Joint Research Program
To Assess and Improve Reliability of AOAC Use Dilution Test Method with *Pseudomonas aeruginosa*

October 24, 2006

Presentation overview

- EPA requires Association of Official Analytical Chemists (AOAC) Use Dilution Test Method (UDT) to demonstrate hospital disinfectant product efficacy

- For 20+ years the UDT has attracted criticism due to deficiencies inherent in the method – all lead to variable test outcomes

- Industry, through CSPA has worked with AD to improve the UDT – but focused on different issues

- This program provides new scientific data

Presentation overview (cont)

- For the QAC RED DCI, EPA and industry need a robust and reliable test method

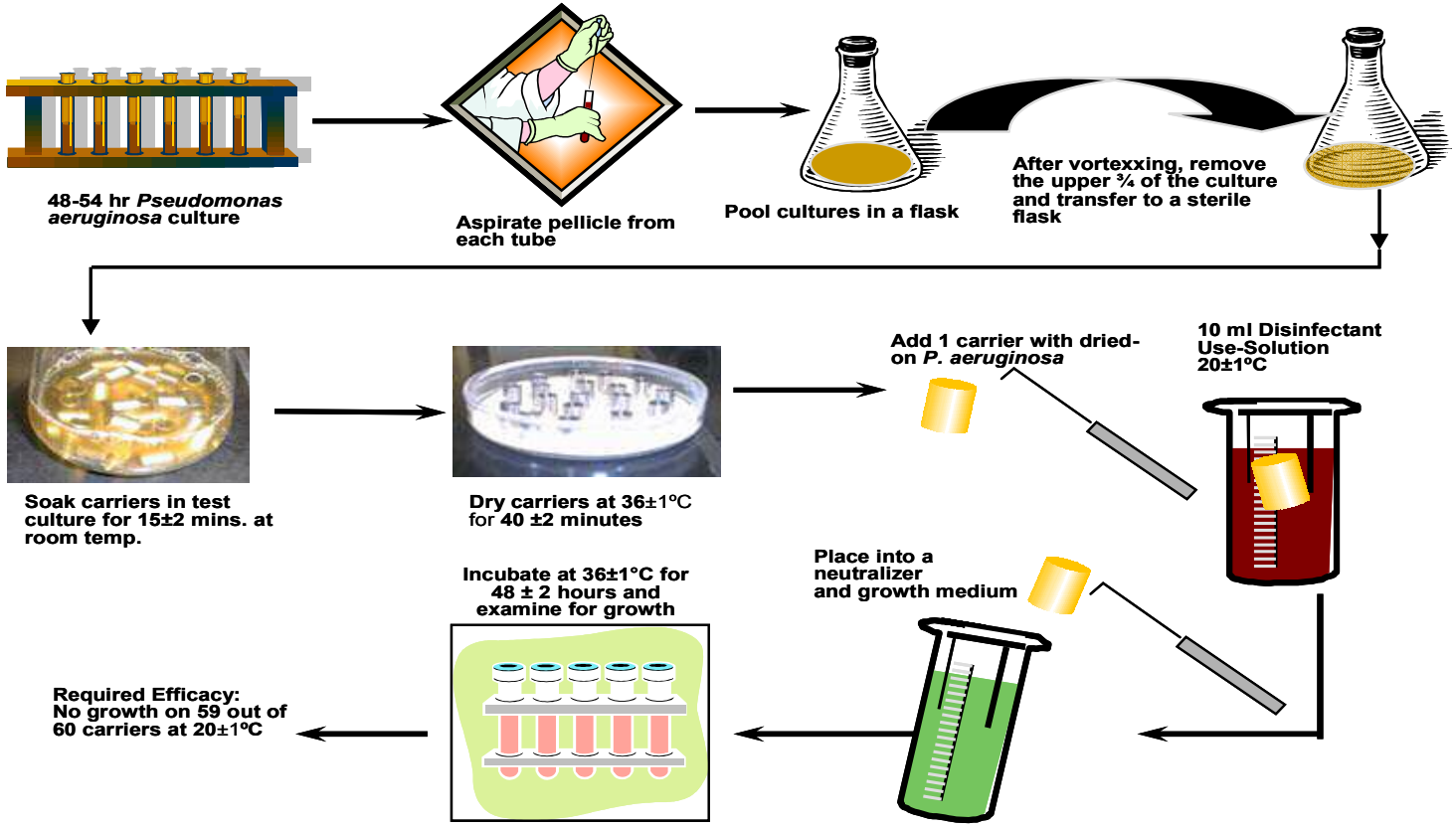
- Both EPA and industry need a robust and reliable test method for enforcement testing

Presentation overview (cont)

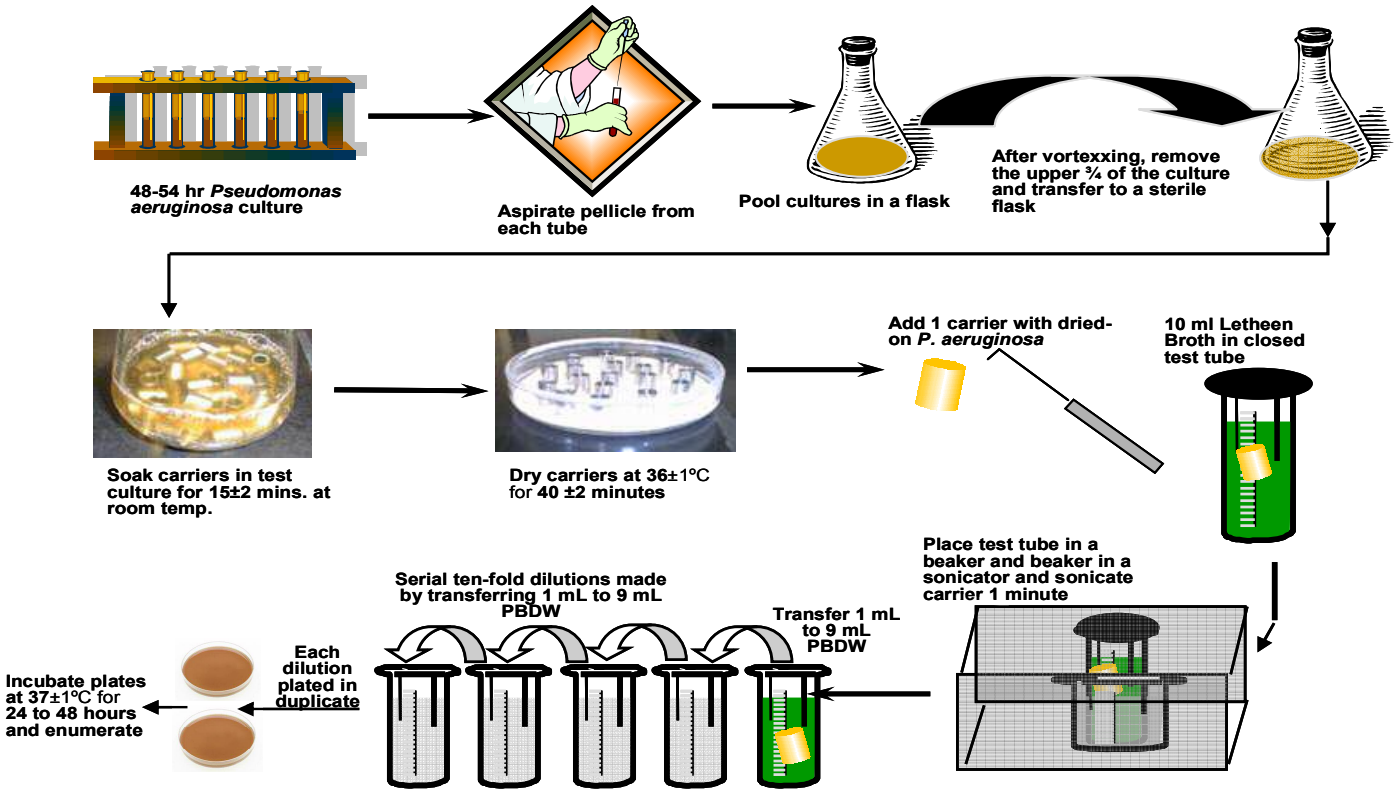
- Ecolab and Lonza initiated a joint internal research program to:
  - Identify the most significant sources of variability in the method
  - Identify ways to reduce these sources of variability

