Principles of Microbiological Testing: Methodological Concepts , Classes and Considerations

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Relating Microbiological Testing and Microbiological Criteria to Public Health Goals

October 31- November 1, 2005

Gallaudet University Kellogg Conference Center

Washington, DC

Basic Principles of Microbiological Testing of Food

- **Collect sample(s) of the food**
- **Prepare homogenates**
- **Analyze homogenates**
  - **Direct Microscopy**
  - **Detection/Enumeration**
    - Quantitative; Colony Counts, MPN
    - Qualitative; enrichment followed by detection technique and isolation of target organism
    - Evidence of growth may be detected by various means; visual, biochemical, immunological, genetic

Detection Technologies

- Enzyme immunoassay
  - Immuno-capture
  - Immuno-precipitation
- Nucleic acid hybridization
  - DNA/RNA
  - PCR & other nucleic amplification methods
- Electrochemical
- Enzymatic amplification
- Electrical separation
- Chromatographic separation

Quantitative Microbial Analyses

- Lower Limit "Rules of Thumb"
  - <10 - 100 cfu/g — MPN
  - >10 - 100 cfu/g — Viable Counts
  - $>10^3$ - 10^4 cfu/g — DEFT
  - $>10E^4$ - 10^5 cfu/g — ELISA, Flow Cytometry, Quantitative PCR
  - $>10^5$ - 10^6 cfu/g — Direct microscopy, Spectrophotometry
- Sensitivity may be increased by methods through enrichment of multiple samples at the limit of detection (calculated MPN)

Precision of Colony Counts

For Poisson distribution, Variance (s^2) = Mean $(\bar{x})$

$$RSD(\%) = \frac{SD}{mean} \times 100 = \frac{\sqrt{\bar{x}}}{\bar{x}} \times 100$$

Mean Colony Count	SD ±	RSD (%) ±	95% Limit Values
400	20.0	5.0	360 - 440
200	14.1	7.1	172 - 228
100	10.0	10.0	80 - 120
50	7.1	14.1	36 - 64
25	5.0	20.0	15 - 35
10	3.2	31.6	4 - 16

Qualitative Detection

- **Enrichment(s) followed by detection, and confirmation (if required)**
  - **Confirmation**
    - differential isolation,
    - Identification of target organism
- **Limit of Detection is dependent on;**
  - **Number of samples**
  - **Size of samples**

Two-class Plans (c = 0): Probabilities of Acceptance

Composition of Lot		Number of sample units tested				
% acceptable	% defective	5	10	20	60	100
98	2	.90	.82	.67	.30	.13
95	5	.77	.60	.36	.05	.01
90	10	.59	.35	.12	<	<
80	20	.17	.11	.01		
70	30	.03	.03	<		
50	50	.01	<			
40	60	<				
30	70					

ICMSF, 1986. *Microorganisms in Foods*, Sampling for microbiological analysis: Principles and applications, University of Toronto Press, Toronto.

Applications of Microbiological Testing at Various Levels

- **Nationally and Internationally**
  - Epidemiological data; e.g., outbreaks, recalls, etc.
  - Baseline studies
  - International Trade (SPS)
- **Industry specific**
  - Trade association studies
  - Retail surveys
- **Company**
  - Across different production facilities and lines
  - Customer/supplier (purchase specifications)
- **Facility/product specific**
  - HACCP
  - Prerequisite programs (GMP, GHP)

Applications of Microbiological Testing in Food Safety Programs

- Microbiological testing plays an essential role in HACCP
  - Hazard analysis
  - Process validation
  - Monitoring of critical ingredients and high risk finished products
  - Verification of CCPs and the overall process

Applications of Microbiological Testing in Food Safety Programs

- GMP/GHP Programs
  - Determine potential for post process contamination
  - Establish adequacy and frequency of cleaning and sanitation
  - Detect difficult areas to clean and sanitize

Applications of Microbiological Testing in Food Safety Programs

- Compliance testing
  - Mandatory regulatory programs
  - Purchase specifications
  - Documentation in case of litigation
- Problem solving
  - Often microbiological data exists but is only being used for acceptance on a given unit of production (batch, lot, day)
  - Trend analysis of this data often identifies the most likely source of a problem, or pin points areas for further investigation

Microbiological Criteria (Codex)

A microbiological criterion defines the acceptability of a product or a food lot, based on the absence or presence, or number of microorganisms including parasites, and/or quantity of their toxins/metabolites, per unit(s) of mass, volume, area, or lot .

