NATIONAL ADVISORY COMMITTEE
ON
MICROBIOLOGICAL CRITERIA FOR FOODS

RESPONSE TO THE QUESTIONS POSED BY THE FOOD SAFETY AND INSPECTION SERVICE REGARDING DETERMINATION OF THE MOST APPROPRIATE TECHNOLOGIES TO ADOPT FOR FSIS ROUTINE AND BASELINE MICROBIOLOGICAL ANALYSIS

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List of acronyms

AFLP	amplified fragment length polymorphism
APC	total aerobic plate count
APHIS	Animal and Plant Health Inspection Service, USDA
ARS	Agricultural Research Service, USDA
CCEHBR	Center for Coastal Environmental Health and Biomolecular Research, of NOAA's National Ocean Service
CDC	Centers for Disease Control and Prevention, DHHS
CSREES	Cooperative State Research Education and Extension Service (now IFA), USDA
CFSAN	Center for Food Safety and Applied Nutrition, FDA
CFU	colony-forming units
CRADA	Cooperative Research and Development Agreement
CVM	Center for Veterinary Medicine, FDA
DHHS	Department of Health and Human Services
DOC	Department of Commerce
ECC	*E. coli* Biotype I count
ECL	electrochemiluminescence
ELISA	enzyme-linked immunosorbant assay
EPA	Environmental Protection Agency
EPEC	enteropathogenic *E. coli*
FAO	Food and Agricultural Organization of the United Nations
FDA	Food and Drug Administration, DHHS
FERN	Food Emergency Response Network
FOBS	fiber-optic biosensors
FRET	fluorescence resonance electron transfer
FTIR	Fourier transform infrared
FSIS	Food Safety and Inspection Service, USDA
HACCP	Hazard Analysis and Critical Control Points
HCV	harmonized collaborative validation
IAEA	International Atomic Energy Agency of the United Nations
ICMSF	International Commission on Microbiological Specifications for Food
IFA	Institute for Food and Agriculture (formerly CSREES), USDA
IMS	immunomagnetic separation
IPA	inter-governmental personnel agreements
ISO	International Organization for Standardization
LOD	Limit of Detection
LRN	Laboratory Response Network
MAAB	multi-analyte array biosensor
MALDI	matrix assisted laser desorption/ionization
MAP	*Mycobacterium avium* subspecies *paratuberculosis*
MEC	Method Evaluation Committee
MLST	multilocus sequence typing
MLVA	multiple-locus variable number tandem repeat analysis
MPN	most probable number
MS	mass spectroscopy

MVT	Method Validation Team
NA	not applicable
NACMCF	National Advisory Committee on Microbiological Criteria for Foods
NAHMS	National Animal Health Monitoring System, USDA-APHIS
NARMS	National Antimicrobial Resistance Monitoring System
NAS	National Academies of Science
NIH	National Institutes of Health, DHHS
NMFS	National Marine Fisheries Services
NOAA	National Oceanic and Atmospheric Administration
NPL	National Program Leader
NRTE	not ready-to-eat
NSIL	National Seafood Inspection Laboratory, DOC-NOAA Fisheries
OFRG	oligonucleotide fingerprinting of rRNA genes
ORA	Office of Regulatory Affairs, FDA Foods Program
PCR	polymerase chain reaction
PEMC	piezoelectric-excited millimeter-sized cantilever
PFGE	pulsed field gel electrophoresis
PR-HACCP	Pathogen Reduction-HACCP
PVM	*Peer-Verified Methods*^{SM}, AOAC
qPCR	Quantitative real-time PCR
RFP	request for proposal
RTE	ready-to-eat
RT-PCR	real-time PCR
SIP	Seafood Inspection Program, NOAA
SLTs	shiga-like toxins
SLV	Single Laboratory Validation
SNP	single-nucleotide polymorphism
SPR	surface plasmon resonance
SSRMP	Seafood Safety Research and Monitoring Program, NOAA
TCC	total coliform count
TOF	time of flight
TRC	Technical Review Committee
USAHA	US Animal Health Association
USDA	United States Department of Agriculture
xMAP	suspension microarray [Table 3]

1. Introduction: Statement of Charge to NACMCF and the Rationale for the Approach to Address the Charge

1.1. Charge to the Committee:

Determination of the Most Appropriate Technologies for the FSIS to Adopt in Performing Routine and Baseline Microbiological Analyses

The National Advisory Committee on Microbiological Criteria for Foods (NACMCF) should provide guidance to assist with the U.S. Department of Agriculture, Food Safety and Inspection Service (USDA/FSIS) Agency's goal of moving into the next generation of microbiological testing methods. To do so, NACMCF should review the current status of molecular methods, including genotyping assays, nanotechnology, and other available or evolving technologies for potential applicability to FSIS microbial analysis and explore their roles for incorporation into FSIS microbiological testing programs at both the laboratory and in-plant level.

The Agency suggested that the charge might best be approached by NACMCF in two stages. The first would focus on **laboratory methods** for pathogen detection, and the second on **in-plant testing** to reliably assess process control. Analyses for use in FSIS laboratories versus within plants are likely to require different technologies. Analyses carried out in FSIS laboratories will be used for baseline monitoring of national microbial trends and regulatory sampling. In-plant sampling may primarily help in assessing process control and real-time monitoring of plant performance.

FSIS requested the NACMCF to examine the merits of available technologies for application to FSIS microbial testing with a focus on:

- Selectivity and sensitivity
- Adaptability to various matrices (including foods, the processing environment, and human clinical samples)
- Scope of analyses (including species identification, serotype equivalence, antibiotic resistance, PFGE equivalence, and additional indicators of microbial hazards, such as virulence factors)
- Enumeration
- Data acquisition and transfer
- Speed
- Ability to be effectively incorporated into FSIS methods
- Cost and resource efficiency

Charge Questions:

1. What are the most appropriate technologies FSIS should consider for improved microbiological analyses? What are the most promising methods that could replace or complement those currently used at FSIS? What are the important parameters to be considered in determining the suitability of a method for a particular application (such as laboratory analyses for pathogens versus in-plant testing for process control, or routine versus baseline testing, and enumeration of pathogens and indicators)?

2. What are the advantages and disadvantages of these newer technologies/methods? When selecting newer technologies/methods consider the FSIS approach of reliance on culture-confirmed positives for target organisms in the context of method correlation, substitution, and degree of confidence. For instance, if the technology does not measure or correlate with viable cell presence, can reasonable decisions be made about the safety of the product?
3. When adopting new technologies and testing platforms, what considerations must be made regarding sampling protocols? How does sampling (size, site, rinse, excision) impact assay sensitivity, specificity, and limit of detection? Are there any practical ways (concentration technologies, etc.) that could be adopted to compensate for potential loss in specificity, sensitivity, and detection limit requirements for microbiological targets?
4. Consider specifically the accuracy, applicability, and validation of an assay capable of detecting thousands of single-nucleotide polymorphisms (SNPs) in a single reaction. Would such an assay be timely, cost-effective, and capable of screening specimens to monitor process control? Would it be capable of differentiating multiple microbial species in a single sample? Could it have application for differentiating bacterial subspecies (particularly relevant for salmonellae, which are currently characterized by serotype), or detecting antibiotic resistance genes and virulence factors? Determine the suitability of incorporating SNPs in meeting the current and future testing needs of FSIS.
5. When selecting a new technology, what factors should be considered, such that the data generated would be useful in an expanded manner to include attribution/risk profiles and models for human illnesses?
6. What issues will need to be considered to make newer and promising technologies a reality in FSIS' future testing for pathogens and indicator organisms? For technologies that may be useful in the future, identify research gaps that need to be addressed prior to implementation.

