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Estimation of Uncertainty of Measurement in Microbiology

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Abstract

Uncertainty of measurement is a quantitative indication of the analytical variability of a result. The uncertainty may need to be taken into account when interpreting data. Systematic assessment of the factors influencing the result and of the uncertainty forms a key part of method validation. This article examines the uncertainty of measurement of urine cultures and how it affects the final patient result.

Introduction

An evaluation, or at least a full consideration, of all of the identifiable components that contribute to the uncertainty of a test result allows valid results to be obtained and indicates the aspects of the test that require attention to improve procedures. In microbiology, it is usually possible to mitigate the combined effects of most, if not all, sources of uncertainty. This is usually accomplished by a robust program of on-the-job training and at least annual competency assessments. However, ISO (International Organization for Standardization) likes to see everything displayed as a mathematical formula. Fortunately or unfortunately, many accrediting agencies are trying to harmonize with ISO so that all testing or medical laboratories throughout the world are held to the same standards. ISO requires the collection of duplicate data for a particular test over a period of time so that the standard deviation can be determined. They call this the intermediate precision of the test method if all possible sources of variation in the method are taken into account when duplicate analyses are performed (1). These sources of variation include transport effects, storage effects, laboratory environmental effects, analyst effects, and the effects of using different items of equipment, different batches of media, etc.

At present, clinical microbiology laboratories are not required to report uncertainty of testing. In fact, the medical community is not yet familiar with this concept. However, testing laboratories accredited under ISO/IEC 17025 are required to incorporate estimation of uncertainty of measurement into all testing (2). Its author, the director of a clinical laboratory accredited by the College of American Pathologists (CAP) and the technical manager of an environmental laboratory accredited to ISO 17025 standards, I wondered what would be required to validate the quantitative results of clinical microbiology cultures. This article reviews how to evaluate a test, using urine cultures as an example.

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Specific Factors Contributing to Uncertainty in Microbiology Quantitative Tests

The following factors should be examined for each applicable test method:

Sampling

- Homogeneity of original sample source
- Source of sample
- Transport time and temperature of sample
- Storage conditions of sample prior to analysis

Method of analysis

- Source of method, e.g., standard versus in-house
- Method validation, if not standard

Homogenization of sample

- Clumping of microorganisms
- Uneven distribution of microorganisms – matrix effect
- Insufficient mixing
- Microbial density

Dilutions

- Accuracy of pre-measured volumes or weights of dilution fluids
- Volume of dilution fluid used
- Degree of mixing at each dilution step
- Number of steps in a serial dilution

  0196-4399/00 (see frontmatter)

- Precision and accuracy of diluting equipment, e.g., Pipetman
- Pipette volume used
- Microorganisms adhering to pipette tip

Media and reagents

- Accurate weighing
- Quality of water
- Performance of media and reagents
- Shelf life
- Storage conditions

Equipment

- Maintenance, repair, and calibration

Personnel

- Validating and maintaining competency
- Hiring

Sample analysis

- Inoculation of media
  - Volume
  - Equipment used to dispense, spread, and filter
- Incubation conditions
  - Duration
  - Temperature
  - Humidity
  - Atmospheric conditions, e.g., CO_2
- Reading and interpretation of results
  - Recognition of target colonies
  - Number of colonies counted
  - Dilution(s) chosen for counting
  - Proportion of colonies confirmed
  - Assessment of intra-laboratory results
  - Assessment of inter-laboratory results

Quantification of Contributions to Uncertainty

Not all identified contributions to uncertainty make a significant contribution to the total uncertainty. Unless there is a large number, contributions that are less than one-third of the largest contribution need not be quantified in detail. In microbiology, we can expect that the precision of the method itself forms the dominant contribution to the uncertainty estimate (1).

Equations for Calculation of Uncertainty in Quantitative Count Tests

Measuring precision

Variance = S^2

$$S^2 = \frac{(X1 - \bar{X})^2 + (X2 - \bar{X})^2 + \ldots + (Xn - \bar{X})^2}{n - 1}$$

X represents each data point
$\bar{X}$ is the mean or average of all data points
Standard Deviation (SD) = $\sqrt{S^2}$
Relative Standard Deviation (RSD) = $S/\bar{X}$
95% confidence interval for population mean: $\bar{X} \pm$ (Students $t \times$ SD/$\sqrt{n}$)

Repeatability

Precision can be measured by repeatability (or replicability), which measures random error of the method for duplicate tests performed under identical conditions (within analyst variation).

