US EPA approved Microbiological Methods for Testing Drinking, Ground, Recreational and Waste Waters

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OBJECTIVES

- Introduction to water microbiology
- Define total coliforms, fecal, E.coli & enterococci
- Overview of Regulations
- USEPA approved methods
- Understanding MPN theory and relationship to MF
- Q&A

Bacteria Hierarchy

- Family
- Genus (type of bacteria)
- Species

- Enterobacteriaceae
- Escherichia, Klebsiella, Citrobacter
- E.coli, E. hermanii,
- K. pneumoniae,
- C. freundii

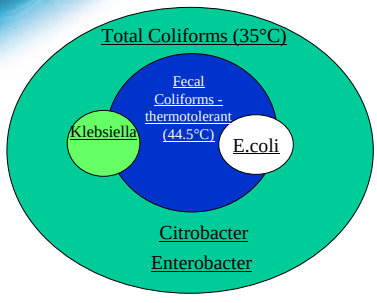
Coliform Genera

- Escherichia- human and animal feces
- Enterobacter- environment, feces
- Klebsiella- environment, feces
- Citrobacter- environment
- Serratia- environment

Thermotolerant Coliforms [aka Fecal Coliforms]

- It is a subset of total coliforms
- Defined as coliform bacteria that can grow at 44.5°C
- Consists of the following coliforms:
  - *E. coli*
  - *K. pnuemoniae*
  - *Enterobacter spp.*

Coliform Bacteria Group



Definitions of Coliforms & E.coli

- Standard Methods for the Examination of Water and Wastewater, 21st Edition
  - **9221 MTF**: Gram negative bacteria that ferment lactose resulting in gas and/or acid formation (turbidity) within 48 h at 35C.
  - **9222 MF**: Gram negative bacteria that develop red colonies with a metallic sheen within 24 h at 35C on m-Endo medium. Some members of the coliform group produce red colonies without a metallic sheen.
  - **9223 Enzymatic:** The total coliform group is defined as all bacteria possessing the enzyme β-D galactosidase cleaving the chromogenic substrate resulting in the release of the chromogen.

Definition of coliforms and *E.coli*

Enzymatic con't

E.Coli is defined as giving a total coliform response and possessing the enzyme β-D glucuronidase, cleaving the flurogenic substrate resulting in the release of the fluorgen.

- Fecal Coliform (thermotolerant bacteria)
  - Bacteria that grow at 44.5°C
    - Produce gas & turbidity in EC medium
    - Produce blue colonies in m-FC media
    - Produce yellow color wells with Colilert-18

Why Test for *E.coli*

Animal	# Tested	*E.coli*	Klebsiella spp	Enterobacter/ Citrobacter
Human	26	96.8%	1.5%	1.7%
Cow	15	99.9	-	0.1
Horse	3	100	-	-
Sheep	20	97	-	3
Pig	15	83.5	6.8	9.7
Average		94.5%		

Source:*E.coli*:Fecal Coliform A.P. Dufour. Special Technical Publication 65, ASRM. Pp48-58, 1977

Commonly Used Indicator Bacteria for Water Testing

- Most commonly used:
  - Total coliform
  - Fecal coliform
  - *E. coli*
  - *Enterococci*
- Total coliform used for >100 years
- Fecal coliform used for >80 years
- *E. coli* >20 years

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Requirements for an Indicator Organism

- Present when pathogens are present
- Absent in uncontaminated waters
- Present in higher numbers than pathogens in contaminated water
- Better survival in water than pathogens
- Easy and Safe to analyze
- Rapid results
- Inexpensive
- Accurate

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Overview of Regulations

- Total Coliform Rule-
- LT2
- Ground Water
- Recreational Water
- Waste Water

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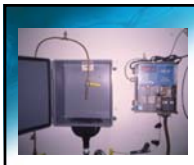
Total Coliform Rule

Promulgated 6/29/89

- To control pathogens
- Monitor for total coliform bacteria in the distribution system (CFR 141)
  - Number of samples dependent on population served
    - i.e.; 8501-12900 population requires 10 samples/month minimum.
- Used as an indicator of treatment reliability & distribution integrity
- If >5% TC/month, violates MLC for TC; if 2 consecutive TC with one E.coli, = acute violation and requires immediate notification



Where is Sampling Done?

