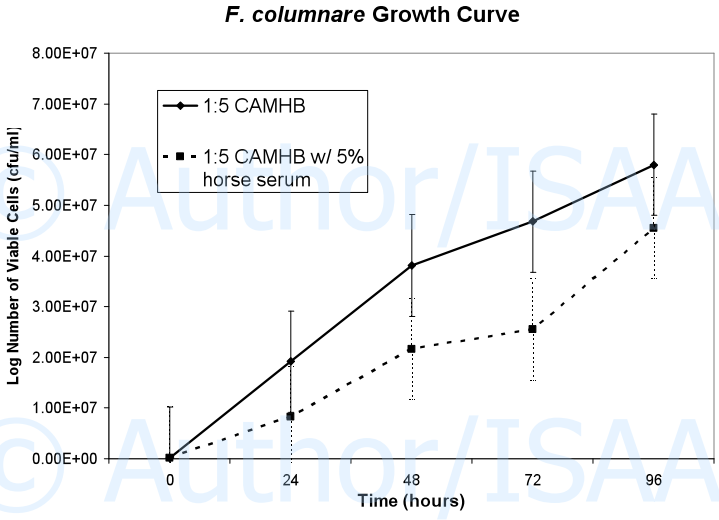
Outline - methods

- broth microdilution
- 1:5 dilute cation adjusted Mueller-Hinton broth (dilute cations)
- *F. columnare*: 28°C, 48 hrs
- *F. psychrophilum*: 18°C, 96 hrs

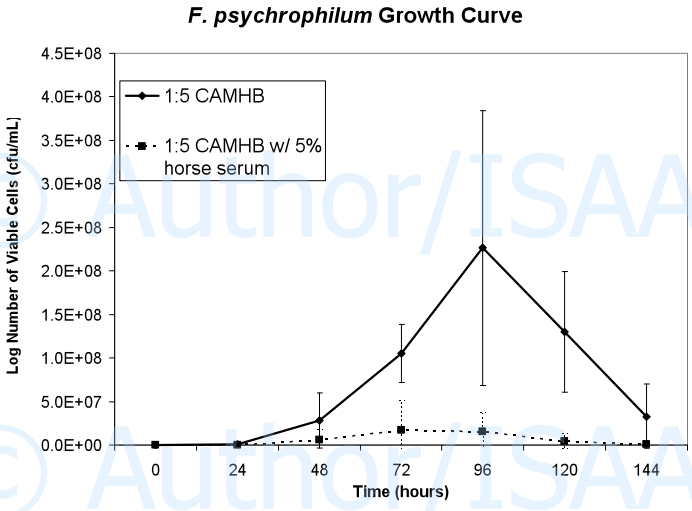
Outline – Method Development

- **Growth curves**
  - Are the incubation times appropriate?
- **Methods for making standard inoculums**
  - Adaptations for fastidious gliding bacteria.
- **Preliminary broth microdilution testing**
  - Will the QC bacteria work for testing these Flavobacteria?
- **QC stability**
  - Are the QC bacteria stable in the modified testing conditions?
- **Agar dilution**
  - Confirm testing results with the gold standard method. Are the results similar?
- **Multi-user Trial**
  - Do different technicians get similar results?

Growth curves



Growth curves



Standard inoculum

Broth culture

Standard cell suspension

Inoculated broth

Agar plate

Problem – *F. columnare*

Cain & LaFrentz, 2007

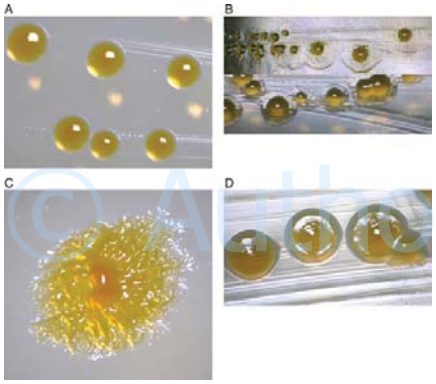


Figure 13B.1.1 Morphology of F. psychrophilum colonies exhibiting a smooth margin (**A**), F. psychrophilum colonies exhibiting thin spreading margins ("fried-egg") (**B**), F. columnare colony exhibiting a characteristic flat rhizoid colony with an irregular margin (**C**), and F. columnare colonies exhibiting smooth margins (**D**). For a color version of this figure, see http://www.currentprotocols.com

Sticky colonies – adheres to media and clumps in solution – hard to make homogeneous, reliable cell suspensions.