- Internal and external data generated in this effort show that significant improvements in the robustness and reliability of the UDT can be achieved when minor clarifications to the current AOAC method are implemented

Overview of AOAC UDT Method



Overview of AOAC Carrier Count Procedure for UDT



Why clarify the AOAC UDT Method?

- "…any pellicle fragment remaining will result in uneven clumping and layering of organism on the cylinders…causing false positive results." (AOAC Method 964.02 - disinfectant testing with *Pseudomonas aeruginosa*)

- Ecolab and Lonza's investigations suggest that pellicle fragment retention in *Pseudomonas aeruginosa* cultures represents a significant source of variability in the method
  - Published literature (Cole et. al., 1989*) supports this thinking
  - Therefore joint testing focused just on methods to minimize interference of *Pseudomonas aeruginosa* pellicle fragments on test outcomes

*Applied and Environmental Microbiology, Feb. 1989, p. 511-513

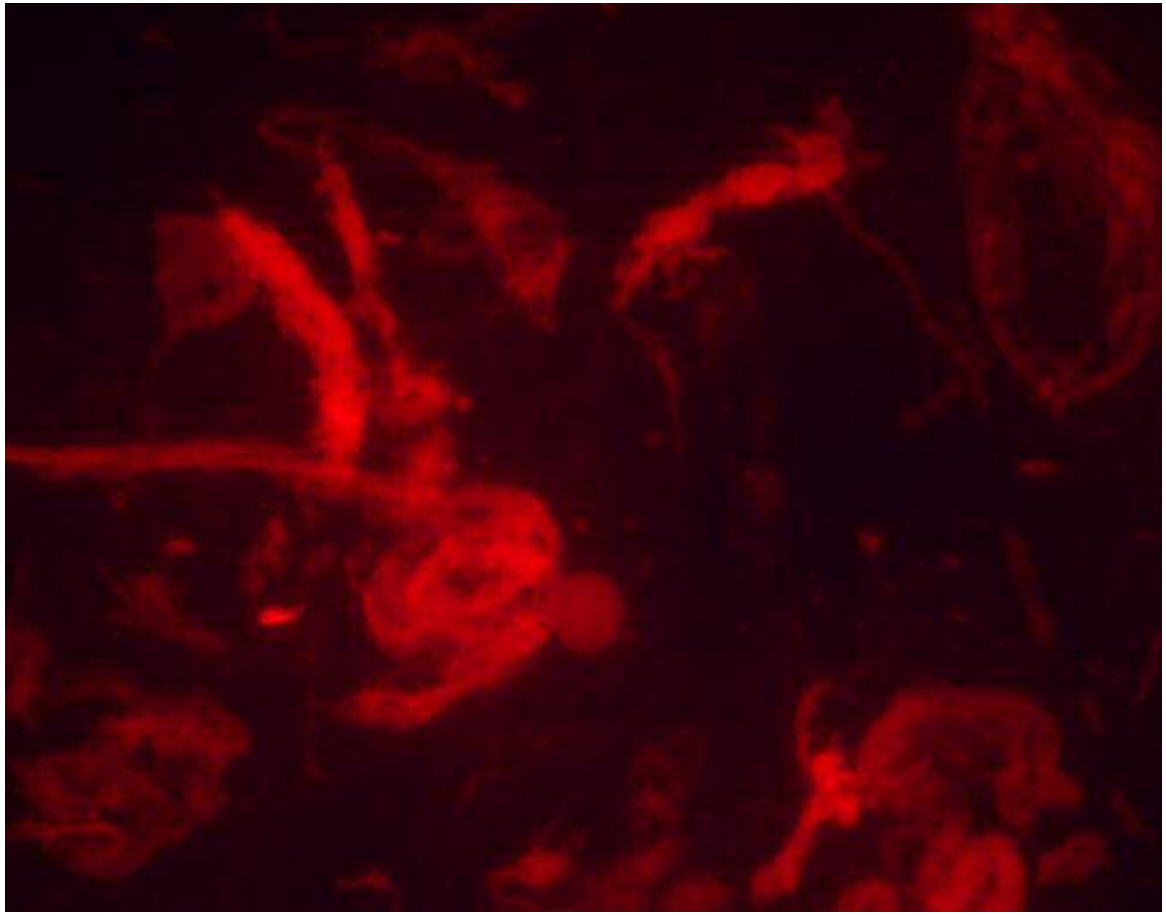
What is pellicle?

- Ecolab and Lonza's evaluations extended into the makeup of *Pseudomonas aeruginosa* pellicle and potential factors that might impact it
  - Pellicle is 75% extra-cellular polysaccharide (EPS)

- Analytical methods for EPS reference solubility in water
  - Methods for EPS extraction are based on water solubilization

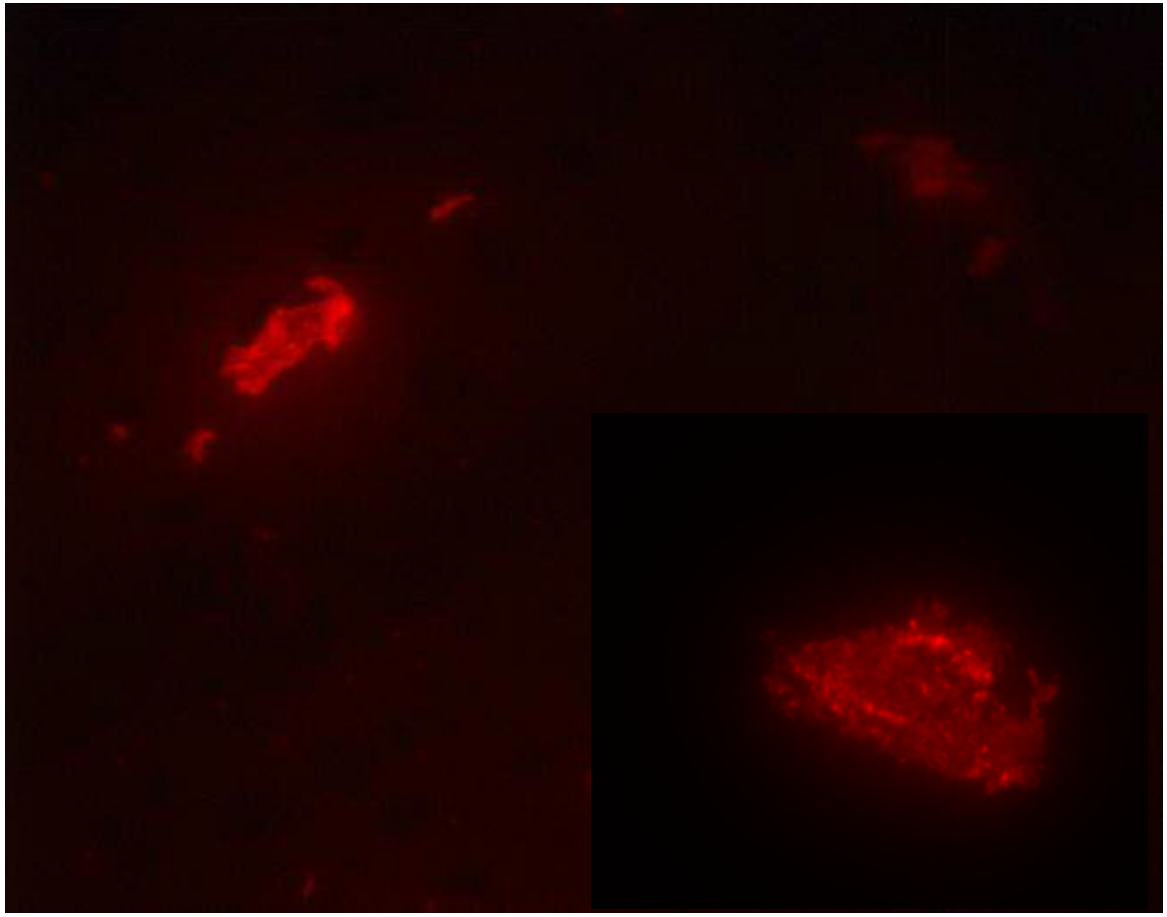
- Residual EPS fragments frequently remain in neat culture after the pellicle aspiration step