1.2. Public Health Focus

Foodborne infections cause an estimated 76 million acute illnesses and 5,000 deaths each year in the United States (Mead 1999). These infections are the result of the contamination of food with a variety of disease-causing bacteria, viruses and parasites that can occur as food moves from the farm to the consumer. The USDA's Economic Research Service (ERS) estimates that illnesses caused by shiga toxin-producing *Escherichia coli (E. coli)*, *Salmonella*, *Campylobacter* and *Listeria monocytogenes* (*L. monocytogenes*) result in $6.9 billion in medical costs and lost productivity each year in the United States (Crutchfield and Roberts, 2000). The Department of Health and Human Services, Food and Drug Administration (DHHS/FDA) further estimates that 2% to 3% of foodborne illnesses result in secondary long-term health consequences (Lindsay, 1997). With the globalization of the food supply and emerging foodborne pathogens, foodborne illness is clearly a serious public health issue that requires continued attention.

Recognizing this threat to the public health, the United States (U.S.) regulatory agencies charged with the oversight of food safety have evolved from a command-and-control (and largely visual) to an increasingly science-based, data-driven inspection approach that shifts significant

responsibility for ensuring the safety of domestic and imported food products to the food industry. In 1996, FSIS adopted Hazard Analysis and Critical Control Points (HACCP), a proactive, preventive system of process control. Microbiological testing plays a critical role in enhancing and verifying HACCP systems. For example, during food production/processing, microbiological testing can be used to continually improve HACCP systems, reducing the likelihood of pathogen contamination, and in so doing, enhance public health. Although end-product testing cannot ensure the safety of food products, microbiological testing data are also pivotal in making policy decisions, guiding compliance/enforcement actions, and developing risk assessments. This is not to say that microbiological testing methods are perfect; in fact, to assure appropriate use, microbiological testing must be accompanied by appropriate sampling techniques which are statistically valid. Taken together, microbiological detection methods employed by regulatory agencies must be robust, dependable and defensible.

Traditionally, FSIS has set public health-based performance goals to assure that the products under their regulatory jurisdiction have a minimal impact on the overall burden of foodborne illness. These goals are based on the Healthy People 2010 (HP 2010) objectives (http://www.healthypeople.gov/document/pdf/Volume1/10Food.pdf) and estimates by the DHHS, Centers for Disease Control and Prevention (CDC) (Mead, 1999). Specifically, FSIS used the HP 2010 goals to establish public health-based performance goals for three pathogen/product pairs: *E. coli* O157:H7 in not-ready-to-eat (NRTE) ground beef products, *L. monocytogenes* in ready-to-eat (RTE) meat and poultry products, and *Salmonella* in NRTE broiler carcasses. Regarding *Campylobacter*, FSIS is in the process of analyzing the results of a year-long baseline study for broiler carcasses, and recently initiated a similar study for turkey carcasses. FSIS expects to establish a quantitative standard for these species in the near future.

Table 1: Healthy People 2010 Objectives*

Pathogen	1997 Baseline Infections	2010 Target
Campylobacter	24.6	12.3
Escherichia coli O157:H7	2.1	1.0
Listeria monocytogenes	0.5	0.25**
Salmonella	13.7	6.8

*****Laboratory confirmed cases/100,000 humans (Food Net)
** Changed to year 2005 by Executive Order (President Clinton)

The impact of FSIS regulatory activities on the HP 2010 goals cannot be measured directly, in large part because of the absence of reliable and detailed foodborne illness attribution data (data which is used to allocate the burden of foodborne illnesses to specific commodities). In recent years FoodNet data have demonstrated that the incidence of reported laboratory-confirmed foodborne illnesses has remained relatively unchanged. Furthermore, the Office of the Inspector General (Semiannual Report to Congress, FY 2003 – Second Half, USDA Office of Inspector

General) has stated that FSIS must develop goals, objectives and methods in support of an effective microbial testing program. Therefore, NACMCF recommends that as a first step, FSIS clearly articulate measurable public health goals and demonstrate how those goals advance the Agency's public health mission to reduce the burden of foodborne illness attributable to FSIS-regulated food products. Once FSIS has articulated its public health goals, FSIS must clearly define its microbiological testing objectives and how they address these goals. Microbiological methods that employ any new technology must then have to fulfill the necessary test criteria that clearly support the FSIS testing objectives and public health goals.

1.3. Committee's Approach to Answering the Charge

Upon reviewing the language in the charge (the title, the preamble, and the charge questions), the NACMCF determined the need to establish a context for the use of the terms "technology/new technology" and "microbiological method/testing/analysis" and then to maintain this context throughout the document for clarity. At this early deliberative juncture, the Committee also believed strongly that FSIS must adopt a longer-term vision which includes development of a process for applying appropriate new microbiological technologies as part of a broad food safety and public health strategy. Thus, the Committee's approach for addressing the charge, that both delineates the terms mentioned above and puts a public health focus front-and-center, emerged as:

> *the recommendation of any new technology for use by FSIS must be presented in an appropriate context to have applicable meaning and utility. The context agreed upon was the application of a new technology as a fully validated microbiological testing method ready for implementation. The method must be rooted in the broader public health goals of FSIS, and further defined by the microbiological testing objectives as applied to an FSIS program activity.*

The NACMCF's full charge and the explicit explanation of the need for public health to be the main driver for how NACMCF addressed the charge (and, in turn, how NACMCF recommended FSIS should develop microbiological testing as part of a food safety strategic plan) is presented above in Sections 1.1 and 1.2, respectively. This Section (1.3) continues below with a description of how the document is further structured to address the charge.

In reviewing the charge questions, NACMCF determined that there was substantial overlap and oftentimes the questions were too prescriptive, making it difficult to address the longer term vision of the Agency. Therefore, the Committee chose to address the charge in a holistic manner, rather than answering the specific charge questions independently. The need for this approach became more apparent as the Committee began its deliberations and recognized that applying new technologies to improve microbiological methods is a dynamic process. Moreover, new technologies emerge at a rapid rate and certain ones may not be practical for use in a food safety testing laboratory, because of expense, operator training needs, ability to transfer into a high throughput testing format, and sample preparation and matrix interference concerns.

Because of these issues, the Committee determined that the best way to structure the document was first to provide a "Background" that discussed the role of testing in the protection of the safety of the food supply (Section 2), particularly in the context of the Federal regulatory system. Next, a

review of the general considerations for the application of various microbiological testing methods to food safety is provided (Section 3). This is followed by a description of new and emerging technologies, including a discussion of the critical performance criteria which need to be considered when selecting, evaluating, and validating new methods that incorporate these technologies (Section 4). A discussion of the advantages and disadvantages of potential emerging methods that employ new technologies follows, which covers multiple issues (*e.g.*, rapid, on-site analysis; discrimination between viable and non-viable cells; the need for an isolate; qualitative versus quantitative results; and multianalyte considerations) in a manner that is relevant to the regulatory "gold standard" of culture-based testing (Section 5). Finally, NACMCF described the critical elements that need to be considered as FSIS seeks to apply a new method that takes advantage of new technologies for an intended food safety and public health-related programmatic purpose (Section 6).