Microbiological Criteria should specify (CODEX)

- the food , and the point in the food chain to which it applies
- any actions to be taken when the Criterion is not met

Microbiological Criteria Components

- Microorganisms and reasons for concern
- Analytical methods to be used
- Sampling plan and size of analytical units
- Microbiological limits
- Numbers of units to be in conformity

Establishment and Application- CAC / GL 21 - 1997

Uses of Microbiological Criteria

- **Assess the safety of food**
- **Verify/validate procedures in HACCP**
- **Demonstrate adherence to GMP/GHP**
- **Demonstrate the utility (suitability) of a food or ingredient for a particular purpose**
- **Establish the keeping quality (shelf-life) of certain perishable foods**
- **As a regulatory tool to drive industry improvement**
- **As verification that a Performance Objective or FSO has be acheived**

Types of Acceptance Criteria

• **Standar**d—a mandatory criterion that is part of a law or ordinance.

• **Guidelin**e—an advisory criterion issued by a control authority, industry association, or food producer to indicate what might be expected when best practices are applied.

• **Specificatio**n—Part of a purchasing agreement between a buyer and supplier of a food; such criteria may be mandatory or advisory according to use.

Sampling Plans

- **Define the probability of detecting a microorganisms or other hazards in a lot**
- **None can ensure the absence of a particular hazard**
- **Should be administratively and economically feasible**

Types of Microbiological Sampling Plans

Attributes plans:

Qualitative analytical results (presence/absence) or quantitative results that have been grouped (e.g. <10 cfu/g, 10 to 100 cfu/g, >100 cfu/g)

Variables plans:

Non-grouped quantitative analytical results

Require distributional assumptions be made

Indicators

- Should indicate something :
  - *Contamination*
  - *Survival*
  - *Recontamination*
  - *Growth*
- Should be easy to determine
- Should behave as pathogen (growth, survival) when used instead of testing for pathogen
- Cannot be relied upon as "proof" that pathogen of concern is absent

Pathogen not measurable

- Example : < 1 *Salmonella* / 10 kg of dried egg-product
- Enterobacteriaceae are good indicators of
  - adequate pasteurisation and
  - control of recontamination

Salmonella criterion for dried egg products

- case 11 : n = 10 c = 0, 25g samples

- lots containing 1 S. per 83 g
  - will be rejected with 95% probability

lots containing < 1 S. per 7.7 kg will be accepted with 95% probability

A producer would need to test 565 end-products to verify that he would meet this criterion

Indicators are measurable

- Example: Absence of Enterobacteriaceae in 1 g of egg-product

a) case 7 : n = 5, c = 2 * (use : biscuit)
b) case 8 : n = 5, c = 1 (dried egg)
c) case 9 : n = 10, c = 1 (use : tiramisu)

*** if adequate heating is assured, no testing is necessary**

Utility and Indicator Testing

Species or groups or microorganisms whose presence may indicate the potential for the presence of pathogens or the extent to which good manufacturing and hygiene practices were adhered to during manufacture

Examples

- **Aerobic plate count**
- **Coliforms or Enterobacteriaceae (EB)**
- ***Escherichia coli***
- **Yeast and mold count**

Salmonella Incidence in Relationship to *E.coli* Most Probable Number (MPN) in Raw Pre-formed Meat Patties

E. coli MPN / g	Samples Within MPN Range	Samples Positive For Salmonella Within MPN Range	% Positive Within MPN Range
<3	270	2	0.7
3-5	406	20	4.9
51-100	54	3	5.6
101-240	96	4	4.1
241-1,100	65	3	4.6
1,101-11,000	56	9	16.1
>11,000	25	5	20.0

No indicators available

- Example : <1 C. botulinum in 1000 ton of low-acid canned meat product

- Reliance on
- Process Criteria (bot cook)
- and GMP

No Microbiological Criteria

To Test or Not to Test ?