Large Systems

- May have dedicated sampling stations.
- In public buildings such as fire and police stations.
- Some are sampled daily M-F while others once a week, or two or three times a week.

Small Systems

- Depending on size as little as one site exists for sampling.
- Usually no dedicated sampling stations
- Maybe a kitchen or laundry sink.

LT2 ESWTR

- Proposed- 7/03
- Promulgated- 1/5/06
- Large water systems must test for Crypto, E.coli & turbidity
- Small water systems test for E.coli
- Small systems may be required to test for Crypto.
- Proposes methods for Crypto & E.coli

Key Features of the LT2 Rule

- Applies to all public water systems using surface water sources (including GWUDI)
- Supplements existing regulations to address *Cryptosporidium* in systems with higher risk
  - Filtered systems with high source water occurrence
  - All unfiltered systems

Analytical Methods for Biological Pollutants in the Ambient Water Rule

- Procedure for enumerating E.coli, enterococci and Crypto & Giardia in ambient water
- Based on 1986 guidelines for E.coli & enterococci
- Methods include- m-TEC, Colilert and Colilert-18 for E.coli, m-EI & Enterolert for enterococci.
- Final rule effective - 8/20/03

Guidelines for Analysis of Pollutants; Analytical Methods for Biological Waste Water

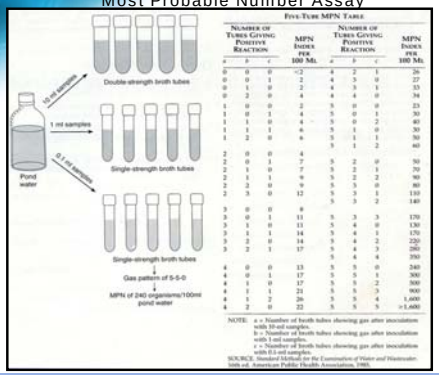
- Federal Register Notice- August 16th, 2005
- Promulgated on March 26th, 2007
- Testing for E.coli and for Enterococci
  - Approved methods include m-TEC, m-ColiBlue, Colilert, Colilert-18 for E.coli, Enterolert & m-EI methods for enterococci
  - E.coli for fresh surface waters and enterococci for marine waters

  June 2010
- US EPA recommends Colilert-18 for testing fecal coliforms in WW AT 45.5°C

Multiple Tube Fermentation (MTF) Most Probable Number (MPN)

IDEXX

Most Probable Number Assay



FIVE-TUBE MPN TABLE

Number of Tubes Giving Positive Reaction			MPN Index per 100 mL	Number of Tubes Giving Positive Reaction			MPN Index per 100 mL
a	b	c		a	b	c	
0	0	0	<2	4	3	1	26
0	0	1	2	4	3	2	27
0	1	0	2	4	3	3	33
0	2	0	4	4	4	0	34
1	0	0	2	5	0	0	23
1	0	1	4	5	0	1	30
1	1	0	4	5	0	2	40
1	1	1	6	5	1	0	30
1	2	0	6	5	1	1	50
				5	1	2	60
2	0	0	4				
2	0	1	7	5	2	0	50
2	1	0	7	5	2	1	70
2	1	1	9	5	2	2	90
2	2	0	9	5	3	0	80
2	3	0	12	5	3	1	110
				5	3	2	140
3	0	0	8				
3	0	1	11	5	3	3	170
3	1	0	11	5	4	0	130
3	1	1	14	5	4	1	170
3	2	0	14	5	4	2	220
3	2	1	17	5	4	3	280
				5	4	4	350
4	0	0	13	5	5	0	240
4	0	1	17	5	5	1	300
4	1	0	17	5	5	2	300
4	1	1	21	5	5	3	900
4	1	2	26	5	5	4	1,600
4	2	0	22	5	5	5	>1,600

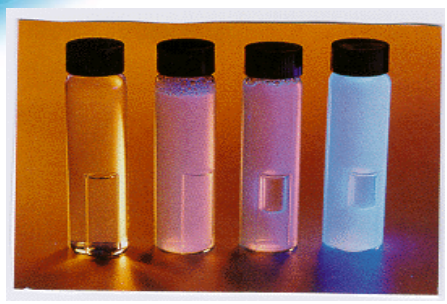
NOTE: a = Number of broth tubes showing gas after inoculation with 10-ml samples.
b = Number of broth tubes showing gas after inoculation with 1-ml samples.
c = Number of broth tubes showing gas after inoculation with 0.1-ml samples.
SOURCE: Standard Methods for the Examination of Water and Wastewater, 16th ed. American Public Health Association, 1985.