One caveat to the NACMCF approach to address the charge is that the discussion on single nucleotide polymorphism (SNP) technology (Question 4) was limited largely to addressing the focus given in the charge preamble "on **laboratory methods** for pathogen detection" and "on **in-plant testing** to reliably assess process control." Therefore, while the Background (Section 2) describes the present status of several methods, including those based on SNP technology, the discussion is confined to the detection function and does not address the use of these technologies in genotyping and subtyping applications. This is not to say that their use in genotyping is not promising, but rather that the Committee believed that this topic was worthy of a wholly separate charge to NACMCF. FSIS did brief the Committee on an extensively researched internal "white paper" on new subtyping technologies that could supplement and/or potentially replace Pulsed-field Gel Electrophoresis (PFGE), the current gold standard typing method employed in the CDC-managed PulseNet program. Both FSIS and FDA fiscally co-support PulseNet with CDC by Interagency Agreements (IAGs).

In summary, NACMCF chose a public health thrust to drive its response to the Charge, gathered and described background information on the current and future detection technologies which could be applicable to a regulatory setting, and used this foundation as the basis upon which to address the broad charge in a holistic manner. The Committee identified, both in the Table of Contents and the Introduction to each section, the location of discussions addressing the specific charge questions. Some questions are addressed in more than one section. It is the opinion of the Committee that the Charge has been adequately addressed in this document.

2. Background: Testing and Methods Development Programs of Federal Food Safety Agencies

In the U.S., a number of Federal and state agencies have complementary roles in ensuring the safety of a myriad of domestic and imported food products. The two major Federal regulatory agencies responsible for the safety of the food supply are the USDA/FSIS and the DHHS/FDA. The DHHS/CDC conducts human disease surveillance for foodborne and other illnesses of public health importance. In addition, the Environmental Protection Agency (EPA) sets limits on the amount of pesticide residues permitted in food, and the National Marine Fisheries Services (NMFS) within the Department of Commerce provides fee-for-service inspections of seafood

safety and quality. In the Department of Defense, the U.S. Army Veterinary Service is the Executive Agency responsible for food safety and defense. The Veterinary Service audits food processors and monitors food safety and quality throughout the supply chain, which is critically important during deployments.

During information gathering for this Background section from FSIS, the NACMCF learned of the restrictions on method development by FSIS, which apparently occurs because this activity is perceived as research and hence outside the purview of USDA/FSIS. This information prompted the Committee to explore in greater depth the method development activities of other agencies relative to their food safety responsibilities.

2.1. Roles and Responsibilities of Food Safety Agencies

The USDA's FSIS is the public health agency responsible for ensuring that the nation's commercial supplies of meat, poultry, and processed egg products are safe, wholesome, correctly labeled and packaged (USDA, 2008). FSIS monitors domestic and imported meat, poultry and processed egg products for bacterial contamination, residues of pesticides, drugs, and other chemicals through implementation of HACCP and verification testing. FSIS is actively involved in recalls and trace-back/forward activities for products that may be adulterated and/or related to foodborne disease outbreaks. FSIS has a pre-market approval process for all labeling applied to meat, poultry and processed egg products. In addition, by statute, FSIS is required to conduct inspection in all regulated facilities each day. In the case of slaughter and processed egg inspection, FSIS personnel must be continually present during the entire operation. FSIS regulated products are regularly tested for foodborne pathogens such as *Salmonella*, *L. monocytogenes*, and *E. coli* O157:H7, to verify and ensure that process controls are effective. For the meat, poultry and egg products regulated by the FSIS, the pathogens with the greatest impact on the public health are the bacterial agents Shiga-toxin producing *E. coli* (such as *E. coli* O157:H7), *Salmonella*, *Campylobacter*, and *L. monocytogenes*, while viral agents such as norovirus, and parasitic agents, such as *Toxoplasma gondii* are also of concern. In addition, zoonotic pathogens such as *Mycobacterium bovis* and *Brucella abortus*, which are now largely controlled as foodborne problems in this country, still occur in food animals and in wildlife animal reservoirs.

The FDA Foods Program consists principally of activities of the Center for Food Safety and Applied Nutrition (CFSAN) and field programs of the Office of Regulatory Affairs (ORA). The Center for Veterinary Medicine (CVM) has a role in animal feed and veterinary drug safety for animals, including those destined for human consumption. FDA's Foods Program mission is to promote and protect the public health and economic interest by ensuring that the food and feed supply is microbiologically, chemically, nutritionally, and toxicologically safe and wholesome and cosmetics are safe; and that food and cosmetic products are honestly and accurately labeled. FDA's Foods Program is unique relative to FSIS (and FDA's own drug, medical device, and biologics centers) because the predominant focus for ensuring food safety relies mostly on post-market activities which require the documentation of risk. To fully appreciate the significance of this food protection mission, however, it must be understood that the underlying assumption of the laws FDA enforces is that foods are safe. Thus, with the exception of certain pre-market food and feed additive and labeling requirements, FDA must rely on post-market surveillance and scientific evidence to prove that a product is a threat to public health to take action against it.

In contrast to FSIS and FDA, the CDC is non-regulatory. In collaboration with local and state public health departments, CDC conducts surveillance for human illness, investigates disease outbreaks, estimates the burden of illness caused by specific agents, and monitors longer term trends as prevention efforts are implemented. Public health surveillance depends on reports from clinical laboratories of the isolation of clinically meaningful microbes from sick persons. Active sentinel site surveillance through the FoodNet platform provides reliable information on the incidence of diagnosed foodborne infections and the trends over time, that are integral to setting and tracking progress towards national disease reduction goals (MMWR FoodNet 2008). For some microorganisms such as *Salmonella*, this surveillance is strengthened by sending the strains isolated from patients to public health laboratories for further testing to characterize and subtype them. This subtyping enhances the capacity of the public health system to detect and investigate outbreaks. Traditional subtyping has depended on tests for microbe characteristics such as serotype and toxin production. In recent years, the public health laboratories have used molecular subtyping methods (or "fingerprinting") for the same purposes. The national network for molecular subtyping of foodborne bacteria, PulseNet, connects all 50 states with the database and methods development hub at CDC, as well as the laboratories of FSIS and FDA. PulseNet makes it possible to detect widespread and dispersed outbreaks that would likely have been missed in the past, and improves the precision of epidemiological investigations (Tauxe 2006). Most outbreaks are investigated by local and state public health authorities. CDC scientists are consulted on many of these, and coordinate or lead investigations of outbreaks that are particularly severe, unusual or widespread. In outbreak investigations, diagnostic and subtyping tests have been critical to define which illnesses are likely to be part of an outbreak, and which are not, and to link isolates from suspected or implicated foods to the clinical cases, as well as to potential upstream or environmental sources of contamination.

Interagency coordination occurs through numerous formal and informal collaborations. The CDC, FSIS and FDA are all connected to PulseNet and participate in FoodNet, as well as other surveillance networks. Interagency liaisons foster communication and coordination. If methods are standardized across the agencies, then sharing microbiological data across the agencies can answer additional questions. This is important to monitoring antimicrobial resistance in foodborne pathogens in people, animals and foods through the National Antimicrobial Resistance Monitoring System (NARMS), (www.fda.gov/cvm/narms_pg.html). Comparing the organisms identified by regulatory product testing with those coming from clinical, environmental and animal sources can help to allocate the disease burden of a pathogen across a variety of potential food sources.