Actual pellicle (extra-cellular polysacharide) fragments at 400x fluorescent microscopy



- System preparation using Concanavalin A Texas Red
- Bacteria are present but not visible
- Pellicle materials are present from hundreds of microns to sub micron fragments

Residual pellicle fragments in neat culture post removal



- System preparation using Concanavalin A Texas Red
- Pellicle debris can be easily found in samples prepared in a conventional hospital disinfectant test system preparation
- Any fragment containing bacteria could translate to a positive carrier
- Particles range in size and shape

Potential methods for reducing pellicle fragment interference

- A number of possible techniques were evaluated in an attempt to reduce pellicle fragment interference
  - culture filtration
  - pellicle aspiration
  - double decant of culture
  - pellicle aspiration & double decant
  - pellicle attenuation through solubilization

- Only pellicle attenuation yielded robust, reliable and reproducible results

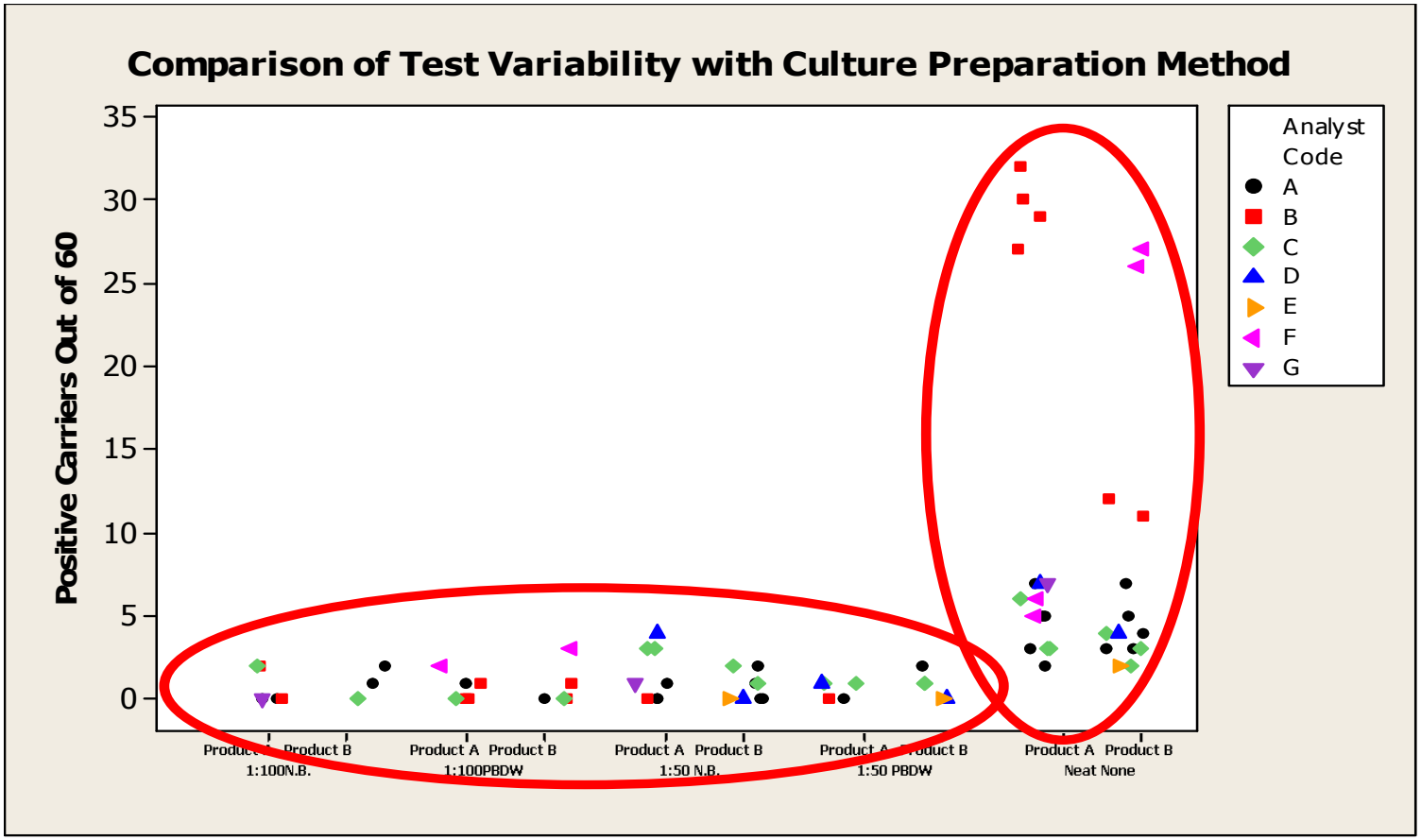
Overview of Ecolab / Lonza joint research program

- UDT parameters:
  - Two Products, a neutral-pH QAC and an alkaline-pH QAC
  - Product diluent, 400 ppm synthetic hard water
  - Organism, *Pseudomonas aeruginosa* (ATCC 15442)
  - Soil, 5% fetal bovine serum
  - Culture prep, neat, pellicle attenuated by dilution
    - Dilution of neat culture 1:50 or 1:100 in phosphate buffered dilution water (PBDW) or nutrient broth (NB)
  - Disinfectant contact time, 9 minutes 45 seconds (outside of label use directions)
  - 30 minute carrier soak time in letheen broth before cell removal

