# Testing Methodologies and Sampling

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# Overview

- Historical Perspective
  - Rationale for the testing recommendations in the 1999 FDA guidance
- Looking Forward
  - What has been learned since then?
  - New issues to consider

## Why test?

- End-product testing of foods does not ensure their safety
  - Low levels of microorganisms
  - Sporadic contamination of units
- Sprouts are a unique food
  - Natural microbial growth to high levels
  - Microorganisms may be uniformly distributed throughout batch

## Pre-guidance questions on microbiological testing

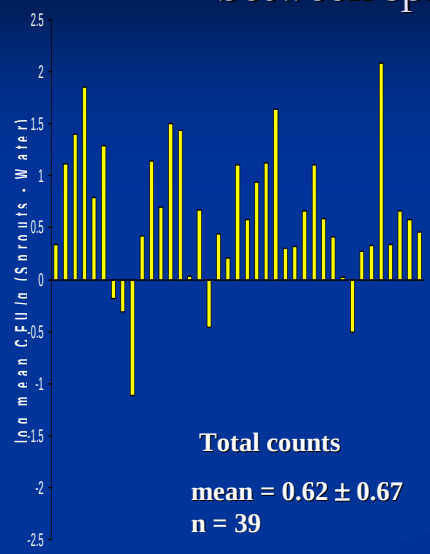
- What should be tested?
- How and when should testing be done?

# What should be tested?

1998 FDA Public Meeting:

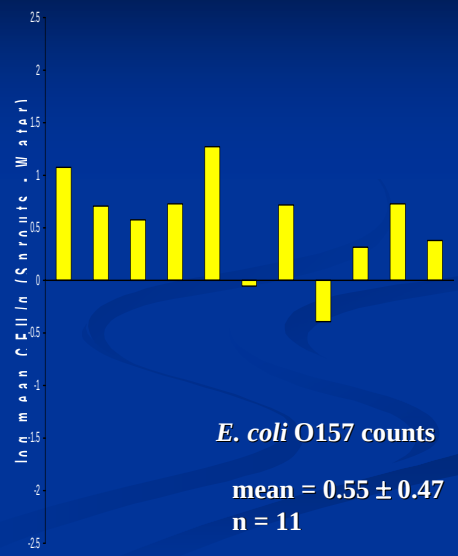
Industry advocates the testing of spent irrigation water to indicate the microbiological condition of the sprouts batch

## Difference in microbial counts between sprouts and water




**Total counts**

**mean = 0.62 ± 0.67**
**n = 39**




***E. coli* O157 counts**

**mean = 0.55 ± 0.47**
**n = 11**

## Detection of *Salmonella* in Sprouts and Water from Outbreak Seeds

| Seed Lot | Box | No. Confirmed / No. Tested | |
|---|---|---|---|
| | | Sprouts | Water |
| X | 1 | 2/2 | 2/2 |
| | 2 | 0/2 | 0/2 |
| | 3 | 0/2 | 0/2 |
| | 4 | 0/2 | 0/2 |
| Y | 1 | 1/2 | 1/2 |
| | 2 | 0/2 | 0/2 |
| | 3 | 0/2 | 0/2 |
| | 4 | 1/2 | 1/2 |
| Z | 1 | 2/2 | 2/2 |
| | 2 | 1/2 | 1/2 |
| | 3 | 2/2 | 2/2 |
| Totals | | 9/22 | 9/22 |

## Pre-guidance questions on microbiological testing

- What should be tested?
- How and when should testing be done?

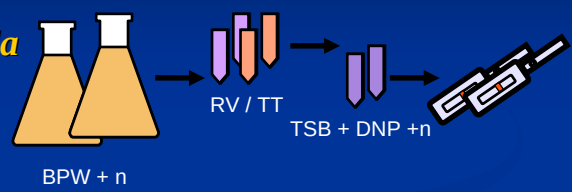
# How to test: requirements for guidance testing methods

- Results 48 hr after sampling
- Validated method
  - *Salmonella* Assurance Gold EIA * and VIP *
  - *E. coli* 0157:H7 VIP * and Reveal