The National Oceanic and Atmospheric Administration (NOAA), through its Seafood Safety Research and Monitoring Program (SSRMP) and the Seafood Inspection Program (SIP), plays an important role in food safety. The SSRMP represents NOAA Fisheries' foundation to proactively and rapidly respond to seafood safety and aquatic animal health issues and episodic events. This program has provided NOAA the capability to respond quickly to environmental disasters and episodic seafood processing malpractices. As part of the SSRMP, the SIP is a voluntary, fee-for-service program for inspection and certification of fishery products for quality and safety. The mission of the SIP is to assist industry and consumers in improving the overall quality and marketability of seafood and ensuring that all processing firms are compliant with FDA and Department of Commerce (DOC) regulations. The SIP supports FDA's mission by enforcing

regulatory requirements and referring non-compliant seafood and processing firms to FDA. A variety of services, including in-plant inspections, product evaluation and grading, HACCP services and consultation for regulatory compliance are offered to the industry.

2.2. Current Microbiological Testing Programs

USDA's regulatory (FSIS and Animal and Plant Health Inspection Service, APHIS) and research (Agricultural Research Service, ARS) agencies are empowered with diverse missions. As a result, these sister agencies differ in resources and capacity to develop and validate detection and subtyping methods. In addition to USDA, other government entities with microbiological testing programs are also discussed below.

2.2.1. FSIS Microbiological Testing Programs and Objectives

FSIS currently has two microbiological testing programs: the Baseline Microbiological Surveys and the Verification Testing Programs. Data from these microbiological testing programs are used to 1) establish microbiological performance standards and testing objectives for specific meat and poultry products, 2) verify process control, 3) improve risk assessments, 4) provide epidemiological information, 5) assess the effectiveness of FSIS inspection programs, and 6) measure the Agency's progress toward meeting its public health goals.

The Microbiological Baseline Surveys were started in the 1990's to provide data as a prelude to the promulgation of the HACCP Final Rule and serve as the basis for the microbial performance standards used in the HACCP Verification Testing Program. These baseline studies sample FSIS-regulated products from federally inspected establishments to determine the presence and levels of specific pathogens and indicator organisms. The intent was to estimate 1) the prevalence of selected foodborne pathogens in select meat and poultry products and 2) the likelihood of exposure of the public to foodborne pathogens of public health concern in meat and poultry products. The number and frequency of samples are driven by statistical considerations as well as the establishment's production volume and within the constraints of existing agency inspection, laboratory and financial resources. Recently, the National Academies of Science (NAS) (Scientific Criteria to Ensure Safe Food, National Academies Press, 2003) reported that the original baseline studies were flawed by significant sampling deficiencies and recommended that FSIS conduct new baseline studies on a periodic basis that are representative and statistically valid. Furthermore, NAS stressed the need for increased transparency in the development of food safety criteria, noting difficulties in reviewing and assessing the validity of the data and assumptions used to create the microbial performance standards. Since then, a number of new baseline studies have been conducted which attempt to address the deficiencies in the original studies. However, only one of the original baseline studies (broilers) has been repeated, and baselines for turkey and hog carcasses have been initiated. Since 2001, NACMCF has provided guidance to FSIS on the design of five baseline studies as they relate to establishing performance standards (www.fsis.usda.gov/OPHS/NACMCF/2004/NACMCF_broiler_4_13_04.pdf).

The HACCP Verification Testing Program is a regulatory program that was designed to verify process control (i.e. effectiveness of in-plant HACCP programs) in federally regulated establishments over a specific interval of time. This includes sample sets of meat and poultry tested for Salmonella, and sampling of selected meat and poultry products for *E. coli* O157:H7 and

L. monocytogenes (see Tables B-1, B-2, and B-3). To verify process control and prioritize future inspection activities, FSIS collects verification samples of products during production and, depending on the purpose of the testing program, conducts microbial testing to detect the presence of *Salmonella*, *E. coli* O157:H7 or *L. monocytogenes*. The number of verification samples collected by FSIS is pre-determined each year for each pathogen/product pair based on the constraints of existing agency inspection, laboratory and financial resources. Different establishments may be tested from year to year and the frequency of sampling is dependent upon a number of factors (e.g., the establishment's production volume, degree of process control and prior FSIS testing history). As pointed out by the Office of the Inspector General and FSIS itself (FSIS, 2003; FSIS, 2004), the HACCP Verification Testing Program was not designed to provide estimates of nationwide prevalence of foodborne pathogens and should not be used to measure the overall effectiveness of HACCP in an establishment or nationally, or to make year to year comparisons. Even so, FSIS tracks the percent positive rate in verification samples quarterly and regularly reports these results to the public as a measurement of its progress toward meeting public health goals. In an attempt to improve its ability to estimate population exposure to pathogens, FSIS calculates the volume-adjusted percent positive rate and has established a new data integration and food protection program (www.fsis.usda.gov/PDF/Strategic_Plan_2008-2013.pdf). FSIS should ensure that the Agency analyses and reports data in a coordinated, efficient and statistically valid manner.

2.2.2. Overview of FSIS Testing Methods

For regulatory food safety testing, the consuming public and the regulated industry expect FSIS test results to be above reproach. Therefore, FSIS uses well known biochemical, serological and genetic criteria for pathogen identification methods that have been historically accepted by the public health and microbiological scientific communities.

For every microbiological testing method, there is a functional limit to the amount of product (sample) that can be accommodated by an analysis. This may be called the "test portion" or "analytical portion." Standard protocols specify the portions of submitted samples that are tested for each type of product and each type of agent. The test portion provides a theoretical limit for detecting a pathogen. The typical test portion specified by most pathogen testing protocols is 25 g but larger test portions are sometimes used, as these can enhance the detection of low levels of the contaminant or facilitate detection when the contaminant is distributed unevenly throughout the food product.

Pathogen testing methods currently in use by FSIS typically employ a one- or two-stage broth enrichment step followed by a rapid screening test, typically based on detection of an antigen (i.e., immunoassay) or genetic determinants (i.e., polymerase chain reaction, PCR). The use of screening tests expedites identification of samples that are negative and enables FSIS to determine potentially contaminated product more quickly. This allows FSIS laboratories to utilize their limited testing resources more efficiently, and industry to expedite disposition of held product.

2.2.3. Methods Development and Validation Capabilities of USDA

USDA's non-fee for service regulatory (FSIS and APHIS) and research (ARS) agencies are empowered with diverse missions. As a result, these sister agencies differ in resources and capacity to develop, optimize, and validate detection and subtyping protocols.

2.2.3.1. FSIS

According to information provided to this Committee, FSIS has no in-house laboratory capabilities at any of their locations to specifically address microbiological methods development. Thus, this regulatory arm of the USDA is reliant upon other sectors (USDA ARS, academia, and industry) to develop candidate microbiological methods.

2.2.3.2. ARS

The USDA-ARS National Program 108 (NP-108), Food Safety, (animal and plant products) (available at: http://www.ars.usda.gov/research/programs/programs.htm?NP_CODE=108) conducts both pre- and post-harvest food safety research, including methods development. ARS provides scientific information and technology to producers, manufacturers, regulatory agencies (APHIS, FDA, FSIS), and consumers to support their efforts to provide a secure, affordable, and safe supply of food, fiber, and industrial products. Included in this mission is the development and validation of methodologies that have regulatory, industry and research use.

To foster interagency collaboration, a formal FSIS/ARS liaison, similar to the FSIS/CDC liaison, and the APHIS/CDC liaison, is in place. The FSIS/ARS liaison meets with the National Program Leader (NPL) for food safety quarterly and annually for the planning of joint FSIS/ARS research projects. Currently, at the national level, FSIS priorities are shared with the ARS NPLs who may assign specific methods development/validation projects to suitable ARS research scientist(s) as the need arises. The ideal time for this to occur is during the drafting of the five-year research project plan. Ideally, the FSIS counterpart should participate as a stakeholder in the planning of such projects.