Severity of the hazard(s)

New information linking the food to illness

Whether the food is
- Commonly involved in disease
- Primarily destined for a sensitive population
- From a country with endemic disease of importance to food safety

History of consistency and compliance

Distribution of contaminant(s)
- Homogenous, heterogeneous, stratified

Ability to sample
- Sufficient numbers
- Random sampling

Depending on the application the requirement for confidence in the data will be different

- Method performance must be appropriate for the "purpose intended" e.g.
  - Industrial process monitoring.
  - Regulatory screening of foods.
  - Industrial process verification.
  - Regulatory compliance with criteria.
  - Forensic investigation.

Greater Accuracy & Precision required

AOAC Method Validations Protocols Suggested for Various Applications

Applications

- **Process & Product monitoring**
  - **Raw materials**
  - **In-process testing**
- **Process Validation & Verification**
  - **Process validation**
  - **HACCP verification**
- **Regulatory Testing**

Validation protocols

- **Single Lab (SLV)**
  - **10-20 samples per matrix/level**
- **Multi-Lab Validation (MLV)**
  - **Similar to SLV but with multiple labs and more samples**
- **Harmonized Collaborative Validation (HCV)**

Method Validation Criteria

- Ruggedness
- Inclusivity
- Exclusivity
- Matrices
- Comparison to standard
  - Correlation
  - Agreement
- Performance
  - False neg.
  - False pos.
  - Repeatability
  - Reproducibility

Comparison of Confidence Intervals for 3 Levels of Validation for a Qualitative Method, assuming the same performance characteristics in each

Validation Level	# Labs	# Rep Samples/ Lab	Total # Rep Samples	Sensitivity (% +ve)	95% CI
SLV	1	20	20	50%	27.6 - 72.4%
MLV	2	20	40	50%	34.2 - 65.8%
HCV	10	6	60	50%	37.1 - 62.9%

Note: CI of HCV would normally be greater than SLV or MLV due to inter-laboratory variance (repeatability), not included in above analysis.

Factors affecting the Confidence (Uncertainty) in Microbiological Data

- Sampling plan, method and size
- Method performance characteristics
  - Sensitivity
  - Repeatability
  - Ruggedness
- Laboratory performance
  - Error rates
  - Reproducibility

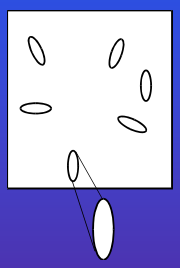
Uncertainty in Microbiological Analyses

- Types of Error
  - Inherent - part of the procedure or method
  - Performance - error associated with laboratory and/or analyst
- Sources of Error
  - Distribution of microorganisms in matrix
  - State of the microorganisms
  - Sampling plan
  - Sampling handling
  - Analysis
  - Reporting

Distribution of microorganisms

- Homogenous
- Heterogeneous
- Random
- Non-random
- Stratified

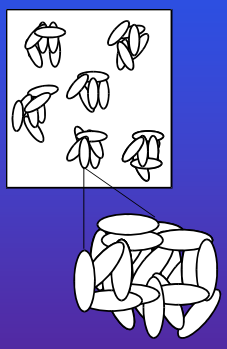
Incidence vs. Level



Example A

Incidence = 6 pos. per 100 x 1 lb. samples

Level = 6 cells per 100 lbs.



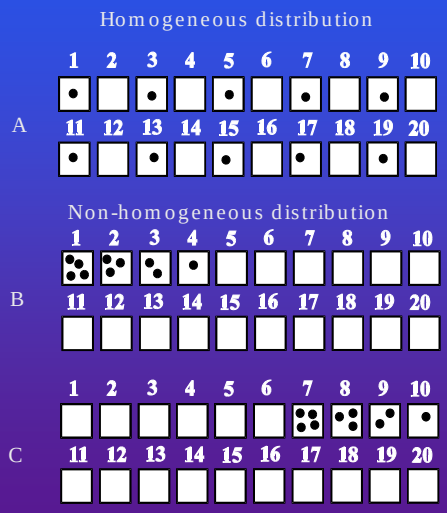
ca. 100 cells /clump

Example B

Incidence = 6 pos. per 100 x 1 lb. samples

Level = 600 cells per 100 lbs.