* AOAC Official Method

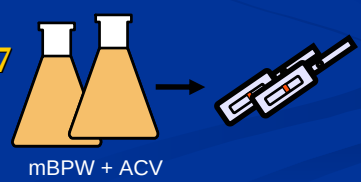
# Testing of Water from Each Production Batch for :

***Salmonella***




**and**

***E. coli* O157:H7**



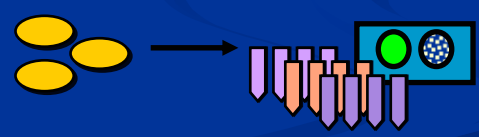
## ….Testing of Water from Each Production Batch

If positive results, then:

- discard sprouts and seed; disinfect

  or

- run confirmatory tests from enrichment



## Detection of Pathogens in Inoculated Sprouts and Sprout Water by Rapid Test Kits

***Salmonella***

| | Sprouts | | | Water | | |
|---|---|---|---|---|---|---|
| Inoc CFU/g | VIP | Gold | Confirm | VIP | Gold | Confirm |
| 1 | + | + | + | + | + | + |
| 10 | + | + | + | + | + | + |
| 100 | + | + | + | + | + | + |
| 1000 | + | + | + | + | + | + |

***E. coli* O157:H7**

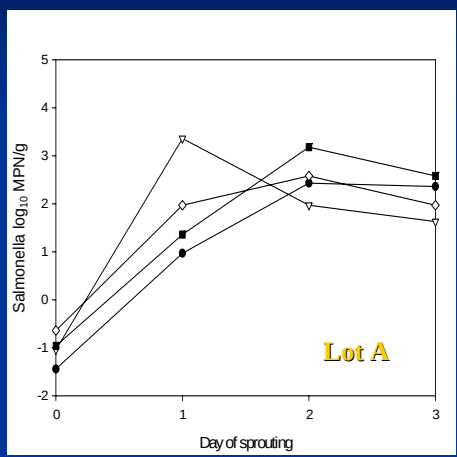
| | Sprouts | | | Water | | |
|---|---|---|---|---|---|---|
| Inoc CFU/g | VIP | Reveal | Confirm | VIP | Reveal | Confirm |
| 1 | + | + | + | + | + | + |
| 10 | + | + | + | + | + | + |
| 100 | + | + | + | + | + | + |
| 1000 | + | + | + | + | + | + |

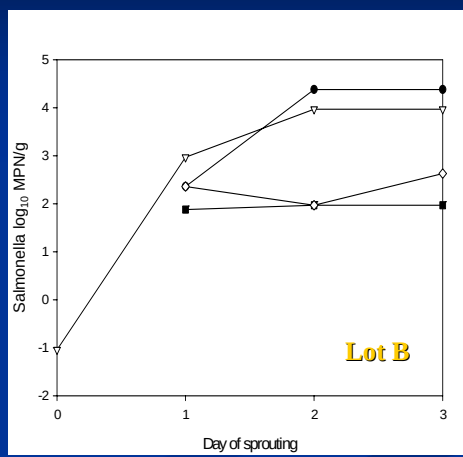
# Detection Limit of Rapid Test Kits Without Enrichment

| | | Approx. Detection Limit $Log_{10}$ CFU/ml | |
|---|---|---|---|
| | | VIP | Reveal |
| Cells in Buffer | | 7 | 6 |
| Lot A | Sprouts | 7 | 5 |
| | Water | 7 | 5 |
| Lot B | Sprouts | 7 | 5 |
| | Water | 7 | 5 |
| Lot C | Sprouts | 6 | 6 |
| | Water | 6 | 6 |