In general, since ARS research is outlined in the five-year project plan, short-term needs tend to fall by the wayside unless they are addressed within the scope of the broadly-written project plans. Less formal collaborations are realized when ARS and FSIS personnel interact with one another at various venues. Again, these collaborations usually fall within the purview of the ARS project plans. Nonetheless, successful projects resulting from ARS/FSIS collaboration have been showcased at annual FSIS/ARS Research Planning Workshops, in the ARS NP-108 annual report, in peer-reviewed journal articles, and by awards to ARS and FSIS staff.

2.2.3.3. APHIS

Although not a food safety agency per se, APHIS, which is the animal health regulatory arm of USDA, has agency-sponsored facilities to support in-house methods development and to evaluate published methods or commercially available systems. In general, APHIS performs its own validation before adopting a method/protocol. In-house developmental projects conducted by APHIS personnel address the Agency's immediate diagnostic needs and yield publishable data. APHIS proactively seeks technical support from ARS investigators, as evidenced by publications resulting from these collaborations. In addition, APHIS enlists the cooperation of government and university partners, updates stakeholders at national meetings, and solicits extramural support. APHIS conducts microbiological testing in response to either disease outbreaks or to producers' needs. For example, the National Animal Health Monitoring System (NAHMS) (USDA-APHIS-Animal Health Monitoring and Surveillance. Available at: http://nahms.aphis.usda.gov/), an APHIS-based initiative, enlists state and Federal veterinarians to distribute questionnaires and

collect field samples (livestock feces), which are then distributed to collaborating laboratories for analysis. Originally, NAHMS samples were processed for *Salmonella* and *E. coli* isolation at the National Veterinary Services Laboratories, Ames, Iowa; recently, testing has been expanded to include other pathogens (*Campylobacter*, *Yersinia*, protozoa, helminths and viruses) with isolations performed in collaborating ARS and academic laboratories, funded in part by extramural initiatives. The US Animal Health Association (USAHA) is a major forum to address the needs of stakeholders and to garner their support and to facilitate ARS-APHIS collaboration (USDA-ARS, US Animal Health Association, available at: http://www.usaha.org/). APHIS is a major contributor to USAHA working committees as evidenced by the annual update summarizing *Salmonella* serotyping and phagetyping results. APHIS also provides support to FSIS in the form of the serotyping of *Salmonella* field isolates and is a participant in studies to evaluate CDC's molecular-based alternatives to traditional serotyping. Finally, APHIS is an active participant in extramurally funded research projects with academic and ARS partners.

2.2.4. FDA

FDA conducts inspections of production, processing, and storage facilities for the food products it regulates. Sampling and testing will occur when violations in good manufacturing practices (GMPs), sanitation, and, where applicable, deviations from HACCP programs are cited. Additionally, CFSAN issues targeted surveillance assignments to ORA for high risk foods, high risk situations (e.g., food service for high profile national events such as political conventions), and certain emergency response situations (e.g., outbreaks) to obtain a short term assessment of pathogen prevalence.

Two relevant research programmatic thrusts for the CFSAN include:

- Development of methods for sampling, detecting and confirming the identity of pathogens in a variety of food types so that FDA can unequivocally establish evidentiary support to its regulatory actions.
- Identification of virulence factors, epidemiological markers, and other determinants that influence the ability of pathogens to use foods as a vehicle for disease transmission, thereby providing enhanced epidemiological investigation, earlier interventions, and more accurate product trace-back.

In addressing each of these needs, FDA has also relied heavily on the basic work of the DHHS' National Institutes of Health (NIH), USDA's ARS and Cooperative State Research Education, and Extension Service (CSREES), commercial entities (e.g. platform technologies), and academia. However, without the ability to augment those studies with the unique capabilities, expertise, and focus of FDA researchers, this scientific knowledge could not have been translated into FDA relevant programs. Although the charge to this NACMCF subcommittee is focused on the FSIS regulatory model and mission, FDA can clearly benefit from the analysis and recommendations cited here and the NACMCF membership considered this in their deliberations.

2.2.5. DOC-NOAA Fisheries

Within NOAA Fisheries, the seafood safety activities are primarily carried out by the Seafood Safety Research and Monitoring Program. Activities include working with CDC, NIH and others to advance the understanding of mercury issues in fish, providing scientific oversight to the SIP,

identifying and characterizing marine pathogens, and improving detection and forecasting of harmful algal blooms.

The SIP conducts inspections of seafood establishments including vessels, processing plants and retail facilities. Validation and audit inspection are conducted in order to assure adherence to all sanitation, HACCP and other regulatory requirements. Inspections often include surveillance and compliance sampling of high risk products which are sent to the National Seafood Inspection Laboratory (NSIL) for microbiological and chemical analysis. The NSIL conducts laboratory analyses using screening methods as well as methods approved by other Federal and international bodies. Samples analyzed at the NSIL include surveillance and compliance samples in support of the SIP, compliance samples from industry and other Federal agencies, and research samples. The laboratory has methods development and validation capabilities.

In addition to NOAA Fisheries activities, NOAA's National Ocean Service conducts seafood safety related activities at its Center for Coastal Environmental Health and Biomolecular Research (CCEHBR). The CCEHBR conducts interdisciplinary research to resolve issues related to coastal ecosystem health, environmental quality, and related public health impacts. Chemical, biomolecular, microbiological, and histological research is done to describe, evaluate, and predict the significant factors and outcomes of natural and human influences on marine and estuarine habitats. Chemical, biomolecular, microbiological, ecological, toxicological and histological methods are developed and used in both laboratory and field studies.

2.2.6. CDC

CDC conducts routine testing to support the network of state and local public health laboratories. This testing function includes identifying problematic organisms, providing specialized diagnostic testing for rare infections (e.g., botulism), and testing clinical specimens (and occasionally, environmental and food samples from outbreak settings), as well as supporting specialized surveillance and research activities. The reference laboratories also develop and validate new methods for diagnosis and subtyping for use in the public health system. In general CDC develops and/or validates its own methods in-house and performs multi-laboratory comparisons with other public health laboratories before adopting a method for surveillance purposes. Specialized protocols for biothreat agents are developed and distributed through the Laboratory Response Network (LRN).

2.2.7. Food Emergency Response Network (FERN)

FERN is a USDA/FDA led activity that comprises over 150 collaborating laboratories. Its mission is to integrate the nation's food testing laboratories for the detection of pathogens and select agents in foods at the local, state and national level. FERN labs use validated methods that have been developed by the FDA, USDA, CDC, or by the military. Laboratories in the FERN also develop and validate methods for targeted analytes that are priority for the network.

2.3. Current Methodological Approaches

There are a number of methodological approaches applied to the detection and further characterization of microorganisms in foods (CCFRA 2006; Emanuel and Fruchey, 2007). Some of the most commonly used technologies are summarized in Table 2 (Feng et al., 2007).