Growth media – initial cultures & colony counts

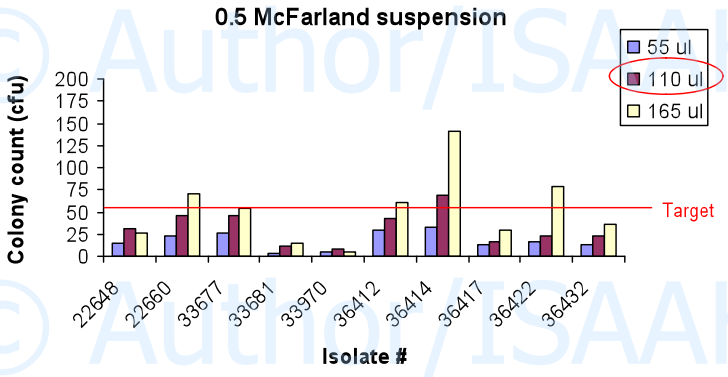
Isolate	Media	% between 100,000 and 900,000 cells
F. columnare - agar		
	CA	47
	eCA	40
	TYE	67
	TYES	20
F. columnare - broth		
static, 3ml 24hrs	CA	53
	eCA	60
	TYE	60
	TYES	73
F. psychrophilum - agar		
	CA	83
	TYE	0
	TYES	50
F. psychrophilum - broth		
static, 3ml 72hrs	CA	0
	TYE	0
	TYES	83

F. columnare

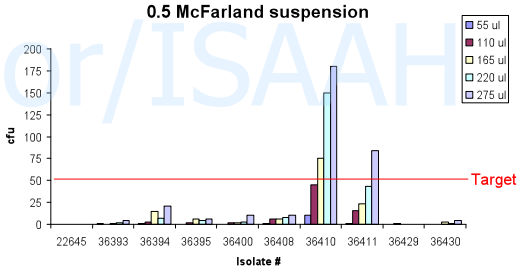
Vortex suspension, let it settle a couple minutes, then remove upper portion and dilute if needed.

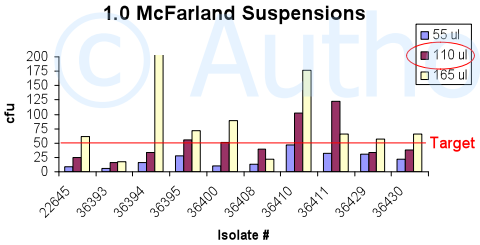
F. columnare suspensions

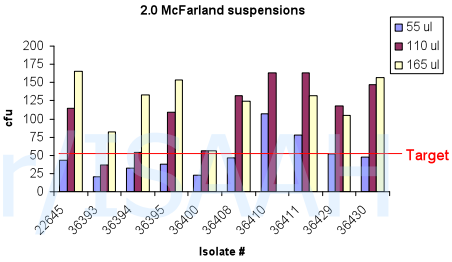
Ideally,
- 0.5 McFarland = 1.5 x 10^8 cells
- 55µl in 11ml CAMHB = 5 x 10^5 cells
- Colony count, 10^4 dilution = 50 cfu



F. psychrophilum suspensions







Good Results:

- 1 McFarland, 110µl into 11ml CAMHB
- 2 McFarland, 55µl into 11ml CAMHB

Choose 1 McFarland - less possible error

Broth microdilution

cell suspension

Broth culture

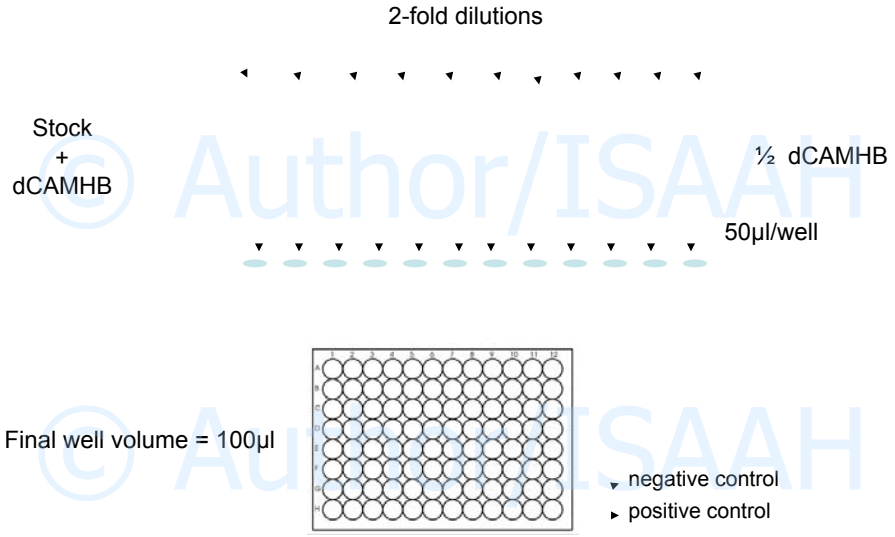
Agar plate

Inoculated broth

incubator

F. columnare: 28°C, 48hrs
F. psychrophilum: 18°C, 96hrs

Custom Frozen Broth Microdilution Plates



F. columnare

Isolate	Colony count	Enrofloxacin	Erythromycin	Flumequine	Florfenicol	Gentamicin	Oxytetracycline	Oxolinic acid	Sulfadimethoxine / ormetoprim	Sulfamethoxazole / trimethoprim
22648	36	0.008	0.12	0.06	0.5	1	0.06	0.12	19/1	4.75/0.25
22650	64	0.004	0.25	0.06	1	1	0.06	0.12	19/1	4.75/0.25
22655	45	0.06	8	2	>16	>8	32	1	19/1	4.75/0.25
22683	28	0.015	0.25	0.06	1	0.5	0.12	0.12	19/1	9.5/0.5
33677	25	0.015	0.25	0.06	2	0.5	0.12	0.12	9.5/0.5	4.75/0.25
33681	53	0.008	0.12	0.06	0.5	0.5	0.06	0.12	9.5/0.5	4.75/0.25
33970	13	0.008	0.25	≤0.004	1	1	0.06	0.12	9.5/0.5	4.75/0.25
33971	10	0.008	0.12	0.06	1	0.25	0.06	0.12	2.38/0.12	0.08/0.004
36413	30	2	64	8	>16	0.25	0.25	>4	152/8	38/2
36416	16	2	64	4	16	>8	2	2	>152/8	76/4
36417	25	0.008	0.25	0.06	1	1	4	0.12	9.5/0.5	19/1
36422	52	0.015	0.25	0.12	2	1	0.12	0.12	9.5/0.5	4.75/0.25
36423	30	0.015	1	0.12	2	1	0.12	0.12	19/1	9.5/0.5
36425	47	0.008	1	0.06	2	1	0.12	0.12	19/1	9.5/0.5
E. coli, ATCC 25922		0.002 - 0.004	8 - 16	0.06 - 0.25	4 - 8	0.25 - 0.5	0.5 - 1	0.06 - 0.12	4.75 / 0.25	0.59/0.03 - 1.19/0.06
A. salmonicida, ATCC 33658		0.008	2 - 4	0.03 - 0.06	1	0.12 - 0.25	0.25 - 0.5	0.015	2.38/0.12	0.59/0.03 - 1.19/0.06