***E. coli* O157:H7**

# Growth of Salmonella During Sprouting of Outbreak Seeds

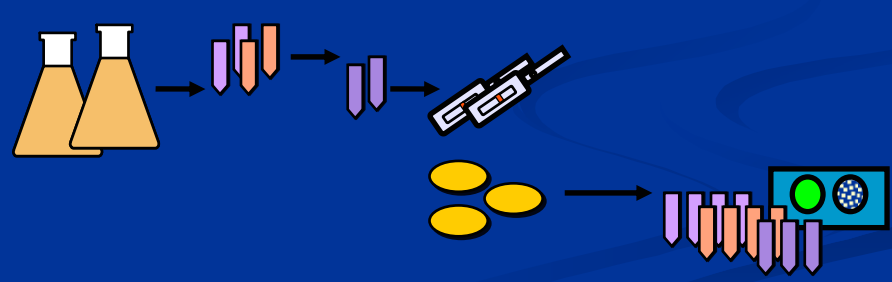


## Detection of *Salmonella* in Sprouts and Water from Outbreak Seeds

| | | | EIA Results on Day of Sprouting | | | |
|---|---|---|---|---|---|---|
| Seed Lot | Rep or Trial | Sample | 0 | 1 | 2 | 3 |
| A | 1 | sprout | + | + | + | + |
| | | water | + | + | + | + |
| | 2 | sprout | + | + | + | + |
| | | water | + | + | + | + |
| B | 1 | sprout | - | + | + | + |
| | | water | - | + | + | + |
| | 2 | sprout | - | + | + | + |
| | | water | - | + | + | + |

## Microbiological testing: validation of rapid tests



## Validation of *Salmonella* Detection by Rapid Tests Assurance Gold[a] and VIP[b]

| Seed Lot C | | Assurance Gold | | VIP | | BAM |
|---|---|---|---|---|---|---|
| Serotype | Inoc CFU/ml | Positive | Confirmed | Positive | Confirmed | Confirmed |
| Cubana | 0.8 | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 |
| Muenchen | 0.7 | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 |
| Tennessee | 0.6 | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 |
| No inoc | 0 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| Totals for 4 seed lots | 0.6-3.6 | 66/66 | 66/66 | 66/66 | 66/66 | 49/60 |

[a] AOAC Official Method 999.08
[b] AOAC Official Method 999.09

## Selected AOAC Performance Tested Methods

| Test Kit Name | Manufacturer | Cert. Date | Analyte | Matrix |
|---|---|---|---|---|
| Gene Trak *Salmonella* DLP | Neogen | 1996 | *Salmonella* | Food, animal feed, environmental samples |
| RapidChek Salmonella | Strategic Diagnostics | 2003 | *Salmonella* | Variety of Foods |
| BAX PCR Assay for Screening *E. coli* O157:H7 | Dupont Qualicon | 2004 | *E. Coli* O157:H7 | Apple cider, orange juice, ground beef |
| Pathigen *E. coli* O157 test | IGEN | 2003 | *E. Coli* O157 | Raw ground beef |

## Detection Limit of Rapid Test Kits for *Salmonella* in Buffer

| | Detection Limit ($log_{10}$ CFU) | | |
|---|---|---|---|
| Serotype | Gold EIA | BAX | GENE-TRAK |
| Tennessee | 6 | 2 | 5 |
| Muenchen | 6 | 2 | 5 |
| Mbandanka | 6 | 2 | 5 |
| Cubana | 6 | 2 | 5 |

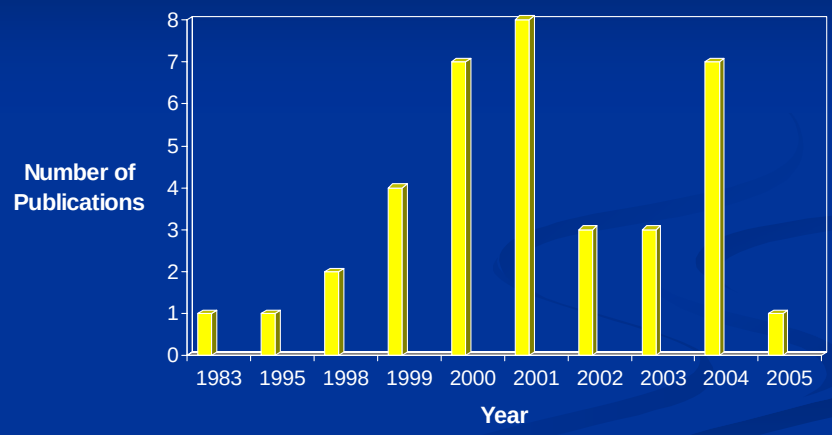
Gold EIA – AOAC Official Method
BAX – unclassified
GENE-TRAK – AOAC Performance Tested Method