Table 2. Existing technologies for the detection and identification of bacterial pathogens, toxins, and indicator organisms in foods*

Technology	**Format**	**Selected Targets**	**Limitations**
Bioluminescence	ATP	viable bacteria	cannot determine species, total count only
Chromogenic and fluorogenic dyes	Media	*Campylobacter*, coliforms, *Cronobacter sakazakii*, *E. coli* O157:H7; *Listeria; Salmonella*, *S. aureus*, *Vibrio*	selective plating media; need incubation; presumptive data; need confirmation
	Assay	*E. coli*, coliforms	automated enumeration; instrument cost
Manual Identification	Biochemical	most bacteria	pure culture required
Auto Identification	Biochemical	most bacteria	pure cultures required
	Fatty acid C oxidation	bacterial communities	limited database
Nucleic acids	DNA Probe	*Campylobacter*, *E. coli* O157:H7, *Salmonella, Listeria*,	need culture enrichment; detects nucleic acid sequences, but not gene expression; cannot

		Yersinia	determine cell viability; confirmation required
	PCR	*Campylobacter, Clostridium, C.*	need some enrichment; detects gene
		Sakazakii, E. coli O157:H7,	sequences, but not gene expression; can't
		Listeria, Salmonella, S. aureus,	determine cell viability; many inhibitors in
		Shigella, , Yersinia,	foods; need confirmation
Antibodies	Latex	many pathogens and serotypes;	pure culture required; good for serotyping
	agglutination	some toxins	not sensitive for detection
	Lateral flow	most pathogens, some toxins	culture enrichment and confirmation required
	Magnetic bead	most pathogens	may not yield pure culture; matrix-dependant
			efficiency; not stand alone
	ELISA	most pathogens & toxins	culture enrichment and confirmation required

[*] Most assays provide presumptive data and will need confirmation for definitive results (Adapted from Feng, 2007)

The following sections describe the three basic categories of microbiological methods as applied to food microbiology, i.e., enumeration of microbiological indicators, detection of foodborne pathogens, and methods for further strain characterization.

2.3.1. Detection of Foodborne Pathogens

There are many widely used culture-based methods for the detection of common enteric pathogens in clinical specimens. Clinical specimens usually have large numbers of the target organism and the sample matrices (urine, blood, feces, etc.) are relatively consistent from sample to sample. Adapting such methods to the detection of the same pathogens in foods can be tricky, but over the last 50 years, food microbiologists have developed well validated and robust culture-based methods. These methods are designed to address several issues unique to the detection of pathogens in foods:

- The ability to detect as little as 1 target cell per sample, with sample sizes ranging from 25-325 g;
- The recovery of pathogens sublethally injured as a consequence of previous treatments applied for food processing and/or preservation;
- A high degree of assay specificity to reduce the likelihood of a false negative result.

Standard cultural procedures for foodborne pathogen isolation and detection encompass the sequential steps of (i) cultural enrichment, (ii) selective and differential plating, (iii) confirmation, and (iv) subtyping. Each individual step in this process takes a minimum of 18-48 h. Sometimes this first phase of testing is referred to as "screening." Based on standard cultural procedures, the screening process is completed after incubation of selective and differential plating media. In this case, two different outcomes are possible. If a characteristic colony is not present after the selective and differential plating steps, there is no need to continue the test and the result is reported as confirmed negative. It typically takes 3-4 days to obtain a confirmed negative test result. On the other hand, if suspicious colonies are identified on selective/differential agar, confirmatory testing is necessary and the sample is designated as a presumptive positive. These samples typically require further testing to characterize the phenotypic properties of the organism that may include: the ability to metabolize specific compounds, antigenic properties associated with the organism which distinguish it by serotype, and biochemical characteristics such as the presence of specific proteins or fatty acids. Confirmatory testing assures that the isolate(s) is indeed the target pathogen; not all presumptively positive isolates are actually confirmed as the pathogen. Depending on additional subtyping needs, (described below), complete characterization of a confirmed positive isolate may require anywhere from a few days to a few weeks.

Over the last two decades, the time to result in screening foods for pathogens has improved with the introduction of detection platforms such as enzyme-linked immunosorbant assay (ELISA) and PCR. These are the approaches most commonly used by FSIS for initial pathogen screening. These methods allow the analyst to bypass selective plating by replacing it with a step that takes only a few minutes to hours to complete. Cultural enrichment is still necessary to bring the target organism up to detectable limits (usually $>10^3$ CFU/ml of enrichment broth). With this approach, a confirmed negative test result can be obtained in 1-2 days. However, a presumptively positive sample must be further processed by selective plating, isolation of suspect colonies, and

subsequent confirmation steps. The process is therefore faster for a negative test result but does not result in more rapid results for those samples screened as presumptively positive.

Cultural enrichment techniques typically provide qualitative presence-absence data but no quantitative estimates of the number of target pathogens that are present in the sample. In recent years, enumerative methods have emerged for some pathogens and indicator organisms. For example, for detecting *Campylobacter* in baseline studies, FSIS uses an enumerative selective plating method which does not require prior cultural enrichment (NACMCF, 2007). The *FDA Bacteriological Analytical Manual* (BAM) (FDA, 2003) describes a colony lift hybridization method for the enumeration of *Vibrio parahaemolyticus* and *V. vulnificus* in molluscan shellfish. Theoretically, any enrichment-based pathogen detection approach can be adapted for quantitative analysis by converting it to the most probable number (MPN) format; however, MPN enrichment is cumbersome and resource intensive. There may be opportunities in the future to combine MPN enrichment with PCR, thereby streamlining the process (Nordstrom et al., 2007). For indicator organisms, a commercial system for automated MPN determinations for coliforms, *E. coli* and *Enterobacteriaceae* claims to provide quantitative results (including confirmation) in 22 hrs vs. the 3 - 4 days needed for traditional MPN (Paulsen et al., 2006; Paulsen et al., 2008).

2.3.2. Non-culture-based Approaches

For detection of organisms of public health importance that are not easily cultured or are difficult to detect, diagnostic methods are almost always based on the detection of nucleic acids specific to the target pathogen. For example, the NACMCF report entitled: Assessment of Food as a Source of Exposure to *Mycobacterium avium* subspecies *paratuberculosis* (MAP) details several PCR-based methods to detect *Mycobacterium avium* subsp. *paratuberculosis*, a fastidious pathogen which is often recalcitrant to growth in culture.

Oligonucleotide fingerprinting of rRNA genes (OFRG) has identified microbial communities in soil by employing DNA probes in a microarray (Valinsky et al., 2002). OFRG, which does not rely on isolation of fastidious microbes, correlated shifts in the microbiota of the turkey intestine with *Campylobacte*r colonization (Scupham, 2007). Using a similar approach, *Salmonella* colonization status of cattle has been correlated with the fecal microbiota (Patton et al., 2008). Sequence-based approaches to characterize entire microbial communities are supplementing culture-based methods and in some instances replacing them. For example, the power of rapid pyrosquencing technology can be applied to entire microbial communities, many of which cannot be cultured (Margulies et al., 2005). Pyrosequencing of entire microbial communities may have applications for detection of population shifts in abused, low quality, or pathogen-contaminated products and ultimately may be more sensitive than screening for indicator organisms or specific pathogens. Theoretically, approaches such as these could be used in detection but as is described in Section 4.1, there are a number of hurdles that must be overcome before their routine use in pathogen screening and confirmation become a reality.

2.3.3. Methods for strain subtyping (Question 4)

The process of strain typing at the subspecies level is often referred to as subtyping. There are four major applications of subtyping: taxonomy, phylogeny, outbreak detection/investigation and risk assessment including attribution. Once definitively isolated and identified, bacterial isolates can be

further subtyped based on phenotypic and/or genotypic characteristics of the organism. The automated Phenotype Microarray Technology™ offers the potential to characterize an isolate by measuring the expression of thousands of genes during growth *in vitro* and *in vivo* (www.biolog.com). Traditional phenotypic subtyping (including serotyping and antibiotic resistance profiling, among others) is still performed for many organisms, but recent improvements in molecular subtyping have replaced some of these methods (Barrett and Gerner-Smidt 2007; Hyttia-Trees et al 2007; Chen and Knabel, 2008b). Of particular interest is PFGE, which is the current "gold standard" method used in CDC's PulseNet Program. Much of the developmental work and subsequent implementation of these sorts of molecular methods has been done by the CDC. For example, CDC developed a molecular equivalent for *Salmonella* serotyping which is based on detection of the genes that encode serotype-specific antigens. This assay is now in final evaluation at state public health laboratories and appears to be faster, easier and more reliable than traditional serotyping (Fitzgerald 2007). Other promising technologies include multiple-locus variable number tandem repeat analysis (MLVA), multilocus sequence typing (MLST), amplified fragment length polymorphism (AFLP), SNPs, microarrays and mass spectrometry, which are described later in this document. MLST, a method based on sequence comparison of the sequences of 5-10 genes, is particularly useful for subtyping of many foodborne pathogens (Hyttia-Trees et al 2007; Chen et al. 2007).