IDEXX

Multiple Tube Fermentation Method (Total Coliforms-LTB & BGLB)

IDEXX

Fecal Coliform/E. coli Multiple Tube Method - EC medium + MUG



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Membrane Filtration Methods

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Membrane Filter Apparatus

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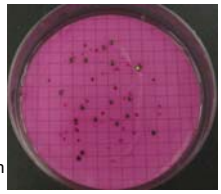
Membrane Filtration- applicable to all methods

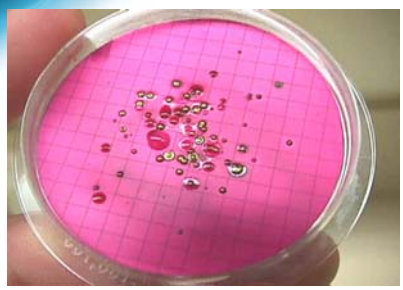
- May required media prep
- Extensive QC
- Minimum of 20 steps
- 20-80 colonies (20-60 for m-FC)
- Risk of confluent growth
- Risk of clogged filters
- Risk of overlapping colonies
- Risk of air bubbles under membrane
- Difficult to read

m-Endo: Method

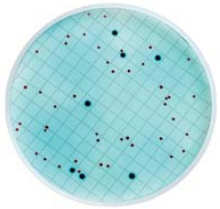


- Standard Methods 9222B&C
- Incubate for 24 hours at 35-37°C
- Read at 24 hours for presumptive results
  - Dark red or pink with metallic/green sheen
- HPC Interference at 120 colonies or more
  - Transfer colonies to BGB to confirm coliforms (up to 48 hours)
    - Positives produce gas and turbidity
- Transfer positives to EC (+Mug) for 24 hours at 44.5°C
  - Positives produce, turbidity/gas, and/or fluorescence
- Results with confirmation- could take up to 3 days

m-Endo Plate

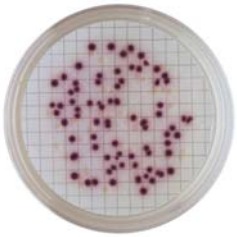


Hach m-ColiBlue



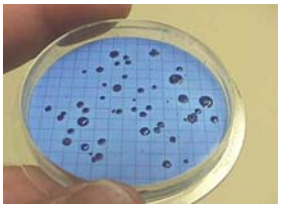
- Reagent storage is at 2-8°C
- Incubate for 24 hours at 35±0.5°C
- Read at 24 hours
  - Red or blue colony = TC
  - Blue colony = *E.coli*
  - *Difficult to interpret colonies (very small)*
- *If count >200 for TC/EC, exclude from calculation*

Modified m-TEC for E. coli



- Contains 5-bromo-6-chloro-3-indolyl-β-D-glucuronide
- Selective inhibitory chemicals that can effect the growth of sub-lethal injured bacteria.
- 2 hours of incubation at 35°C followed by 22 hours at 44.5°C
- Positive reaction is red to magenta color colonies
- Extensive QC requirements

m-FC

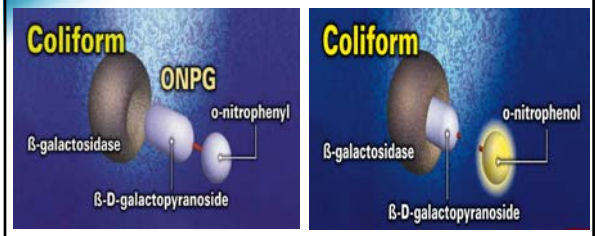


- Enriched lactose medium
- Contains aniline blue
- Larger colonies than m-Endo; range is 20-60 colonies
- Incubate in water bath at 44.5 ±0.2°C for 24 hours in plastic bag
- Blue colonies are positive
- Atypical- grey to green colonies
- Confirm as per section 9020 Standard Methods