## Detection of *Salmonella* in Sprouts and Water from Outbreak Seeds by Rapid Test Kits and BAM

| | Detection by Assay | | | |
|---|---|---|---|---|
| | BAM | Gold EIA | BAX | GENE-TRAK |
| Lot A | 17/20 | 18/20 | 3/20 | 18/20 |
| Lot B | 12/20 | 16/20 | 6/20 | 12/20 |
| Lot C | 4/20 | 7/20 | 4/20 | 6/20 |
| **Totals** | **33/60[a]** | **41/60[b]** | **13/60[c]** | **36/60** |

[a] 3 false negatives
[b] 5 false positives
[c] 23 false negatives

## Publications on Pathogen Detection Methods in Sprouts




## Method Considerations

- Has the method been tested for sprouts / water?
- Has the entire method been tested against a standard method:
  - enrichment – detection - confirmation
- Has the method been evaluated or *validated?*

# Methodologies and Sampling Looking Forward

## Sampling and Testing: New Issues

- Sampling/testing during sprouting
  - Sampling plan
    - When to sample
      - 24 h vs. 48 h
    - How to sample
      - 1 L per batch vs. pooling
      - Considerations for mung bean sprouts
- Sampling/testing of seeds
  - Sampling plan
    - How to sample
  - Testing
    - Testing seeds vs. testing sprouts
- Detection methodologies
    - Additional rapid methods are available

# Sampling and Microbial Testing of Spent Irrigation Water

## When to Sample: 24 h vs. 48 h

- To allow earlier determination of presence/absence of pathogens in sprouts
- Additional growth studies have been conducted since 1999

## Growth of pathogens during sprouting of alfalfa seeds

| Apparatus | Pathogen | 0 h (log CFU/g) | 24 h (log CFU/g) | 48 h (log CFU/g) |
|---|---|---|---|---|
| **Naturally contaminated seeds** | | | | |
| Mini drum | *Salmonella* | -1.5 | 2.0 | 2.0 |
| Mini drum | *Salmonella* | -2.4 / -1.5 | -0.6 / 0.5 | -1 / 1.8 |
| Glass jars | *Salmonella* | -1 | 1.4 | 2.3 |
| Glass jars | *Salmonella* | -1.6 | 2.6 | 3.1 |
| Glass jars | *Salmonella* | -1.2 | 1 | 2.5 |
| Glass jars | *Salmonella* | < detection limit | 1.7 to 2.2 | 2 to 4.5 |
| **Artificially inoculated seeds** | | | | |
| Plastic box | *Salmonella* | 3.2 | 6 | 7 |
| Tray | *Salmonella* | 3.4 | 5 | 5.4 |
| Test tube | *Salmonella* | 1.8 | 4.7 | 6.3 |
| Easy Green™ | *Salmonella* | 0 | 5.7 | 5.7 |
| Screen tray | *Salmonella* | 3.6 | 6.9 | 7.9 |
| Plastic box | *E. coli* O157:H7 | 3.1 | 5.0 | 4.8 |
| Tray | *E. coli* O157:H7 | 3.1 | 5.7 | 5.7 |
| Glass jars | *E. coli* O157:H7 | 3.9 / 1.9 | 6 / 4.5 | 5.5 / 5.3 |
| Test tube | *E. coli* O157:H7 | 2.7 | 3.8 | 4.3 |