Development and future prospects of subtyping foodborne bacterial pathogens are beyond the scope of this document and have been reviewed elsewhere (Hyttia-Trees et al., 2007). Of particular relevance is the recent FSIS document entitled: *Analysis of Molecular Subtyping Methods for FSIS Regulatory Testing: The Present and Future of FSIS Regulatory Subtyping.* This internal "white paper" offers recommendations for future molecular subtyping to be undertaken by FSIS. Consult this document for further details about potential molecular typing methods which could be applied by the Agency but are currently beyond the scope of this document.

3. Purposes of Microbiological Testing (Questions 1, 3 & 5)

Multiple factors must be taken into account when considering new testing methodologies for regulatory laboratories. Not only should the method's appropriateness for meeting a particular public health goal be a factor, but method reliability and validation are critical issues for results that may become legal evidence in court. This section reviews a few of the criteria that must be taken into consideration. The limitation of using pathogen indicator organisms is mentioned in the Philadelphia report (Expert Panel's Summary Report and Recommendations, 1995).

3.1. Microbiological Testing for Public Health

Microbiological testing is an essential tool for protecting consumers from contaminated food. The various roles for microbiological testing are described below.

3.1.1. Surveillance and Investigation

Public health surveillance is the routine reporting of health events in a defined population. Surveillance data are used to estimate the burden of a disease (Mead et al., 1999) to set public health objectives (e.g. Healthy People 2010 goals), to detect and investigate outbreaks, and to track trends over time.

Public health surveillance depends on standard diagnostic testing in clinical laboratories to identify cases of reportable infectious diseases. This is supplemented by routine characterization and subtyping of those organisms for public health purposes, which is conducted largely by the local and state public health laboratories. The use of standardized subtyping methods for foodborne pathogens, and the linking of the results through the PulseNet database has enhanced the ability of the public health network to detect, investigate and control outbreaks. For example, outbreaks can be identified sooner when there is a cluster or an increase in the number of cases caused by one particular subtype. Epidemiological investigation can be targeted to those clusters, and to those instances in which matching clinical and environmental/food isolates have been obtained, improving the ability to identify vehicles of transmission and ultimately, the source(s) of contamination.

When a foodborne illness outbreak is identified, it is likely that more than one Federal agency, as well as state and/or local authorities, will be involved in the investigation. Analytical methods applied by multiple agencies tend to reflect the perspective and mission of each of the agencies. While this can be very useful, it is important that there be coordination and communication between agencies with respect to methodological issues.

3.1.2. Estimating Prevalence

Estimating the prevalence of pathogens in the food supply is critical to understanding and addressing the public health risk of foodborne disease in the United States. Prevalence estimates can provide 1) a mechanism for measuring performance against public health goals, 2) data for risk assessment and 3) the basis for regulatory performance standards. Currently, the Baseline Microbiological Surveys are used to estimate the prevalence of pathogens in specific meat and poultry products and serve as the basis for FSIS microbiological performance standards. However, a number of parameters including method sensitivity and limit of detection significantly affect the reliability of the results and must be taken into consideration when evaluating analytical data.

3.1.3. In-Plant Process Control

Microbiological testing can be used to assess in-plant process control. The key to success in process control is implementing a cost-effective, real-time, on-site testing platform that can be used to rapidly identify a process deviation trend relative to the established acceptable limits. Rapid testing allows for swift correction of process deviations, reducing the likelihood of contaminated finished product. For ease and reduced expense, testing for microbial indicators is often chosen as an alternative to pathogen testing. It is likely that a number of emerging technologies might be applicable to monitoring and verifying process control.

3.1.4. Providing Data for Risk Assessment and Attribution.

Quantitative risk assessment is a prelude to the promulgation of food safety regulations and relies on valid microbiological data to support risk estimates. One component of risk assessment, i.e., exposure assessment, requires data on the prevalence and levels (numbers) of a select pathogen in the food in question. Quantitative data obtained from microbiological testing provide this type of information which can then be used to populate risk models, increasing their scientific rigor and relevance (Malorny et al., 2008). The introduction of enumerative methods for pathogen detection, which might be associated with some of the emerging technologies, would provide much needed quantitative data for risk assessment.

Food attribution, or the ability to attribute the proportion of specific foodborne diseases associated with specific food commodities, is of great interest as food safety agencies move toward risk-based management approaches. Current epidemiological and microbiological data that are used to inform attribution estimates are limited and subject to uncertainty; microbial data are currently limited to *Salmonella* strains from meat, poultry and some egg products. Application of standardized subtyping methods to build libraries of isolate data from diverse sources including foods, environments, animals and humans, would improve attribution estimates.

3.2. Indicators vs. Pathogens

Direct testing for specific pathogens is not always practical when considering technical requirements, cost and the low prevalence of pathogen contamination in many food products. While indicator methods are rapid, inexpensive, and often enumerative, the most important question is whether the chosen indicator is a valid representative of the conditions conducive to the presence of the pathogen of concern (2004 NACMCF report- Performance Standard Reports). Detection of one or more microbiological indicators may be applied in place of specific pathogen detection. Although not a direct measure of pathogen contamination, indicators have historically been used as part of process control systems, to assess the hygienic status of processing operations, and to monitor the efficacy of anti-microbial interventions at critical control points in production and processing of foods. Indicators have also been used to evaluate the overall microbiological quality of finished products and to estimate product shelf life. Typical indicator systems include total aerobic plate count (APC), total coliform counts (TCC) and *E. coli* Biotype I count (ECC). Total *E. coli*, fecal coliforms, and *Enterococcus* spp. of fecal origin have been used extensively as indicators for the potential presence of enteric pathogens (Mossel, 1991; FAO/WHO, 1979).

3.3. The Concept of "Zero Tolerance"

Some microorganisms are considered so hazardous to the public health that they are not allowed in certain foods at any detectable level. This principle has led to the concept of "zero tolerance", which can be defined as the inability to detect the target organism in a certain number of samples of a specified size. Both statistical sampling and microbiological methodology play key roles in the practical application of the concept of "zero tolerance". As only a small number of samples are likely to be contaminated, and pathogens in contaminated foods tend to be distributed in a non-homogeneous manner, the sampling design and method will influence the likelihood of collecting a pathogen in any given sample, if present. While technological advances have resulted in microbiological methods with improved (lower) limits of detection, the performance of these methods in detecting low-prevalence pathogens is inherently impacted by sampling. Therefore, "zero tolerance" provides some protection of public health but cannot guarantee that the product in question is completely free of the pathogen of concern. It is clear that microbial testing alone cannot ensure food safety. Negative and positive pathogen test results do not necessarily indicate absence or presence, respectively, of the target in the sample due to the possibility of false reactions.