F. psychrophilum

Isolate	Colony count	Enrofloxacin	Erythromycin	Flumequine	Florfenicol	Gentamicin	Oxytetracycline	Oxolinic acid	Sulfadimethoxine / ormetoprim	Sulfamethoxazole / trimethoprim
36391	5	0.004	0.5	0.03	0.25	0.06	0.03	0.06	1.19/0.06	0.59/0.03
36392	7	0.004	0.5	0.03	0.5	0.25	1	0.06	2.38/0.12	0.59/0.03
36395	21	0.03	2	8	1	0.5	0.06	0.12	4.75/0.25	2.38/0.12
36396	34	0.06	1	0.5	1	0.25	0.06	2	4.75/0.25	2.38/0.12
36397	26	0.004	1	0.03	1	0.12	4	0.06	9.5/0.5	2.38/0.12
36398	3	0.004	0.5	0.03	0.5	0.12	≤0.015	0.06	1.19/0.06	0.59/0.06
36400	51	0.008	1	0.06	1	0.5	4	0.25	4.75/0.25	2.38/0.12
36401	3	0.12	0.5	2	0.25	0.06	≤0.015	1		
36403	17	0.008	0.5	0.06	0.5	0.12	16	0.12	2.38/0.12	1.19/0.06
36404	17	0.008	0.5	0.06	0.5	0.25		0.06	9.5/0.5	1.19/0.06
36408	33	0.008	1	0.06	1	0.5	16	0.12	9.5/0.5	1.19/0.06
36409	66	0.008	1	0.06	1	0.5	0.06	0.12	4.75/0.25	1.19/0.06
36410	120	1	1	8	1	0.5	8	>4	38/2	9.5/0.5
36411	79	0.25	1	8	1	0.12	4	4	4.75/0.25	2.38/0.12
36429	81	0.015	2	0.06	1	0.25	0.06	0.12	1.19/0.06	0.59/0.03
36430		0.008	2	0.06	1	0.25	0.06	0.12		0.08/0.004
E. coli, ATCC 25922		≤0.001	4	0.06 - 0.12	16	0.06 - 0.25	0.5	0.06	9.5/0.5 - 4.75/0.25	1.19/0.06
A. salmonicida, ATCC 33658		0.008	4	0.03	0.5 - 1	0.12 - 0.25	0.25	0.015	2.38/0.12	1.19/0.06

QC stability

28° C		Colony count	Enrofloxacin (0.001-2mg/mL)	Erythromycin (0.06-128mg/mL)	Flumequine (0.06-128mg/mL)	Florfenicol (0.008-16mg/mL)	Gentamicin (0.004-8mg/mL)	Oxytetracycline (0.015-32 mg/mL)	Oxolinic acid (0.002-4mg/mL)
E. coli, ATCC 25922	5th pass	17	0.004	4	0.12	4	0.5	1	
			0.004	4	0.12	8	0.25	1	0.03
			0.004	8	0.12	8	0.5	1	0.03
			0.004	8	0.12	8	0.25	1	0.03
	10th pass	22	0.004	32	0.12	8	0.25	1	0.06
			0.008	16	0.25	8	0.5	1	0.12
			0.004	16	0.12	8	0.5	1	0.06
			0.004	16	0.12	8	0.5	1	0.06
	15th pass	24	0.004	16	0.12	8	0.25	2	0.12
			0.008	16	0.12	8	0.5	2	0.12
			0.004	16	0.25	8	0.5	2	0.12
			0.004	16	0.12	8	0.25	2	0.12
	20th pass	30	0.004	16	0.12	8	0.5	1	0.12
			0.004	16	0.25	8	1	1	0.12
			0.004	16	0.25	8	0.25	1	0.12
			0.004	16	0.25	8	0.25	1	0.12
	25th pass	11	0.004	8	0.12		0.25	1	
			0.004	8	0.12		0.25	1	
			0.004	8	0.12		0.25	1	
			0.004	8	0.12		0.25	1	
	30th pass	53	0.004	16	0.12	8	0.25	1	0.06
			0.004	16	0.12	8	0.5	1	0.12
			0.004	16	0.25	8	0.5	1	0.12
			0.004	16	0.25	8	0.5	1	0.12