# Summary of Literature Data

## Pathogen growth at 24 h and 48 h of sprouting

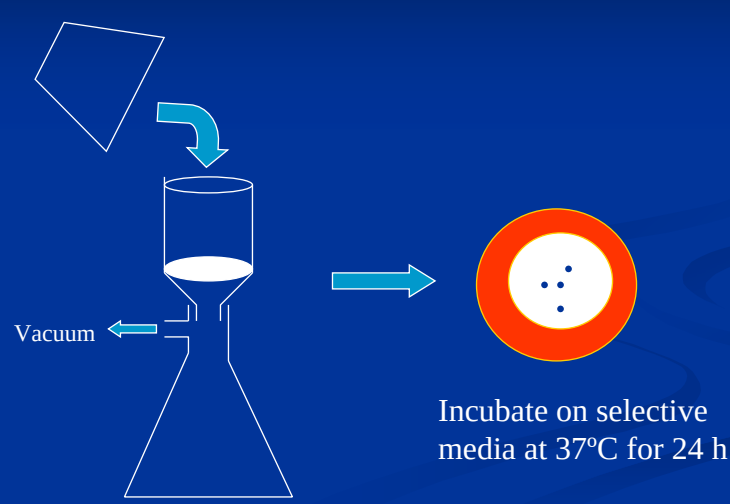
| | Increase at 24 h (log CFU/g) | Increase at 48 h (log CFU/g) | Log increase at 48 h |
|---|---|---|---|
| Naturally contaminated seeds | 1.8 - 4.2 | 1.4 - 4.7 | 0.85 ± 0.27 |
| Artificially inoculated seeds | 1.1 - 5.7 | 1.6 - 5.7 | 0.88 ± 0.22 |

# How to Sample

- 1999 FDA guidance
  - Recommended 1 L of spent irrigation water be collected per production batch
  - Pooling was not advised due to concerns of sample dilution
- Microbial counts in mung bean spent irrigation water may be much lower than those of bean sprouts
- Sample pre-concentration
  - Allow concentration of mung bean sprout water
  - Allow pooled samples to be tested with a single test

# Sample Pre-Concentration

## Standard Membrane Filtration



# Tangential Flow Filtration System

| | |
|---|---|
| **Volume processed** | Up to 10 L |
| **Time required** | < 2 hours |
| **Volume reduction** | 100 fold (10 L – 100 mL) |
| **Methods that can be used for analysis** | Culture methods, dipstick tests, ELISA, biosensors |

# Evaluation of TFF for Concentration of Pathogens

- Determine whether the increase in background microflora in the concentrated samples affects the enrichment and subsequent detection of pathogens by conventional and/or commercially available rapid methods.
- Determine the lowest level of pathogens that the system can recover.

## Validation of tangential flow filtration as part of sample treatment for detection of *Salmonella* in spent sprout irrigation water

| Seedlot | Total Counts (CFU/ml) | Inoculation level (CFU/10 ml conc. sample) | Sample:media = 10ml : 90ml | |
|---|---|---|---|---|
| | | | Assurance Gold™ EIA | Confirmed |
| A | $5.4 \times 10^7$ | 0 | 0/5 | 0/5 |
| | | 1.2 – 4.4 | 18/20 | 20/20 |
| | | 12 – 44 | 20/20 | 20/20 |
| | | 120 – 440 | 20/20 | 20/20 |
| B | $3.0 \times 10^7$ | 0 | 0/5 | 0/5 |
| | | 3.3 – 3.8 | 19/20 | 19/20 |
| | | 33 – 38 | 20/20 | 20/20 |
| | | 330 – 380 | 20/20 | 20/20 |
| C | $2.1 \times 10^7$ | 0 | 0/5 | 0/5 |
| | | 2.8 – 4.7 | 16/20 | 19/20 |
| | | 28 – 47 | 20/20 | 20/20 |
| | | 280 – 470 | 20/20 | 20/20 |

Number of positive samples / Number of samples tested

## Recovery of *Salmonella* from the TFF system

| Inoculation level (CFU/10 L sprout water) | Actual counts (CFU/10 L sprout water) | Assurance Gold™ EIA | Confirmed |
|---|---|---|---|
| 0 | 0 | 0/2 | 0/2 |
| 1 | 2.8 | 0/3 | 0/3 |
| 10 | 28 | 3/3 | 3/3 |
| 100 | 280 | 3/3 | 3/3 |