Key Results 1

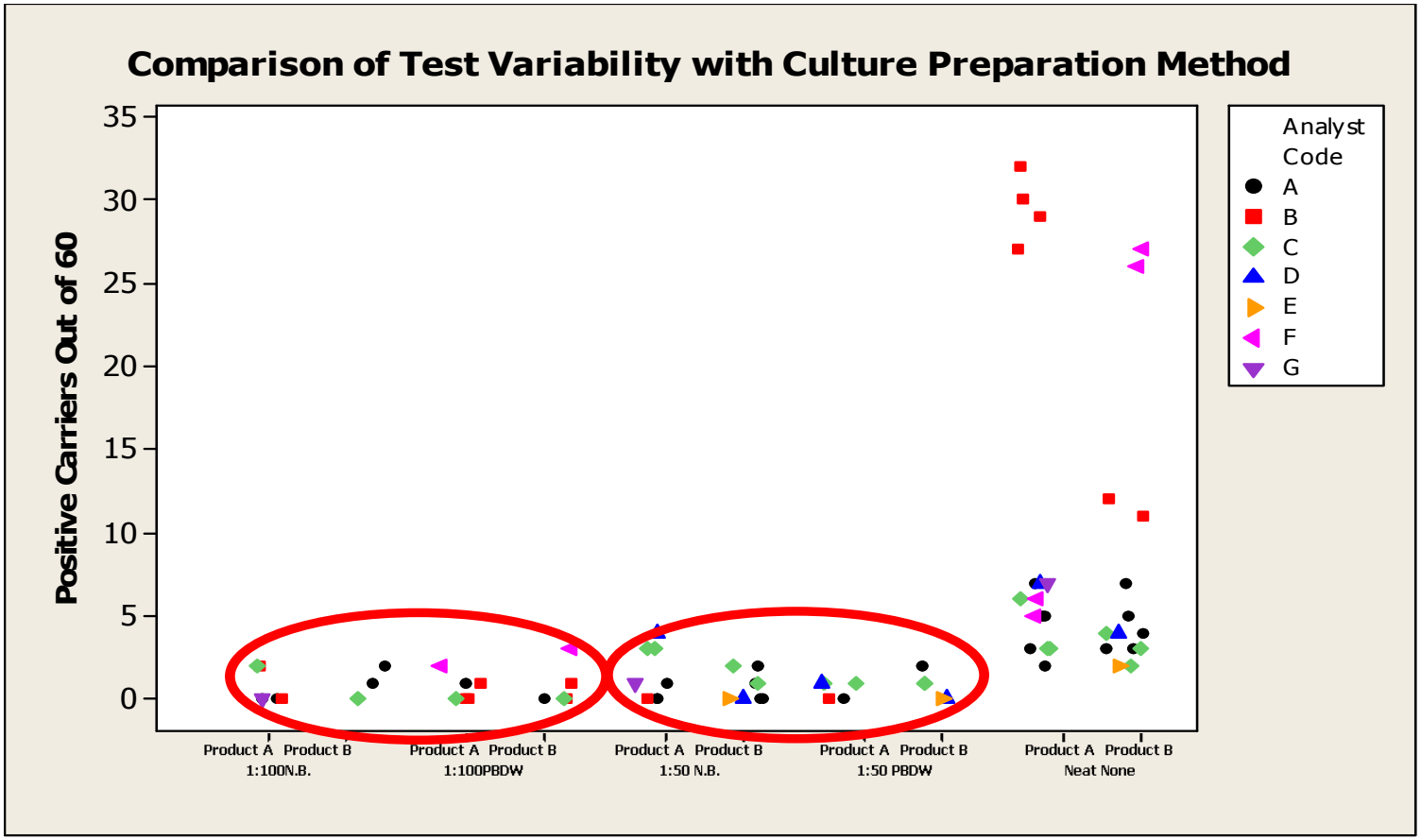
Test variability was significantly reduced through pellicle fragment attenuation

"Variability in test outcomes among analysts is dramatically reduced when a culture dilution step is implemented c.f. use of neat culture"



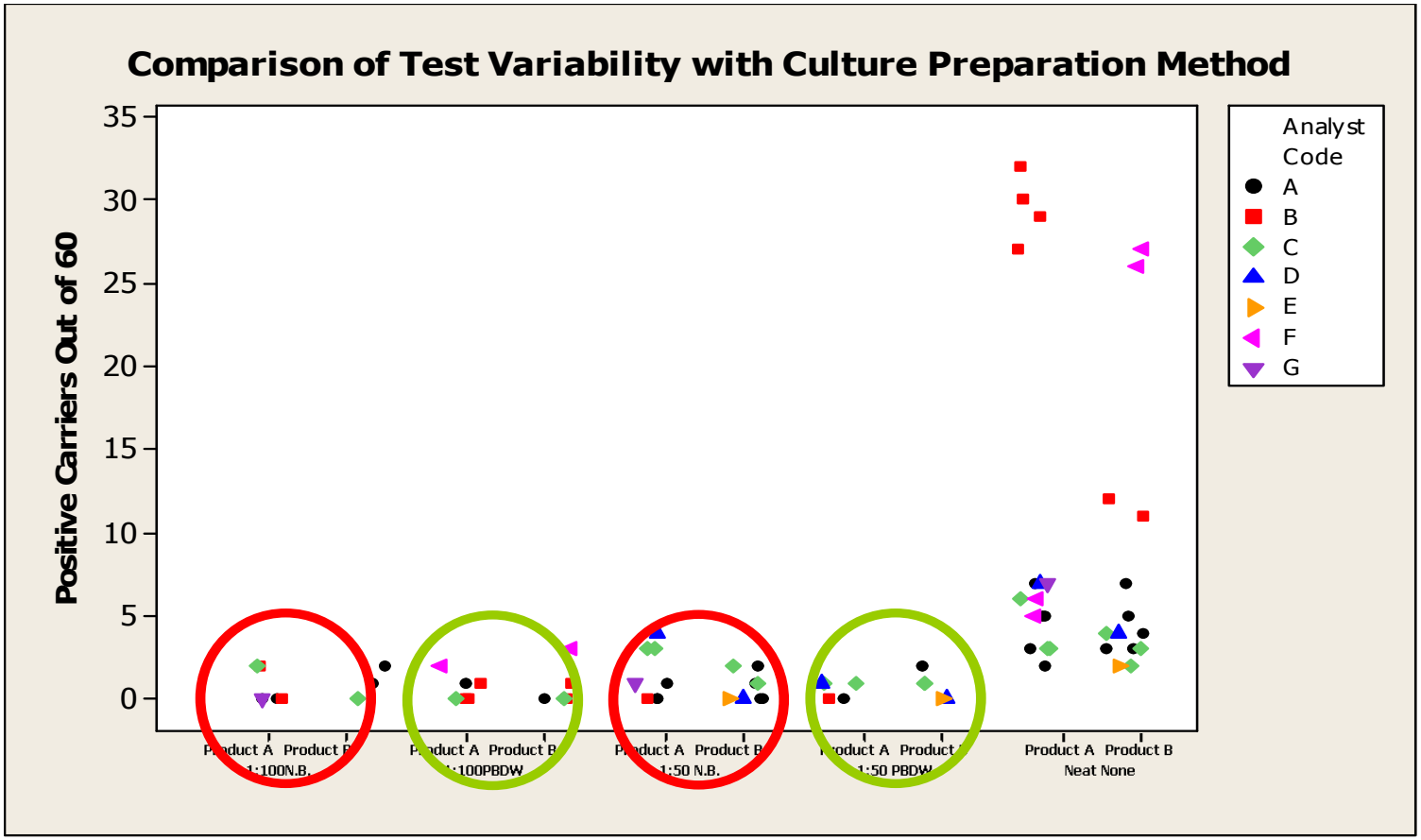
Testing across 4 laboratories (n = 6,015 carriers, p-value < 0.0001)