QC stability

28° C		Colony count	Enrofloxacin (0.001-2mg/mL)	Erythromycin (0.06-128mg/mL)	Flumequine (0.06-128mg/mL)	Florfenicol (0.008-16mg/mL)	Gentamicin (0.004-8mg/mL)	Oxytetracycline (0.015-32 mg/mL)	Oxolinic acid (0.002-4mg/mL)
A. salmonicida subsp. salmonicida, ATCC 33658	5th pass	16	0.008	8	0.03	1	0.5	0.5	0.008
			0.008	16	0.03	1	0.25	0.5	0.008
			0.008	32	0.03	1	0.25	0.5	0.015
			0.008	16	0.03	1	0.12	0.5	0.015
	10th pass	19	0.008	32	0.03	1	0.5	0.25	0.015
			0.008	32	0.03	1	0.5	0.25	0.015
			0.008	16	0.03	1	0.5	0.25	0.015
			0.008	32	0.03	0.5	0.25	0.25	0.015
	15th pass	20	0.008	4	0.03	1	0.25	0.25	0.015
			0.008	4	0.03	1	0.5	0.5	0.015
			0.008	4	0.03	1	0.5	0.5	0.015
			0.008	4	0.03	1	0.5	0.5	0.015
	20th pass	14	0.004	4	0.03	1	0.25	0.25	0.015
			0.008	4	0.03	1	0.25	0.25	0.015
			0.008	4	0.03	1	0.25	0.5	0.015
			0.008	4	0.03	1	0.25	0.5	0.015
	25th pass	14	0.008	4	0.03		0.25	0.5	
			0.008	4	0.03		0.25	0.5	
			0.008	4	0.06		0.25	0.5	
			0.008	2	0.03		0.25	0.5	
	30th pass	15	0.008	8	0.06	1	0.5	0.5	0.015
			0.008	8	0.06	1	0.25	0.25	0.015
			0.008	8	0.06	1	0.5	0.25	0.015
			0.008	8	0.06	1	0.5	0.25	0.015

QC stability

18° C		Colony count	Enrofloxacin (0.001-2mg/mL)	Erythromycin (0.06-128mg/mL)	Flumequine (0.06-128mg/mL)	Florfenicol (0.008-16mg/mL)	Gentamicin (0.004-8mg/mL)	Oxytetracycline (0.015-32 mg/mL)	Oxolinic acid (0.002-4mg/mL)
E. coli, ATCC 25922	5th pass	24	≤0.001	4	0.12	16	0.06	0.5	0.06
			0.002	4	0.12	16	0.12	1	0.06
			≤0.001	4	0.12	16	0.12	0.5	0.12
	10th pass	21	≤0.001	4	0.12	16	0.12	0.5	0.06
			≤0.001	4	0.12	16	0.12	0.5	0.06
			≤0.001	4	0.12	16	0.12	0.5	0.06
			≤0.001	4	0.12	16	0.12	0.5	0.06
			≤0.001	4	0.12	16	0.12	0.5	0.06
	15th pass	10	≤0.001	2	0.06	16	0.06	0.5	0.06
			≤0.001	2	0.06	16	0.12	0.5	0.06
			≤0.001	4	0.06	16	0.06	0.5	0.06
			≤0.001	4	0.06	16	0.06	0.5	0.06

18° C		Colony count	Enrofloxacin (0.001-2mg/mL)	Erythromycin (0.06-128mg/mL)	Flumequine (0.06-128mg/mL)	Florfenicol (0.008-16mg/mL)	Gentamicin (0.004-8mg/mL)	Oxytetracycline (0.015-32 mg/mL)	Oxolinic acid (0.002-4mg/mL)
A. salmonicida subsp. salmonicida, ATCC 33658	5th pass	22	0.008	4	0.06	1	0.5	0.5	0.03
			0.008	4	0.06	1	0.5	0.5	0.03
			0.008	4	0.06	1	0.5	0.5	0.03
			0.015	4	0.06	1	0.12	0.25	0.03
	10th pass	14	0.008	4	0.03	1	0.25	0.25	0.015
			0.008	4	0.03	1	0.25	0.25	0.015
			0.008	4	0.03	1	0.12	0.25	0.015
			0.008	4	0.03	1	0.12	0.25	0.015
	15th pass	38	0.008	4	0.03	1	0.25	0.25	0.015
			0.008	4	0.03	1	0.25	0.25	0.015
			0.008	4	0.03	1	0.25	0.25	0.015
			0.008	4	0.03	1	0.12	0.25	0.015