Number of positive samples / number of sample tested

# Validation of the TFF system: *E. coli* 0157:H7

| Seedlot | Total Counts (CFU/ml) | Inoculum (CFU/ml) | Enrichment media: Reveal 20 h media | | | Enrichment media: mBPW+acv | | |
|---|---|---|---|---|---|---|---|---|
| | | | device | confirmed | confirmed with IMS | device | confirmed | confirmed with IMS |
| A | 1.8–6.5 x $10^7$ | 0 | 0/10 | 0/10 | - | 0/10 | 0/10 | - |
| | | 1.0 – 2.4 | 5/20 | 5/20 | - | 3/20 | 5/20 | - |
| | | 10 – 24 | 20/20 | 14/20 | - | 7/20 | 9/20 | - |
| | | 100 – 240 | 20/20 | 20/20 | - | 17/20 | 19/20 | - |
| | | | | | | | | |
| B | 1.3–9.3 x $10^7$ | 0 | 0/10 | 0/10 | - | 0/10 | 0/10 | - |
| | | 1.3 – 1.7 | 3/20 | 7/20 | - | 0/20 | 0/20 | - |
| | | 13 – 17 | 20/20 | 15/20 | - | 0/20 | 3/20 | - |
| | | 130 – 170 | 19/19 | 19/19 | - | 20/20 | 13/20 | - |
| | | | | | | | | |
| C | 1.3–10.7 x $10^7$ | 0 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 |
| | | 1.0 - 2.6 | 1/20 | 2/20 | 11/20 | 1/20 | 0/20 | 10/20 |
| | | 10 - 26 | 20/20 | 15/20 | 20/20 | 3/20 | 6/20 | 18/20 |
| | | 100- 260 | 20/20 | 20/20 | 20/20 | 17/20 | 9/20 | 20/20 |

# Recovery of *E. coli* O157:H7 from the TFF system

| Inoculation level (cfu/10 L sprout water) | Actual counts (cfu/10 L sprout water) | Confirmed IMS+TC-SMAC |
|---|---|---|
| 0 | 0 | 0/3 |
| 1 | 1.1-3.1 | 3/3 |
| 10 | 11-31 | 3/3 |
| 100 | 110-310 | 3/3 |

Number of positive samples / number of sample tested

# Seed Sampling and Testing

- Outbreak investigation
- Screening seeds used for sprout production

## Isolation of pathogens from outbreak associated sprouts/seeds

| Pathogen | Year | Location | Type of Sprout | No. of Cases | Isolated from Sprouts/Seeds |
|---|---|---|---|---|---|
| *E. coli* O157:NM | 1998 | CA | Alfalfa/Clover | 8 | Yes/No |
| *S.* Bovismorbificans | 1994 | Sweden, Finland | Alfalfa | 595 | Yes/No |
| *S.* Enteritidis | 2000 | CA | Mung | 45 | No/No |
| *S.* Gold-Coast | 1989 | U.K. | Cress | 31 | Yes/No |
| *S.* Havana | 1998 | CA, AZ | Alfalfa | 18 | No/Yes |
| *S.* Havana/Cubana/ Tenn. | 1998 | CA | Alfalfa | 34 | Yes/Yes |
| *S.* Mbandaka | 1999 | OR,CA, ID,WA | Alfalfa | ~ 68 | Yes/Yes |
| *S.* Montevideo/ Meleagridis | 1996 | CA | Alfalfa | >500 | Yes/No |
| *S.* Newport | 1995 | Denmark | Alfalfa | 154 | Yes/Yes |
| *S.* Newport | 1995-96 | OR, Canada, Denmark | Alfalfa | 133 | Yes/Yes |
| *S.* Saint-Paul | 1988 | U.K. | Mung | 143 | Yes/Yes |
| *S.* Senftenberg | 1997-98 | CA | Alfalfa/Clover | 52 | Yes/No |
| *S.* Stanley | 1995 | US, Canada, Finland | Alfalfa | >272 | No/No |

IFT/FDA Report, 2001

## Detection of *Salmonella* in Naturally Contaminated Seeds (Inami et al., 2001)

- Sampling Plan
  - Sample size: 500 g
  - Sample unit: 100 g
  - 60 sample units per lot were analyzed
- Testing methodologies
  - Sample processing
    - Shredding vs. Sprouting
  - Detection
    - BAM