“There is no significant difference in variability between a 1:50 and a 1:100 dilution”



Testing across 4 laboratories (n = 6,015 carriers, p-value < 0.0001)

“There is no significant difference in variability with the use of either nutrient broth or phosphate buffered diluent water”

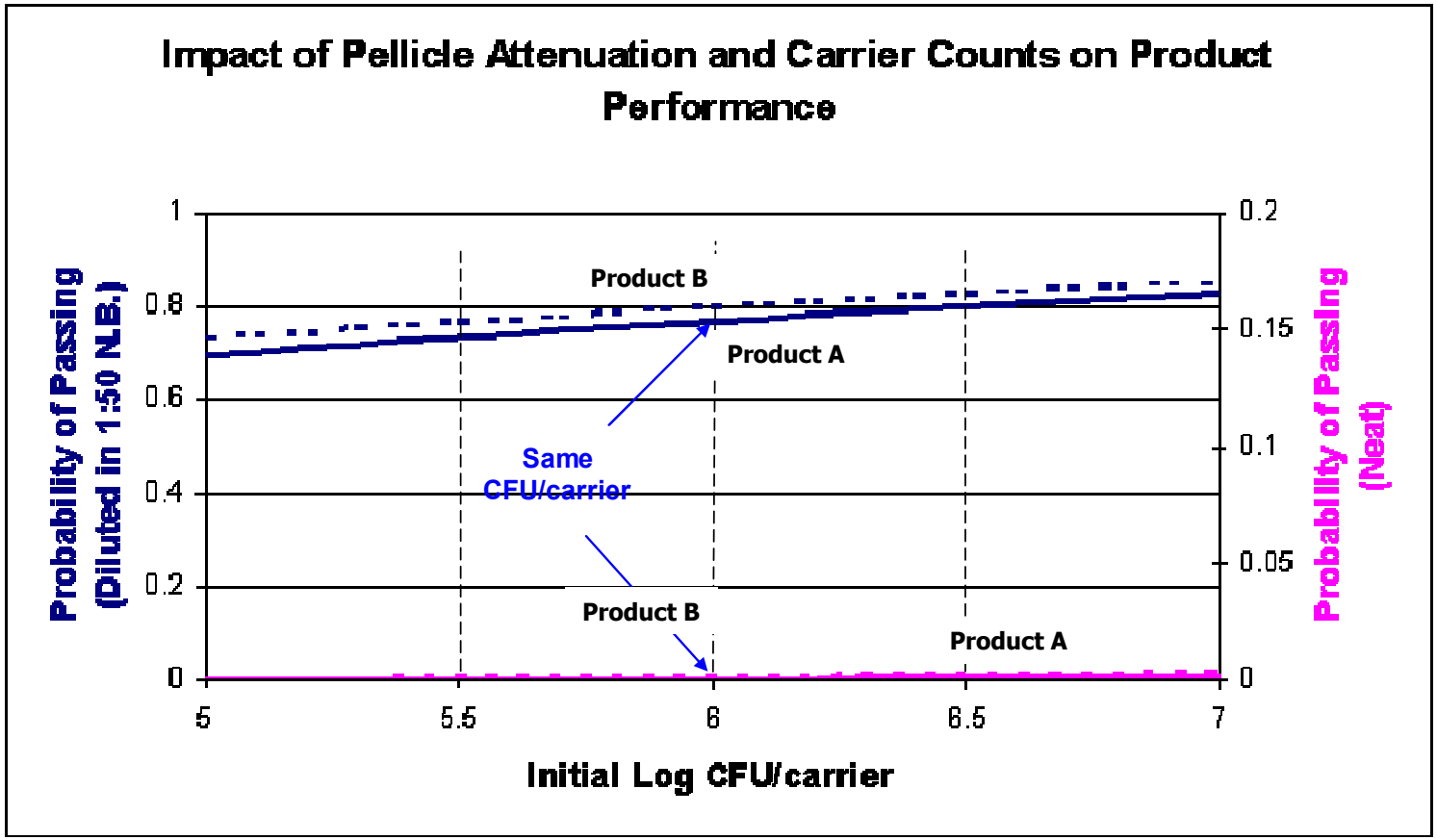


Testing across 4 laboratories (n = 6,015 carriers, p-value < 0.0001)

Key Results 2

Pellicle fragment impact is independent of carrier counts

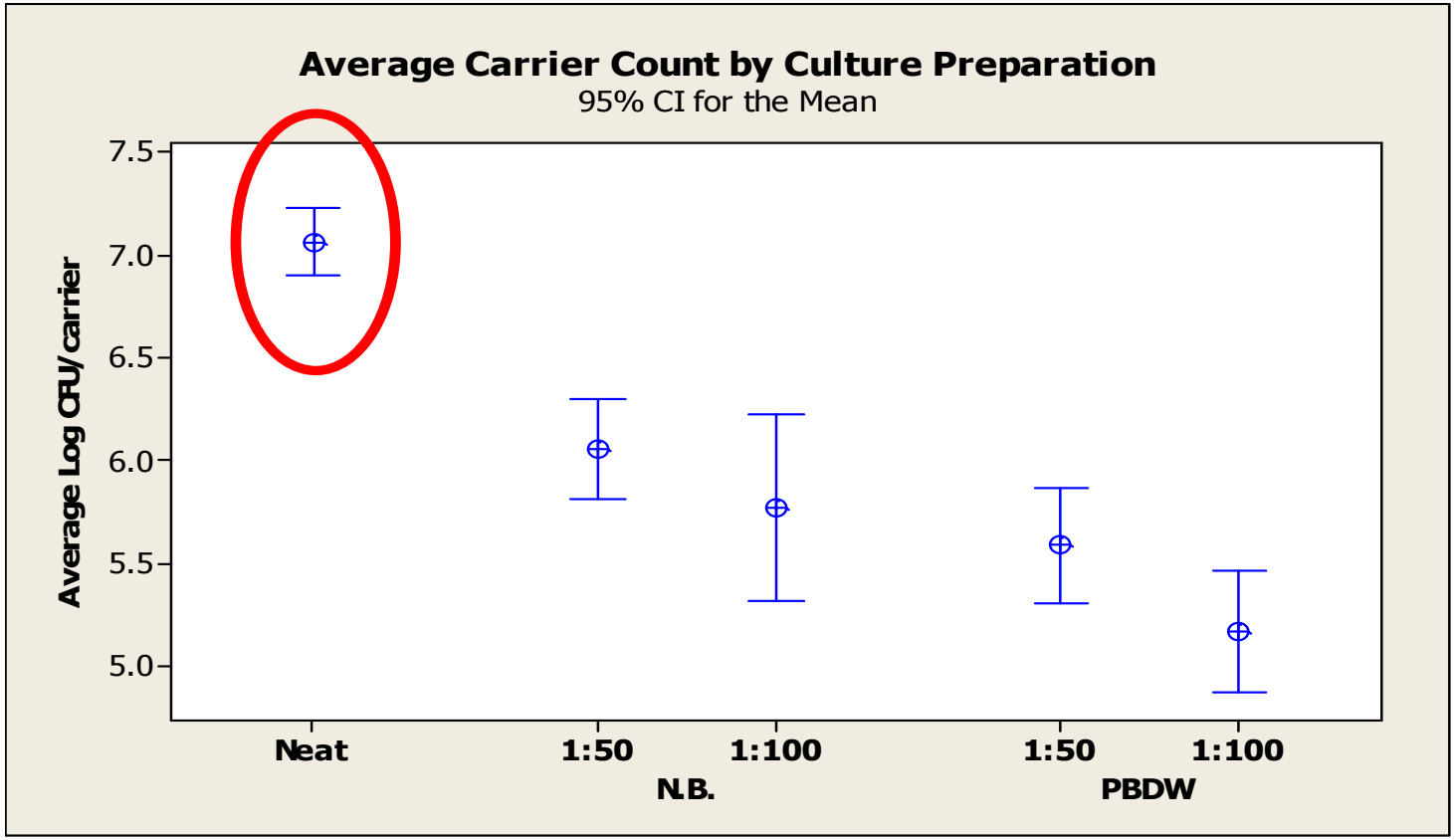
"Within each test system there is no significant relationship between average initial $\log_{10}$ CFU / carrier and the probability of observing a positive carrier"