Defined Substrate Technology- Colilert & Colilert-18

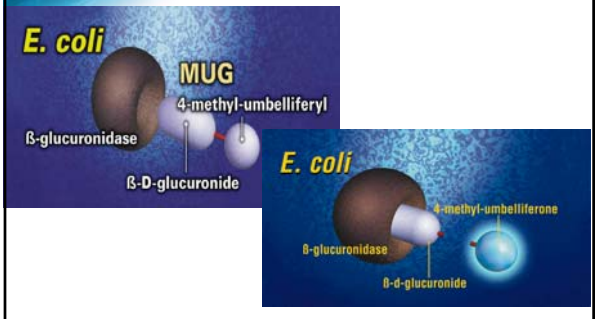
ONPG Positive Reaction

Colilert & Colilert-18



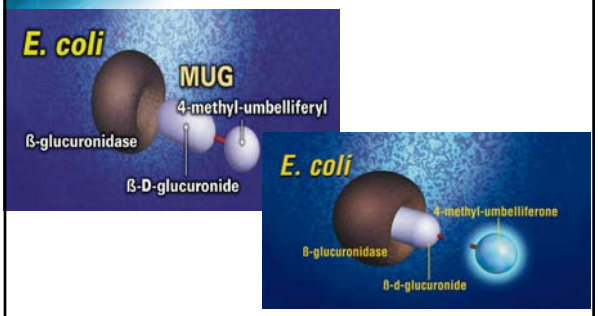
MUG Positive Reaction

Colilert & Colilert-18



MUG Positive Reaction

Colilert & Colilert-18



Quanti-Tray Demonstration

Add Colilert to sample and shake to dissolve

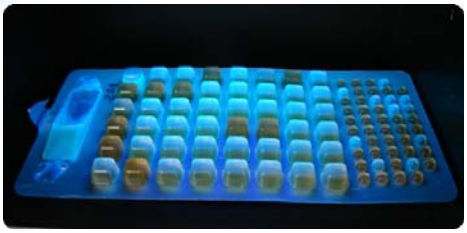
Pour mixture into a Quanti-Tray

Quanti-Tray Demonstration

Seal and incubate at 35°C for 18 or 24 hours for total coliforms and 44.5°C for fecal coliforms for 18 hours

Count positive wells and refer to MPN table

***E.coli*- Blue Fluorescence- Quanti-Tray under a 365nm UV Light at 35°C for 18 or 24 hours for E.coli**



Studies

- AWWARF Study : **Significance of Methods and Sample Volumes for EC &TC Measurements- Fricker et al**
  - **Methods and volume of samples evaluated for drinking water testing cited in Sept. 2010 RTCR meeting.**
    - Glactosidase methods will detect more coliforms (higher concentration and a broader range) than lactose based methods
    - Study found Colilert-18 and Colilert had a confirmation rate of 97.7 and 95.1%. False negative rate of 1.8 and 2.8%
    - Confirmation rates for m-Endo was 84.6% & false negative rate of 13.2%
    - Verification of E.coli for Colilert-18 & Colilert are 100 & 99.1%, m-FC is 79.7%

Studies con't

- Choice of a specific method is often a personal preference within a lab. Factors such as sensitivity, specificity, accuracy, simplicity & time to results are most important factors in choosing an appropriate method

Studies con't

- Comparison of m-Endo, MacConkey & Teepol Agar Methods for MF Counting Total Coliform bacteria in Water: Grabow & Preez AEM, Sept'79 Vol 38 #3 p 351-355
  - 341 coliform like colonies picked from m-Endo membranes for identification
    - 186 /55% true coliforms
    - 138/40% Aeromonas hydrophilia
    - 17/5% other species
- A comparison of ten USEPA approved total coliform/E. coli tests: Jeremy Olstadt, WQTC 2005
  - 2 different strains of Aeromonas spike into different waters at 3 different sites- Colilert & Colilert 18 negative at 10^5/mL

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Studies con't

- Evaluation of Two Colilert Formulations and Quanti-Tray for the Assessment of the Bacteriological Quality of Water: Fricker et al. Water Research 1997 Vol 31 pp 2495-2499
  - Identified 296 wells for total coliforms for Colilert & Quanti-Tray
    - All 296 wells confirmed as coliforms compared to 257 colonies/363 (71%) for the UK MF method.
- Evaluation of the Autoanalysis Colilert for Determination & Enumeration of Coliforms: Covert et al AEM Oct 1989 Vol 55 #10
  - No false positives from Pseudomonas, 1 + Aeromonas* at $7.5X10^4$/ml
  - Flavobacterium + at 10^7-10^8/mL
  - *All above label claim

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Enterococci MF and Defined Substrate Technology®