## What to Test: Seeds vs. Sprouts

### Inami et al., 2001

- Shredding
  - 100 g dry seeds blended for 2 min
  - Shredded seeds mixed with 900 ml lactose broth
  - Followed BAM for sample pre-enrichment, enrichment and confirmation
- Sprouting
  - 100 g seeds soaked in 200 ml sterile water for 18-20 h at 25ºC.
  - Sprouted for 3 days at room temperature
  - 100 g germinated sprouts mixed with 900 ml lactose broth and blended for 2 min
  - Followed BAM for sample pre-enrichment, enrichment and confirmation

| | positive / total analyzed | | # positive shredding / # positive sprouting |
|---|---|---|---|
| Seed lot | Sprouting | Shredding | |
| A | 3/30 | 2/30 | 75% |
| B | 10/30 | 9/30 | 90% |
| C | 27/30 | 24/30 | 89% |

# What to Test: Seeds vs. Sprouts

## Fu et al., 2005 (unpublished)

- Soak water vs. Day 2 water
  - 100 g seeds soaked in 500 ml sterile tap water for 3 h. Soak water collected for analysis.
  - Soaked seeds were sprouted in glass jars at 25 ºC and rinsed with 500 ml sterile water daily. Day 2 water collected for analysis.

| | # positive sample / # sample analyzed | | # positive on Day 0 / # positive on Day 2 |
|---|---|---|---|
| | Soak water | Day 2 water | |
| Seedlot CoA98<br>1999 outbreaks in WI | 12/18 | 16/18 | 75% |
| Seedlot 36120<br>1998 outbreaks in CA | 2/18 | 5/18 | 40% |
| Seedlot 45197<br>1999 outbreaks in OR, WA | 2/24 | 6/24 | 33% |

# Effect of Sampling Size

| | Sample size | Soak water<br>(# positives / # analyzed) |
|---|---|---|
| Seedlot CoA98<br>1999 outbreaks in WI | 100 g | 12/18 |
| | 1 kg | 5/5 |
| Seedlot 45197<br>1999 outbreaks in OR, WA | 100 g | 2/24 |
| | 1 kg | 5/5 |

## Screening Seeds Used for Sprout Production: Industry Practice

- Six-step procedure developed by ISS and Jonathan's Sprouts
  - Seed sampling
    - 25 g subsamples from each bag . At least 3 kg per seed lot
  - Seed inspection
  - Sprout growing
    - Follow normal sprouting procedures
  - Spent irrigation water sampling
    - 48 h
  - Enrichment of sampled water
  - Pathogen testing
- Prevented at least one potential outbreak of *E.coli* 0157:H7 and prevented shipment of contaminated seeds to sprouters.

## Detection Methodologies

Validation performed in sprout system is critical

## Automated Immunomagnetic Separation System

The Pathatrix System
(Matrix MicroScience, CO)

- AOAC performance certified for detection of *E. coli* O157:H7 and *Salmonella*
- Capable of isolating a single pathogen from 25 g of sample

## Evaluation of the Pathatrix IMS system for isolation of *E. coli* O157:H7 from spent irrigation water

| | Pathatrix protocol BPW @ 37 °C w/ shaking | | Modified protocol mBPW+ACV @ 37°C w/ shaking | | Modified protocol Reveal 8h @ 42°C w/out shaking | |
|---|---|---|---|---|---|---|
| Inoc. level (CFU/sample) | Unconc. | TFF-conc | Unconc. | TFF-conc | Unconc. | TFF-conc |
| 0 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| 1 | 0/3 | 0/3 | 2/3 | 0/3 | 3/3 | 3/3 |
| 10 | 0/3 | 0/3 | 3/3 | 0/3 | 3/3 | 3/3 |
| 100 | 0/3 | 0/3 | 3/3 | 2/3 | 3/3 | 3/3 |

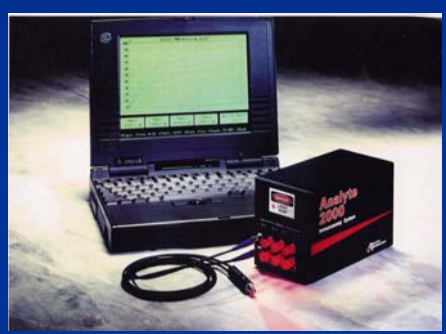
Sample size: 25 ml; TFF samples correspond to 2.5 L of unconcentrated sprout water