For an individual analyst's repeatability:

$$S_R^2 = \frac{(X1 - \bar{X})^2 + (X2 - \bar{X})^2 + \ldots + (Xn - \bar{X})^2}{n - 1}$$

Repeatability variance (S_r^2)

$$(S_r^2) = \frac{[\Sigma \text{ between duplicate variances}]}{n}$$

or

$$(S_r^2) = \frac{[\Sigma \text{ differences between duplicates}^2]}{2n}$$

Where
n = number of samples or duplicate pairs
Repeatability standard deviation $SD_r = \sqrt{S_r^2}$
Relative standard deviation of repeatability (RSD_r) = $SD_r/\bar{X}$

Reproducibility

Reproducibility measures random error under changed conditions of measurement ("ruggedness of the test") (between-analyst or -laboratory variation)

$$S_R^2 = \frac{(X1 - \bar{X})^2 + (X2 - \bar{X})^2 + \ldots + (Xn - \bar{X})^2}{n - 1}$$

If between-analyst duplicates are used to measure reproducibility, reproducibility variance (S_R^2) may be determined in the following manner:

$$(S_r^2) = \frac{[\Sigma \text{ variancess of between-analyst duplicates}]}{n}$$

or

$$(S_r^2) = \frac{[\Sigma \text{ differences of between-analyst duplicates}^2]}{2n}$$

Where
n = number of samples or duplicate pairs
Reproducibility standard deviation (SD_R) = $\sqrt{S_r^2}$
Relative standard deviation of reproducibility (RSD_R) = $SD_R/\bar{X}$

Table 1. Sample of an Excel worksheet for the calculation of intermediate precision for two analysts doing replicate counts[a]

Date	Sample no.	First analyst name	First analyst count	Second analyst name	Second analyst count	RSD_R
	1		61		63	0.00553
	2		85		86	0.00186
	3		120		103	0.02293
	4		121		132	0.01271
	5		164		65	0.00263
	6		65		73	0.01939

RSD reproducibility	
RSD_{RC} minimum	0.01357
RSD_R maximum	0.00186
RSD	0.02293

Application of RSD_{RD}	
Count	61
Lower limit	57
Upper limit	65

RSD, relative standard deviation
RSD_R, relative standard deviation of reproducibility
RSD_{RC}, relative standard deviation of replicate counts

[a]The counts are made by two different analysts within a laboratory. Counts for each analyst are entered into the indicated columns. The relative standard deviation of reproducibility (RSDR) for each pair is then calculated after a $\log_{10}$ transformation of the count. These calculations are then combined to obtain the "average" RSD_{RC} expressed as a $\log_{10}$ value.

Table 2. Sample of Excel worksheet for calculation of intermediate precision for a single analyst doing duplicate calculations[a]

Analyst: _____

Date	Sample	Result 1	Result 2	Log R_1	Log R_2	Log R_1 – log R_2	(Log R_1 – log R_2)^2
	1	61	63	1.7853	1.7993	-0.0140	0.000196
	2	85	86	1.9294	1.9345	-0.0051	0.000026
	3	120	103	2.0792	2.0128	0.0663	0.004402
	4	121	132	2.0828	2.1206	-0.0378	0.001428
	5	64	65	1.8062	1.8129	-0.0067	0.000045
	6	65	73	1.8129	1.8633	-0.0504	0.002541
	7						
	8						
	9						
	10						
							Sum = 0.008638

0.000720	$S^2 = \Sigma(\log R_1 - \log R_2)^2$ divided by 2 times test no.
0.02683	S = square root of S^2 = 1 standard deviation
0.05366	$2S$ = 2 standard deviations

To calculate antilog on Microsoft calculator, hit inv and then log.

R_1, first result
R_2, second result
Σ, sum
S, standard deviation
$\Sigma (\log R_1 - \log R_2)^2$, sum of the squared differences between each set of duplicate results divided by the number of tests multiplied by 2.

[a]Calculation of intermediate precision (relative standard deviation [RSD]) for duplicate plate counts.

0196-4399/00 (see frontmatter)

Periodicity of Estimating Uncertainty

Estimation of uncertainty should be assessed at least annually to ensure that it still reflects the conditions in the laboratory, such as staffing and equipment in use.

It is advisable to estimate personnel uncertainty by having an occasional plate read twice in the daily routine. It is best to pick the plates randomly for the second counting after the initial count has been made. Moreover, both readings must be made on the same day to avoid problems with new organisms or overgrowth on the plate. The uncertainty estimate becomes fairly reliable after more than 30 duplicate counts have been included.

A minimum of 5% inter-analyst analysis should be completed to assess the accuracy of the analysis within the laboratory. The count difference between analysts should be within 10%.