Key Results 3

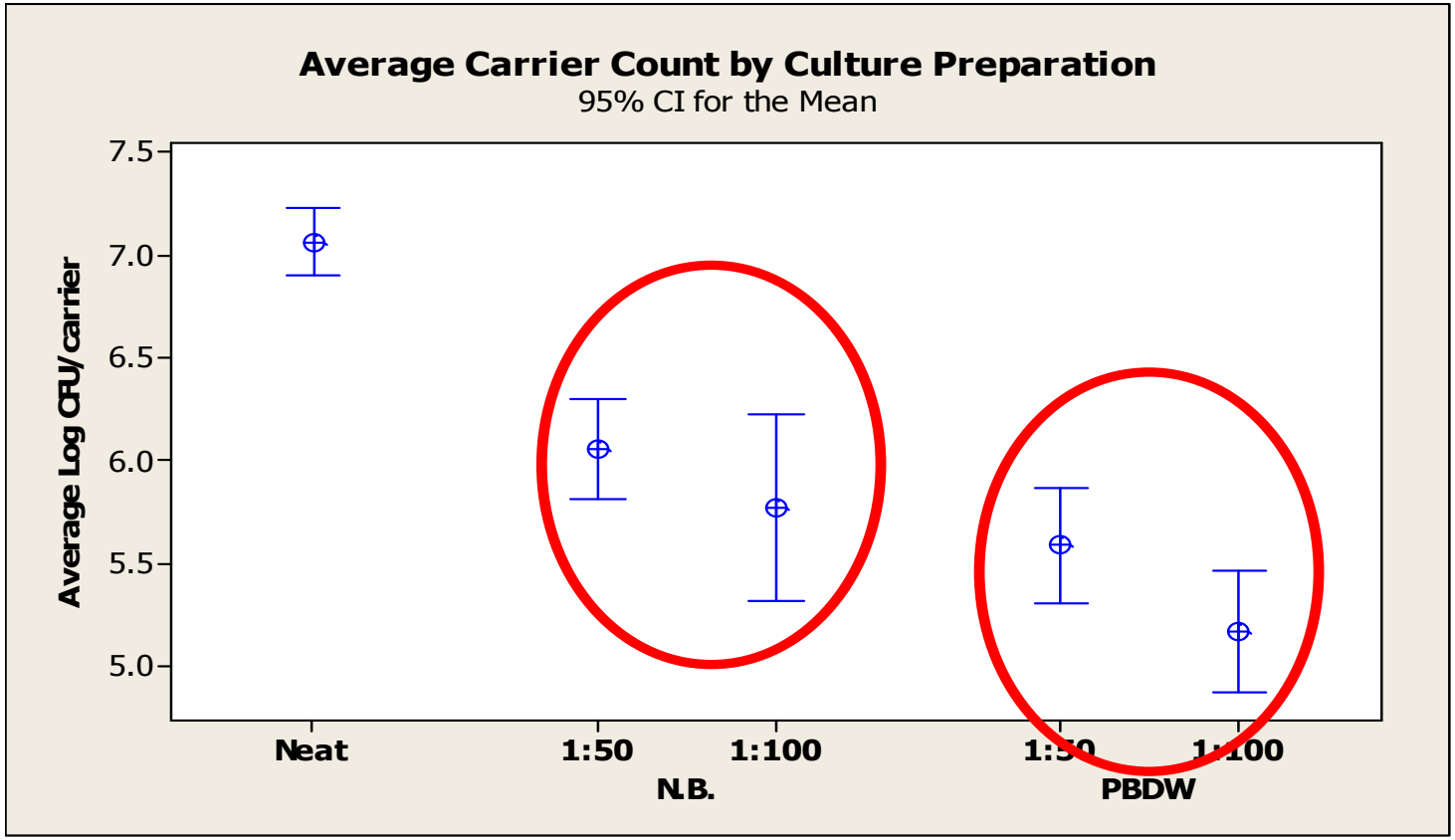
Use of a pre-determined dilution step (attenuation) does not preclude achieving sufficiently high carrier counts

"Average initial carrier counts are significantly greater for neat cultures"



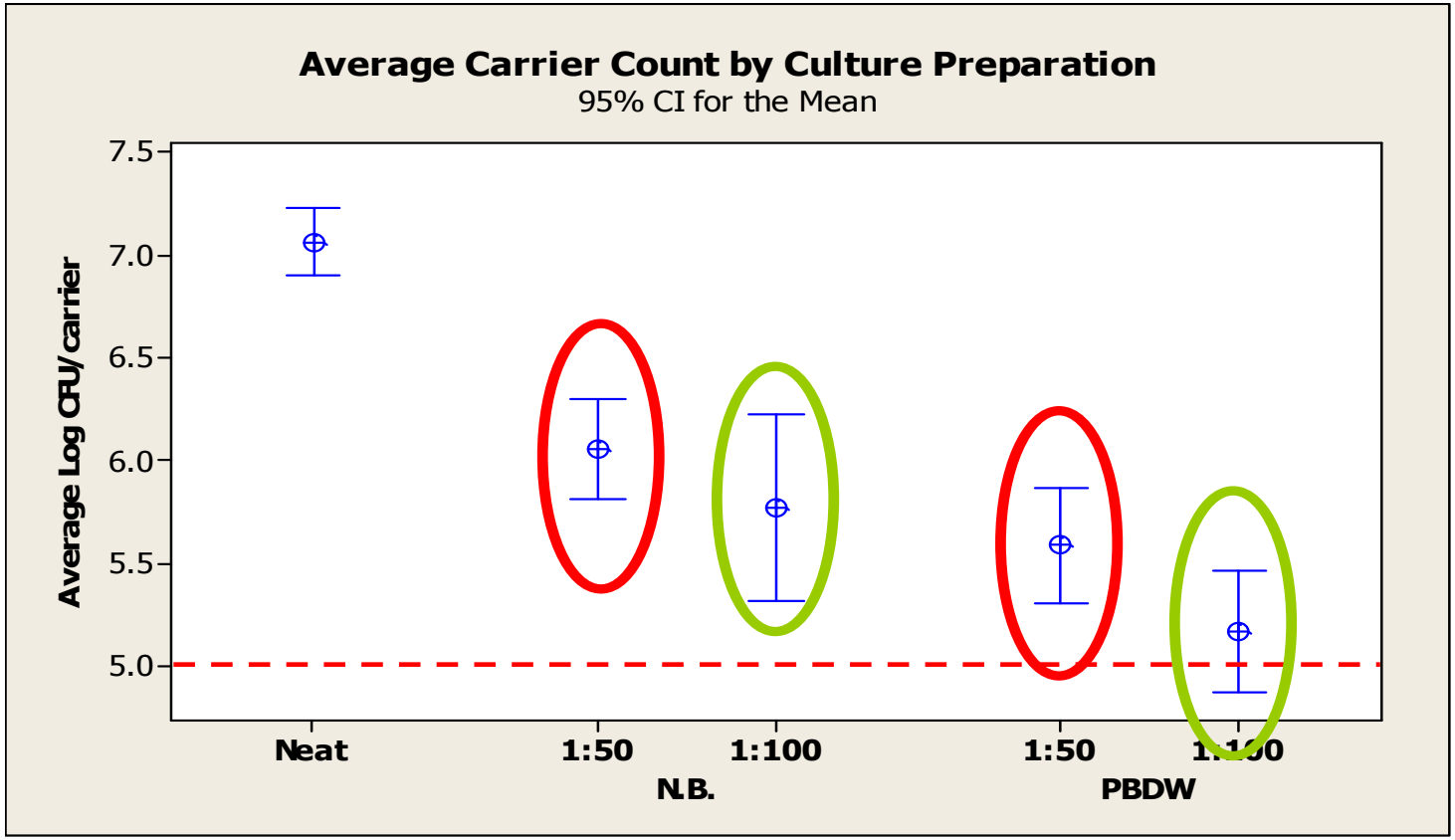
Testing across 4 laboratories (n = 6,015 carriers, p-value < 0.0001)