## Evaluation of the Pathatrix IMS system for isolation of *Salmonella* from spent irrigation water

| | Pathatrix protocol BPW at 30°C, plated on XLD/HE/BS | | Modified protocol TT at 42°C, plated on XLD/HE/BS | |
|---|---|---|---|---|
| Inoc. level (CFU/sample) | Unconc. | TFF-conc | Unconc. | TFF-conc |
| 0 | 0/3 | 0/3 | 0/3 | 0/3 |
| 1 | 0/3 | 0/3 | 3/3 | 3/3 |
| 10 | 2/3 | 1/3 | 3/3 | 3/3 |
| 100 | 2/3 | 3/3 | 3/3 | 3/3 |

Sample size: 25 ml; TFF samples correspond to 2.5 L of unconcentrated sprout water

## Real Time Pathogen Monitoring
### Fiber-Optic Biosensor




Analyte 2000™

RAPTOR™

# Literature Data

- Analyte 2000™ was shown to be able to detect the presence of *E. coli* O157:H7 in ground beef extract at a level of 3-30 CFU/ml (DeMarco et al., 1999)
- RAPTOR™ was shown to be able to detect the presence of *Salmonella* in spent irrigation water collected at 67 h of sprouting from seeds inoculated with 50 CFU/g of the pathogen (Kramer et al., 2004).

## Evaluation of the Analyte 2000™ Fiber-Optic Biosensor for Detection of *E. coli* O157:H7 in Spent Sprout Irrigation Water

| *E. coli* O157:H7 Concentration (CFU/ml) | Signal Change, Mean ± Stdev (pA) | Range of 3xStdev (pA) | #Positive / #Tested |
|---|---|---|---|
| 0 | 7.61 ± 9.79 | 1.8-29.7 | 2/12 |
| $1.9 \times 10^0$ | 17.13 ± 32.77 | 0-36.3 | 4/12 |
| $2.1 \times 10^1$ | 3.54 ± 3.95 | 3.0-11.7 | 3/12 |
| $2.4 \times 10^2$ | 13.02 ± 9.18 | 3.0-50.4 | 7/12 |
| $1.8 \times 10^3$ | 21.42 ± 49.75 | 0.9-55.2 | 3/12 |
| $1.8 \times 10^4$ | 36.01 ± 53.27 | 8.1-40.8 | 5/12 |
| $1.8 \times 10^5$ | 85.82 ± 118.97 | 3.6-28.2 | 11/12 |
| $1.6 \times 10^6$ | 39.18 ± 46.28 | 3.9-39.3 | 12/12 |

# Conclusions

- A great deal of information has been accumulated since 1999, some of which may help to improve the efficacy as well as the compliance of microbiological testing of sprouts.
- Sample pre-concentration by tangential flow filtration would allow 10L of spent irrigation water to be tested with a single test and achieve a detection limit of 1 - 10 CFU/10 L.
- Seed screening provides additional control to prevent sprout associated outbreaks. It also provides additional protection for consumers who produce sprouts at home.
- Testing sprouts/spent irrigation water appears to provide better detection than testing seeds.
- Many new detection methods have been developed. Evaluation of their efficacy in sprout system is critical.


# Acknowledgements

- **Historical overview**
  - Joseph Schlesser, Ph.D., NCFST/FDA
  - Diane Stewart, NCFST/FDA
  - Karl Reineke, NCFST/FDA
  - Jodie Ulaszek, NCFST/IIT
- **Validation of the TFF system for sample pre-concentration**
  - Karl Reineke, NCFST/FDA
  - Olive VanPelt, NCFST/IIT
  - Thomas Krupinski, NCFST/IIT
- **Evaluation of the automated IMS system**
  - Peter Slade, Ph.D., NCFST/IIT
  - Nicole Maks, NCFST/IIT
  - Brian Parisi, NCFST/IIT
- **Evaluation of the fiber optic biosensor**
  - Kay Lin, NCFST/IIT
- **Seed Sampling**
  - Karl Reineke, NCFST/FDA
  - Olive Van Pelt, NCFST/IIT
  - Thomas Krupinski, NCFST/IIT