Distribution of Microorganisms



Sampling plan (others will cover in detail)

- **Sample selection**
  - **random**
  - **stratified**
  - **specified frequency**
- **Sampling method**
- **Number of samples**
- **Size of samples (analytical unit)**

Note: Statistical considerations of sampling plans generally assume that the method ection is error free

Analysis

- Method Selection and Performance
  - inherent accuracy and precision of the method assuming all procedures are done perfectly
- Analyst Performance
  - training
  - proficiency
- Laboratory Performance
  - quality systems
  - proficiency

Method validation and proficiency testing are essential components of a laboratory's quality system and are necessary to determine Uncertainty of a microbiological data result

Method Validation

- Why
  - It sets the performance parameters of the method and demonstrates laboratory competency
- When
  - Upon introduction of a new method, the revision of a method, or addition of a new test matrix

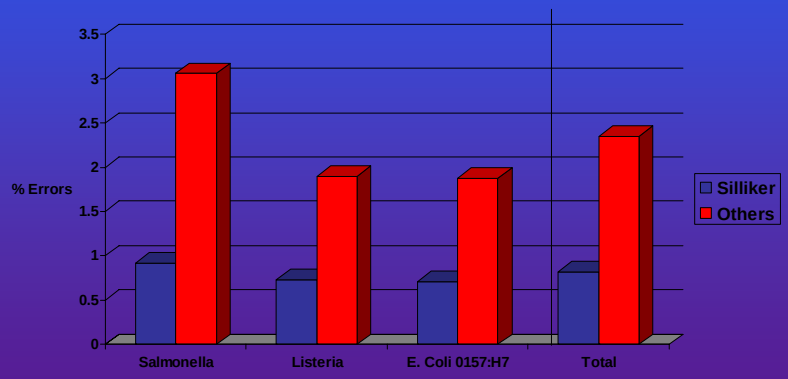
Proficiency Testing

Laboratory proficiency measures a laboratory's ability to generate data that is comparable to other laboratories for identical samples.

Analyst proficiency measures a analyst's ability to generate data that is comparable to other analysts for identical samples.

Pathogen Testing Errors
AOAC Split Sample Program (Jan 01 - Oct 03)

There are 240 private and hundreds more in-plant and corporate food testing labs in the U.S. Approximately 100 labs participate in the AOAC Split Sample Program. This program allows them to benchmark their proficiency against other labs. The Silliker lab data represents 22% of the test volume, and shows significantly better accuracy than the rest of the population.



Quality Requirements
Summary and Conclusions

- Microbiology is not an exact science
- Many sources of variance not related to mistakes
- Many manual steps subject to human error
- Interpretation of data must consider this variation
- Product specifications and process guidelines should be based on sufficient data to know the coefficient of variation
- Uncertainty of the analytical result must be considered when establishing microbiological criteria, including variance associated with the sampling plan, method of analysis and laboratory performance.

Summary and Conclusions

Limitations of Microbiological Testing

- Often it is not practical to test a sufficient number of samples for confidence in lot acceptance
- Non-random sampling may cause incorrect conclusions to be drawn
- Finished product testing determines outcomes, not causes or controls
- No feasible sampling plan can ensure absence of a pathogen

Summary and Conclusions

Uses of Microbiological Testing

- Establish baseline data
- Control ingredients
- Identify highly contaminated lots
- Assessing control of the environment
- Verify compliance of PO and FSO (within limits of sampling and testing)
- Verify control of within HACCP/GHP systems
- Validate a HACCP/GHP system provides a desired level of control

Summary and Conclusions

- If FSOs or POs are within a range allowing practical application of sampling plans and testing protocols, then
  - ➔ Microbiological criteria may be useful to verify acceptability of the lot relative to PO and FSO
- Across Lot/Batch testing may be useful to verify that a food safety system or process is continuing to function as intended