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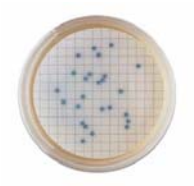
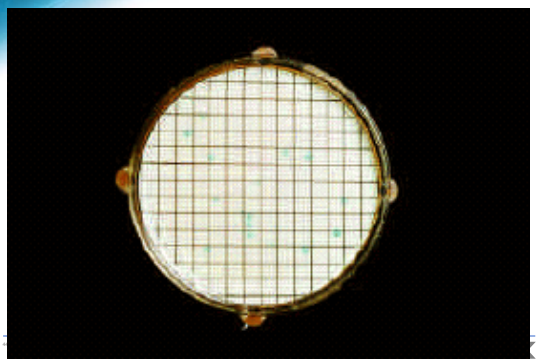
m-EI Method for Enterococci

- 24 hour MF Test
- All colonies regardless of color with a blue halo
- 20-60/plate
- Colonies <0.5mm difficult to read
- Media is expensive (Indoxyl-β-D-glucoside)
- Magnification required to read plates



Method 1600- m-EI



Enterolert™ Demonstration

Add reagent

Seal in a Quanti-Tray
Incubate at 41.5C for 24 hrs

Enterolert™ Demonstration

Count fluorescent wells
and refer to MPN table

Estimation of Bacterial Densities by MPN

- Dates back to 1915 when the concept was introduced by M.H. McCrady (J of Infectious Disease Vol 17, 1915*)
- Prior to this novel concept; no means of direct counting
  - Only had presence-absence of fermentation tubes
- The method is a means for estimating without any direct count, the density of organisms in a liquid.
- Multiple samples of the liquid are taken and incubated in suitable media
  - Record presence or absence of growth in each sample tube
  - Ingenious application of probability theory
  - Estimate the number of organisms from the number of negative tubes

*The Numerical Interpretation of Fermentation Tube Results

MPN Theory – a short math lesson

Basic assumptions:

- The organisms are distributed randomly throughout
- the liquid
  - Sample is well shaken (important and often neglected)
- A sample will exhibit growth (in the culture media) whenever one or more of the target organisms is present
  - Media should be selective and sensitive
- Requires that at least 1 tube shows no growth (sterile)

If n samples, each of volume v mL are taken from a liquid, and s of these are sterile, then an estimate of the organism density d per mL in the original sample is:

$$d = -2.303/v \log(s/n)$$ **[Poisson distribution]**

d can be shown statistically to have the highest probability of estimating the actual organism density – the "**Most Probable Number" - MPN**

Single dilution – Maximum counting range

Number of Tubes	Maximum MPN/100mL	Number of Tubes	Maximum MPN/100mL
5	8	35	125
10	23	40	148
15	40	45	171
20	60	50	196
25	80	75	324
30	102	100	461

$d = -2.303/v \log(s/n)$

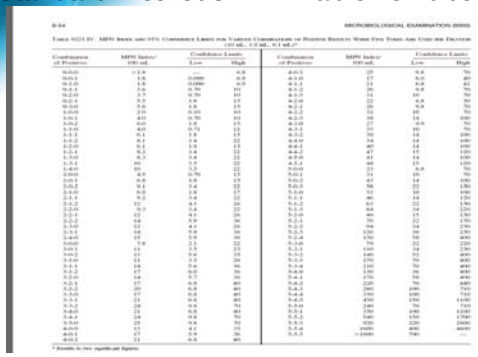
n = 50, v = 2 mL, s = 1

d = -2.303/2 log (1/50)
= 1.956 MPN/mL
= 195.6 MPN/100 mL

Uncertainty of MPN

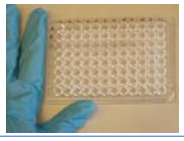
- Skewed distribution & Uncertainty of Results
  - For microbiological tests, because of the complications of the skewed distribution, the estimate of uncertainty from a well-designed precision experiment will be regarded as a reasonable estimate of the uncertainty of results
- When MPN tables are used to obtain results, the 95% CL in the table will be regarded as a reasonable estimate of the uncertainty of the results
  - Labs are encourage to identify and question unusual combinations of positive tubes in the tables.
    - –15 tube MPN combination of + tubes
      - »1-2-3
      - »3-5-4