"Average initial carrier counts are significantly greater for samples diluted in nutrient broth rather than phosphate buffered dilution water"



Testing across 4 laboratories (n = 6,015 carriers, p-value < 0.0001)

"Average initial carrier counts are significantly greater for samples diluted 1:50 rather than 1:100"



Testing across 4 laboratories (n = 6,015 carriers, p-value < 0.0001)

Key Results 4

Of all variables evaluated, probability of observing a positive carrier is only significantly reduced when culture is attenuated (1:50 or 1:100)

"The probability of observing a positive carrier is significantly reduced when culture is diluted (1:50 or 1:100) to attenuate residual pellicle fragments"

Dilution	Diluent	Product	Probability of Observing a Positive Carrier	Predicted Probability of Passing Registration	Observed Probability of Passing Registration
Neat	None	A	13.3%	0.2%	0/16 = 0.0%
1:100	N.B.	A	1.7%	73.6%	4/6 = 66.7%
1:50	N.B.	A	1.9%	68.5%	5/8 = 62.5%
1:100	PBDW	A	1.7%	73.6%	5/6 = 83.3%
1:50	PBDW	A	1.0%	88.4%	5/5 = 100.0%
Neat	None	B	12.1%	0.4%	0/14 = 0.0%
1:100	N.B.	B	1.5%	77.5%	2/3 = 66.7%
1:50	N.B.	B	1.7%	72.9%	6/8 = 75.0%
1:100	PBDW	B	1.5%	77.5%	4/5 = 80.0%
1:50	PBDW	B	0.9%	90.3%	4/5 = 80.0%

Testing across 4 laboratories (n = 6,015 carriers, p-value < 0.0001)

"The probability of observing a positive carrier is not significantly different when using either nutrient broth or phosphate buffered dilution water or when using a 1:50 or 1:100 dilution step"

Dilution	Diluent	Product	Probability of Observing a Positive Carrier	Predicted Probability of Passing Registration	Observed Probability of Passing Registration
Neat	None	A	13.3%	0.2%	0/16 = 0.0%
1:100	N.B.	A	1.7%	73.6%	4/6 = 66.7%
1:50	N.B.	A	1.9%	68.5%	5/8 = 62.5%
1:100	PBDW	A	1.7%	73.6%	5/6 = 83.3%
1:50	PBDW	A	1.0%	88.4%	5/5 = 100.0%
Neat	None	B	12.1%	0.4%	0/14 = 0.0%
1:100	N.B.	B	1.5%	77.5%	2/3 = 66.7%
1:50	N.B.	B	1.7%	72.9%	6/8 = 75.0%
1:100	PBDW	B	1.5%	77.5%	4/5 = 80.0%
1:50	PBDW	B	0.9%	90.3%	4/5 = 80.0%

Testing across 4 laboratories (n = 6,015 carriers, p-value < 0.0001)

"The probability of observing a positive carrier is not significantly different when comparing Products A or B"

Dilution	Diluent	Product	Probability of Observing a Positive Carrier	Predicted Probability of Passing Registration	Observed Probability of Passing Registration
Neat	None	A	13.3%	0.2%	0/16 = 0.0%
1:100	N.B.	A	1.7%	73.6%	4/6 = 66.7%
1:50	N.B.	A	1.9%	68.5%	5/8 = 62.5%
1:100	PBDW	A	1.7%	73.6%	5/6 = 83.3%
1:50	PBDW	A	1.0%	88.4%	5/5 = 100.0%
Neat	None	B	12.1%	0.4%	0/14 = 0.0%
1:100	N.B.	B	1.5%	77.5%	2/3 = 66.7%
1:50	N.B.	B	1.7%	72.9%	6/8 = 75.0%
1:100	PBDW	B	1.5%	77.5%	4/5 = 80.0%
1:50	PBDW	B	0.9%	90.3%	4/5 = 80.0%

Testing across 4 laboratories (n = 6,015 carriers, p-value < 0.0001)

Why does a dilution step attenuate the presence of pellicle fragments?

- Residual EPS fragments are not being removed during the pellicle attenuation step
  - Belief is that they are being solubilized

Summary and conclusions

- Data generated in this joint research program and information extracted from peer-reviewed studies demonstrate significant variability with existing AOAC UDT method

- Method variability is an especially acute problem for testing QAC-based products against *Pseudomonas aeruginosa*

- Residual pellicle fragments remaining in culture are the most likely source of method variability

Summary and conclusions (cont)