A minimum of 5% intra-analyst analysis should be completed by each analyst to assess the precision of the analyst. The count difference for a single analyst should be within 5%.

Quantification of Contributions to Uncertainty for Urine Cultures

Table 3 is an example of a potential method to evaluate contributions to uncertainty for urine cultures. In viewing this table, one must keep in mind that some aspects of traditional microbiology can be regarded as an art rather than a science and that no two people analyzing the test would likely assign exactly the same value to each variable in the test. This is ultimately not as important as analyzing whether any of the contributing factors cannot be negated when performed by competent individuals. In the example shown in Table 3, results were assessed based on the original paper by Kass (3) indicating that cultures from 95% of 74 hospitalized patients with clinical signs and symptoms of pyelonephritis (fever, rigors, back pain, and dysuria) grew $\geq 10^5$ CFU/ml of voided urine.

In this example, there are 3 major potential contributors to uncertainty. The first is the angle at which the loop is withdrawn from the urine. If the loop is not withdrawn exactly perpendicular to the surface of the urine, Albers and Fletcher (4) showed that counts could be off by as much as ±50%. The second and third potential factors were the ability of the analyst to (i) recognize and (ii) accurately count separate target organisms. Together, none of the contributions are more than one-third of the largest contributor, so it would not be necessary to report uncertainty for this test (5), assuming all analysts are deemed competent to perform the test.

Summary

The total uncertainty of a test typically consists of multiple components. In microbiology, at least three factors are always involved: (i) the uncertainty of the inoculum volume, (ii) random scatter due to particle distribution, and (iii) the uncertainty of reading the result. An uncertainty associated with method bias is not relevant, as quantitative microbiological tests are empirical tests with the result of an analysis being dependent on the media, incubation times, temperatures, and all other steps included in the measurement process. On the whole, quantitative microbial analysis (e.g., urine cultures and bronchial alveolar lavage) should be fairly straightforward. The same principles in the preanalytical stage (specimen collection, transport, and storage) and the analytical stage, dilution, if applicable; plating; incubation; counting; identification of target organism[s]; and calibration of results) apply for all specimen matrices.

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Table 3. Factors contributing to uncertainty in medical microbiology

Test method: Urine culture Date: 6/15/10

Evaluator: Alice S. Weissfeld Is uncertainty reportable? ☐ Yes ☑ No

Source of uncertainty	Contributory factor	Non-contributory	Not applicable	% Contributable[a]	Additional comments (Ref.)
Sampling					
• Homogeneity of original sample source			√		
• Source of sample		√			Midstream only in this example
• Transport time and temperature of sample	√			3%	30 minutes at 4°C (6)
• Storage conditions of sample prior to analysis	√			2%	24 hours at 4°C (6)
Method of analysis					
• Source of method, e.g., standard vs. in-house		√			Cumitech 2C (6)
• Method evaluation, if not standard			√		
• Homogenization of sample					
• Clumping of microorganisms			√		
• Uneven distribution of microorganisms – matrix effect		√			
• Insufficient mixing	√			2%	Must swirl to mix prior to sampling
• Microbial density	√				
Dilutions					
• Accuracy of loop to deliver appropriate volume		√			.001-ml platinum loops no longer used. Plastic loops come with certificate of analysis.
• Angle at which loop is withdrawn from the specimen	√			30%	Result may be off by ±50% (4); however, analyst should be trained to remove loop perpendicularly.
Media and reagents					
• Accurate weighing			√		
• Quality of water			√		
• Performance of media		√			QC'd prior to use
• Shelf life		√			Expiration date on all media
• Storage conditions		√			Temperature check daily
Equipment					
• Maintenance, repair, calibration	√			2%	Incubator checked daily
Personnel					
• Validating and maintaining competency		√			Performed a minimum of once annually
• Hiring		√			Screen for competency prior to employment.
Inoculation of media					
• Volume	√			4%	
• Equipment used to dispense, spread and filter	√			2%	
Incubation conditions					
• Duration	√			2%	Must be a full 18 hours
• Temperature	√			2%	
• Humidity			√		
• Atmospheric conditions, e.g., CO_2			√		
Reading and interpretation of results					
• Recognition of target colonies	√			20%	Analyst competency testing critical
• Number of colonies counted	√			20%	Analyst competency testing critical
• Dilution(s) chosen for counting			√		
• Proportion of colonies confirmed			√		
• Assessment of intra-laboratory results	√			5%	
• Assessment of inter-laboratory results	√			5%	

[a]Factors that contribute less than one-third of the largest contributor to uncertainty need not be quantified in detail.

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