Standard Methods MPN Table 15 Tube



Overcoming the tube numbers problem

- To increase counting range need more tubes
- For 100mL sample could use 50 tubes x 2mL
- Would give counting range of ~200MPN/100mL
  - Big headache for lab – lots of tubes – lots of pipettes -lots of washing! Not really practical.
  - Microtitre plate (96 wells) – MPN range 438/100mL



Quanti-Tray®

- Use multiple well concept but automate the sealing

51 well Quanti-Tray
Total sample volume 100mL

$d = -2.303/v \log(s/n)$

$n = 51$, $v = 1.96$ mL, $s = 1$

$d = -2.303/1.96 \log (1/51)$
= 2.006 MPN/mL
= 200.6 MPN/100 mL

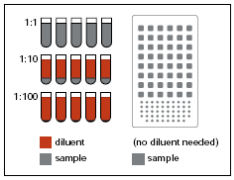
Multiple Dilutions

- Not practical to extend the counting range by increasing the number of wells beyond a certain point.
- Sample serially diluted and each dilution is inoculated into a similar number of tubes. Commonly used configurations:
  - 3 tubes x 3 decimal dilutions – food industry
  - 5 tubes x 3 decimal dilutions – food, water wastewater (Max MPN = 1600/100mL)
- With multiple dilutions the statistical calculation of the MPN value becomes more complex. **Thomas** provided an **approximation** that could be used for any combination of tubes

$$\text{MPN/100mL} = \frac{\text{\# of positive tubes} \times 100}{\sqrt{(\text{\# of mL in negative tubes}) \times (\text{\# of mL in all tubes})}}$$

Quanti-Tray 2000®

- Innovative approach to provide a multiple dilution system without adding dilution water
- Small wells are 1/10th the volume of the large wells
  - Effectively a 49 x 48 tube serial dilution



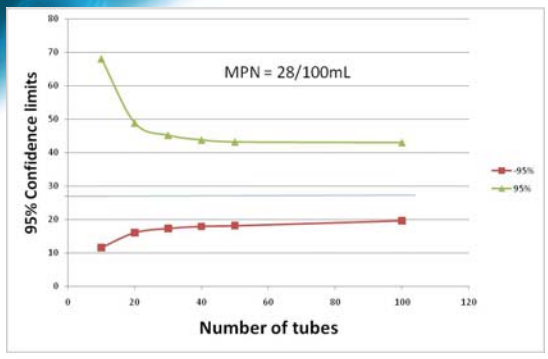
Using the Thomas formula:
(MPN is an approximate, deviates from the MPN table and is not significant)
Number of +ve tubes = 96
Volume of –ve tubes = 0.186mL
Volume in all tubes = 100mL

$$MPN_{max} = \frac{96 \times 100}{\sqrt{(0.186) \times (100)}}$$

= 2232/100mL vs 2419

Confidence Limits

- Distribution of MPN estimates is approximately log normal (distribution).
- Confidence limits can be calculated (95% limits = +/- 1.96σ)
- Precision is low when the number of tubes is small.
  - For 10 x 10mL tubes with 9 positive: MPN = 28/100mL
  - the 95% confidence limits are: 11.5 – 68.1 MPN/100mL
- Confidence limits for 51 well Quanti-tray
  - When increased to 51 x 1.96mL with 22 positive: MPN = 28.8/100mL
  - The 95% confidence limits are: 19.0 – 44.3 MPN/100mL
  - Interesting fact: If had 100 x 1mL with 25 positive: MPN = 29/100mL
  - The 95% confidence limits are: 19.6 – 43.0 MPN/100mL
  - Not much improvement – 50 tubes is about optimum



95% Confidence Limit Comparison

Quanti-Tray MPN/100mL		Quanti-Tray 2000 MPN/100mL		MTF MPN/100mL		MF CFU/100mL	
Value	CL	Value	CL	Value	CL	Value	CL
5.3	2.3-12.3	5.2	1.8-10.8	4.5	0.79-15	5	1.6-7.2
17.8	10.8-29.4	17.9	10.7-28.2	17	6.0-40	17	9.9-27.2
20.7	13-33.3	20.6	12.7-31.8	20	6.8-40	20	12.2-30.8
50.4	35.4-72.5	50.4	35.0-69.1	49	15-150		
78.2	56.4-111.2	78.0	55.6-103.8	79	22-220		

IDEXX Support

1-800-321-0207

- #1 Customer Support
- #2 Technical Service
- #3 Select extension
- www.idexx.com/water

Thank You

Questions