Confirmation – Agar Dilution

***F. columnare*, 28°C**

Isolate	Florfenicol - BMD	Florfenicol - AD w/5% horse serum
22648	1	1
22650	2	1
22655	2	2
22657	>32	64
22660	2	2
22683	2	1
33677	2	1
33681	0.5	0.5
33970	2	1
33971	1	1
36412	>32	64
36413	32	>64
36414	>32	>64
36415	1	2
36416	32	64
36417	1	1
22659	1	1
36422	1	2
36423	2	4
36425	2	2
36428	0.12	2
36432	0.5	0.5
36433	2	2
33682	1	1
11-1 E. coli	8	16
13862 A. salm.	1	1

***F. psychrophilum*, 18°C**

Isolate	Florfenicol - BMD	Florfenicol - AD w/5% horse serum
36391	0.25	0.25
36392	0.25	0.25
36393	0.25	0.25
36394	0.5	0.25
36395	0.25	0.5
36396	0.5	1
36397	0.5	0.5
36398	0.25	0.25
36400	0.5	0.25
36401	0.25	0.25
36402	0.25	≤0.12
36403	0.5	1
36404	0.25	0.25
36406	0.5	0.5
36408	0.25	1
36409	0.5	1
36410	0.5	1
36411	0.25	1
36429	0.25	0.25
36430	0.25	≤0.12
36431	0.25	0.5
36439	0.25	0.5
36440	0.25	1
36441	0.25	<0.12
11-1 E. coli	≥16	32
13862 A. salm.	0.5	1

Multi-user Trial

- Intra laboratory testing
- 4 separate technicians
- 4 drugs: Ampicillin, Oxytetracycline, Florfenicol, Trimethoprim/Sulfamethoxazole
- *F. columnare*, n=6
- *F. psychrophilum*, n=6

	Isolate #	Antimicrobial	% agreement with mode	% agreement within 1 dilution of mode
F. columnare	22650	Ampillicin	100	100
	22655		83.3	100
	22683		91.7	100
	33971		91.7	100
	36413		83.3	100
	36434		50	66.7
	22650	Florfenicol	75	100
	22655		75	100
	22683		91.7	100
	33971		50	100
	36413			
	36434		91.7	100
	22650	Oxytetracycline	91.7	100
	22655		83.3	100
	22683		91.7	91.7
	33971		83.3	100
	36413		100	100
	36434		100	100
	22650	Sulfadimethoxine / ormetoprim	75	100
	22655		75	100
	22683		58.3	75
	33971		58.3	100
	36413		25	50
	36434		58.3	100

	Isolate #	Antimicrobial	% agreement with mode	% agreement within 1 dilution of mode
F. psychrophilum	36391	Ampillicin	58.3	58.3
	36396		50	100
	36400		91.7	100
	36403		58.3	91.7
	36410		100	100
	36429		50	91.7
	36391	Florfenicol	75	100
	36396		75	100
	36400		66.7	100
	36403		50	91.7
	36410		75	100
	36429		50	100
	36391	Oxytetracycline	66.7	91.7
	36396		58.3	83.3
	36400		75	91.7
	36403		91.7	91.7
	36410		100	100
	36429		83.3	91.7
	36391	Sulfadimethoxine / ormetoprim	75	91.7
	36396		50	83.3
	36400		58.3	91.7
	36403		50	83.3
	36410		50	75
	36429		41.7	75

Conclusion

- Reliable methods
  - QC bacteria stable in modified testing conditions
  - Broth microdilution results confirmed by agar dilution
  - Similar results among different technicians
- CLSI Standardization Trial
  - To set expected MIC ranges for QC bacteria at conditions needed to test *F. columnare* & *F. psychrophilum*
    - Dilute broth (4g/L)
    - Custom commercial broth microdilution plates
    - Temperature & incubation times
  - 10 drugs from M42 & M49 guidelines
  - 11 international laboratories
  - 10 replicates: both QC bacteria tested in 3 separate lots of media

Questions?