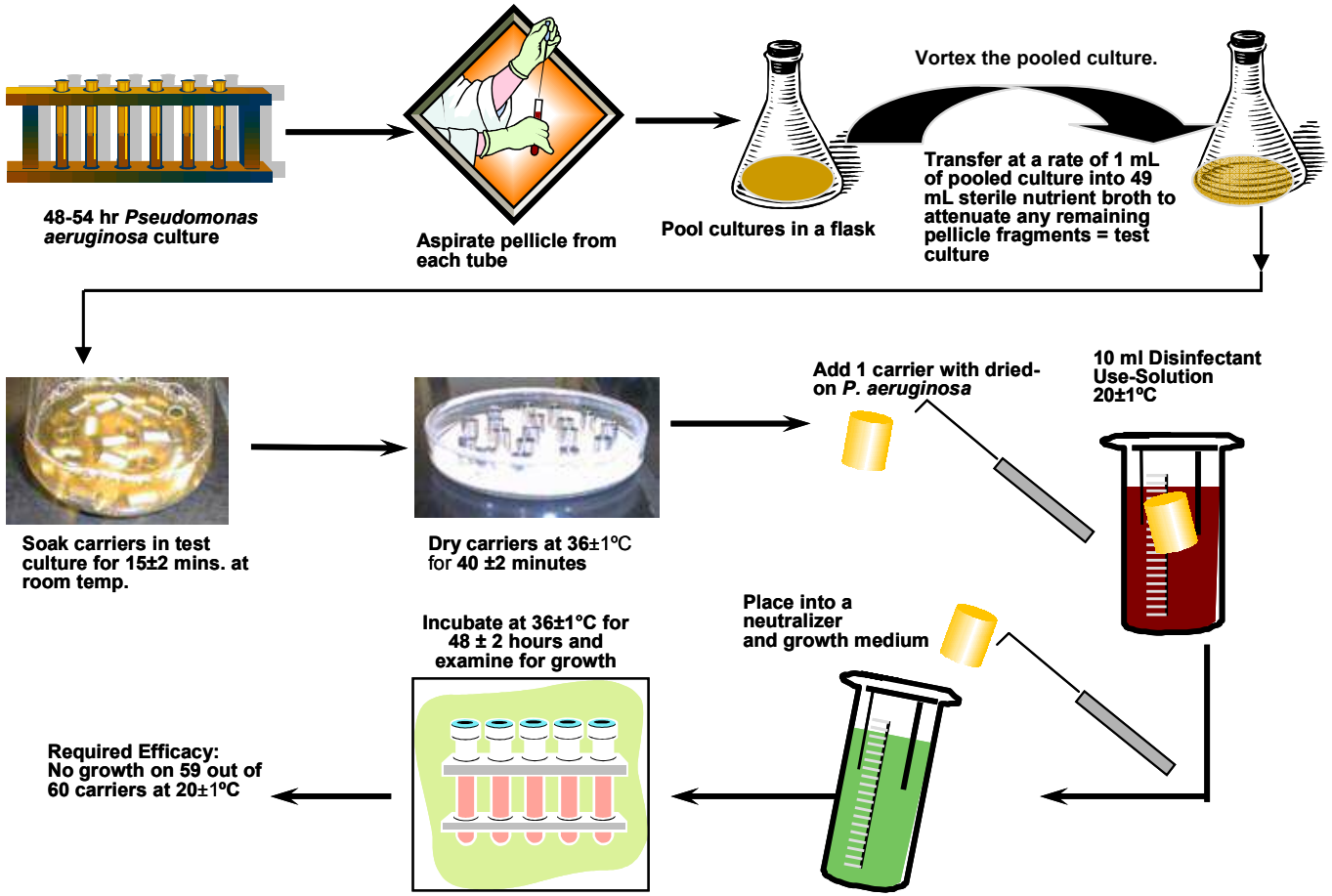
- Attenuation of residual pellicle fragments through a dilution step minimizes the effects of pellicle on the test
- Even with a dilution step, carrier counts can still be reliably generated in the range 1.0 x 10^5 CFU / carrier or greater
- Ecolab & Lonza proposed:
  - That EPA implement clarifications in the Agency's Microbiology Laboratory SOPs for use-dilution testing of disinfectants against *Pseudomonas aeruginosa*
  - To submit the data generated for publication in a peer-reviewed journal

Proposed clarifications to EPA / OPP Microbiology Laboratory SOPs

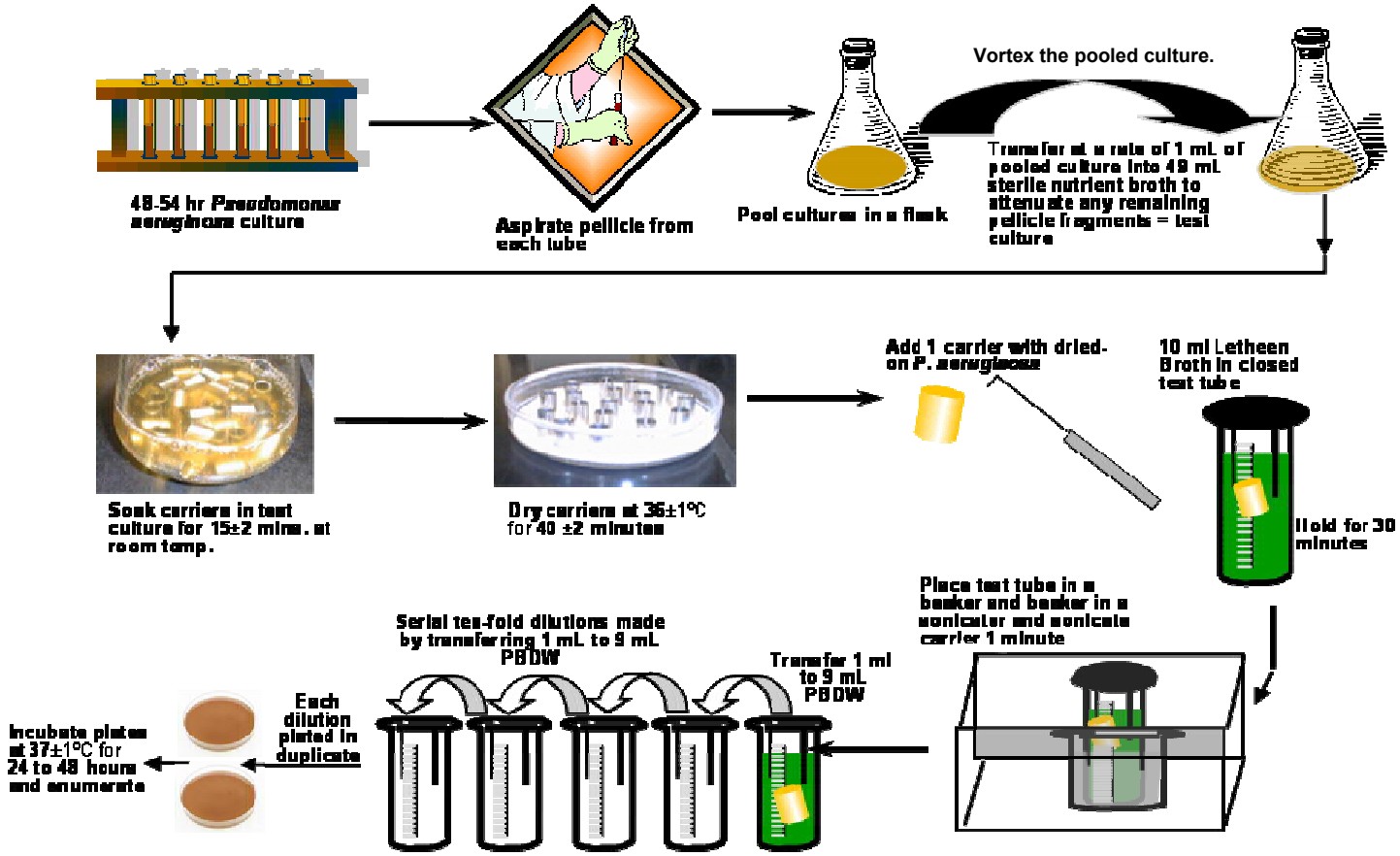
- Use 1:50 culture dilution step in nutrient broth to attenuate pellicle fragment effects
  - Section 10.3.1 of SOP MB-05-04 Use Dilution Method for Testing Disinfectants; and
  - Section 10.3.8 of SOP MB-06-02 Testing of Spray Disinfectants

- Use 30 min carrier soak step
  - Section 10.1.1.1 of SOP MB-04-03 Determining Carrier Counts

AOAC UDT Method with proposed method clarification



AOAC Carrier Count Procedure for UDT with proposed method clarification



Proposed Clarifications to EPA / OPP Microbiology Laboratory SOPs (cont)

- The proposed clarifications are within the scope of the current AOAC UDT method

- These refinements serve to:
  - Improve pellicle fragment elimination from the test system, which is a fundamental requirement in the AOAC UDT Method
  - Optimize recovery of cells from the carrier surface, further improving the accuracy and reproducibility of counts