3.4. Sampling/Statistical Considerations in Microbiological Testing

As stated above, the ability of microbiological methods to detect foodborne pathogens is intimately dependent upon sampling. The term "sampling" refers both to the statistical methods used to determine which and how many samples to test in order to represent a larger amount of product,

and to the technical methods used to collect, preserve and process that sample for microbiological testing. Although the Charge to the Committee explicitly focused on the non-statistical issues of sampling, the Committee nonetheless recognizes that the questions in the Charge raise statistical issues related to sampling.

According to the International Commission on Microbiological Specifications for Food (ICMSF), "the purpose of sampling a food is to collect a representative sample to obtain information on its microbiological status" (ICMSF, 2002). Sampling plans, when designed properly using sound statistical concepts, provide a systematic means for assessing the microbiological status of food with a high degree of confidence (National Academies of Science, 1985). A sound sampling plan should specify the number of samples collected; the methods use to select and collect them; the laboratory testing methods; and criteria for interpreting the results. All of these factors depend on the purpose for the microbiological testing.

1. Sampling can provide an estimate for the parameter of interest, however it does introduce uncertainty. To reduce uncertainty, the sampling must be representative of the population of interest and the sample size must be sufficient to provide a high degree of confidence that the sampling results correctly characterize the parameter of interest. For example, in routine microbiological testing, there is a risk that a lot will be misclassified: lots with acceptable levels of pathogens are rejected (producer risk) and lots with unacceptable levels of pathogens are accepted (consumer risk). While it is not possible to eliminate these risks, the probability that misclassification occurs can be minimized. The extent to which these risks can be minimized depend on a number of factors, including sample size and representativeness as well as the sensitivity, specificity, repeatability and reproducibility of the laboratory methods (Scientific Criteria to Ensure Safe Food, National Academies Press, 2003). An appropriate statistical sampling plan will address these issues and minimize producer and consumer risk. From a public health perspective, it is more important to minimize consumer risk.

2. Obtaining representative samples is crucial to the interpretability and generalizability of the sampling results. A representative sample should reflect the composition of the population of interest, which will affect the number of samples taken as well as the sampling methods. For example, a one one-pound sample from a production lot of 10,000 pounds may be representative if pathogens were distributed uniformly (i.e. homogeneous population). However, it is well established that microorganisms and pathogens are unevenly distributed in food (National Academies Press, 2003; FSIS 2003a; FSIS 2003b). With a heterogeneous population, there is increased risk that sampling results will not accurately characterize the parameter of interest. Increasing the sample size and using appropriate sampling methods, such as stratified sampling, will minimize this risk and provide a greater degree of confidence in the sampling results. There are mechanisms for determining the appropriate number of samples needed for maintaining an acceptable level of risk. An appropriate statistical sampling plan will address heterogeneity within the population to be sampled.

In short, the entire testing spectrum from sampling through laboratory analysis must be considered when determining the most appropriate technologies for performing routine and baseline microbiological analyses. Sampling plans should include an explicit description of the tradeoffs in sample size and statistical power that were considered during design and implementation.

3.5. Performance Criteria for Methods Selection and Evaluation

The basic assumption of microbiological testing is that it will result in some protection to public health. The purpose for testing will influence the criteria used in method selection. For example, for assays intended to provide presumptive identification (screening), the foremost characteristics are sensitivity, reliability in many hands, cost and speed. For confirmatory tests, sensitivity and specificity must be considered to minimize false negative and false positive results. For assays that are used to subtype isolates, it is necessary to demonstrate that, in addition to being practical and reliable assay, the method reliably separates outbreak-related strains from the background of sporadic infections, and provides data that are epidemiologically meaningful. Validity, reliability, feasibility, effectiveness, and validation, all of which are important considerations in choosing and evaluating candidate methods, are described briefly below.

3.5.1. Validity

Validity is a measure of the ability of the test to do what it is intended to do under specific conditions of use, i.e., to detect the organism(s) of interest if it is present, and to not detect it if it is absent. The components of validity are described below.

3.5.1.1 Sensitivity – Imprecise use of the term sensitivity causes confusion in the interpretation of microbiological test results. The reason for this confusion is that there are two distinct types of sensitivity, Analytical Sensitivity and Diagnostic Sensitivity (Saah et al., 1997). Analytical Sensitivity, also known as Limit of Detection (LOD), represents the smallest amount of an analyte in a sample that can be accurately measured by a platform or assay. Therefore, Analytical Sensitivity relates only to the detection platform or assay. In contrast, Diagnostic Sensitivity is the probability of detecting an analytical target (i.e., pathogen, toxin) in a sample from a population of samples (i.e., a production lot) which is contaminated. Therefore, Diagnostic Sensitivity measures the ability to detect ("diagnose") contamination in environments and foods. Significant progress has been made in enhancing the Analytical Sensitivity of various cultural and molecular detection platforms and assays. For example, some cultural methods can detect one viable cell in a 25 gram sample and PCR can theoretically detect one molecule of target DNA in a very small PCR tube. Unfortunately, little progress has been made in improving Diagnostic Sensitivity, which remains a major barrier to the detection of pathogens in foods and thus represents a major research gap (see Number 2 in Section 7).

3.5.1.2. Specificity – Specificity is a performance characteristic that judges the ability of a laboratory test method to exclude non-target analytes in chosen matrices, and it is therefore a reflection of "false positive" rate. As with sensitivity, there are also two distinct types of specificity: Analytical and Diagnostic (Saah et al., 1997). Analytical Specificity is defined as the ability of an assay to exclusively identify a target rather than other similar analytes in a sample. Diagnostic Specificity is defined as the probability that the sample tests negative when the pathogen is absent from the sampled population. Therefore, a highly specific test will rarely be positive in the absence of the contaminant. Specificity is highly influenced by test method and sample matrix, as well as by the presence of closely related species. Like sensitivity, specificity is often established under controlled laboratory conditions and this may not adequately represent the real analytical challenges to the method. For efficient sample processing and use of laboratory resources,

methods should minimize the generation of false positive results that require additional laboratory work.

3.5.1.3. Predictive Value- Sensitivity and specificity define the operating characteristics of an assay, but it is the predictive value (positive or negative) of the assay that is of most importance to FSIS and public health. Positive predictive value is the probability that a sample whose test result for a specific pathogen is positive truly contains that viable pathogen, which can be calculated as one minus the false-negative rate. Negative predictive value is the probability that a sample whose test result is negative does not have the viable pathogen, which can be calculated as one minus the false-positive rate. It is important to apply the concepts of positive and negative predictive value to the entire lot of food being produced, not just to the sample being tested (see Section 7, especially Number 2). High Diagnostic Sensitivity improves negative predictive values and high Diagnostic Specificity improves positive predictive values, regardless of Analytical Sensitivity or Analytical Specificity, and vice versa. Therefore, it is important to realize that assays having very high Analytical Sensitivity and Specificity, but low Diagnostic Sensitivity and Specificity have poor predictive value. This is currently often the case in the field of pathogen detection, and thus represents a major barrier and research gap (see Number 2 in Section 7).

While the ideal test method will be both highly sensitive and highly specific, there is an inherent tradeoff between these two. In order to protect public health, the false negative rate should be minimized. However, a low false negative rate results in a corresponding higher false positive rate which can create unnecessary follow-up testing and consume laboratory resources. Clearly, altering the criteria for positivity will influence both the sensitivity and specificity of the test. Therefore, any decision regarding specific criteria for acceptable levels of sensitivity and specificity must be made by weighing the consequences of both false negative and false positive results. This also needs to be considered when trying to achieve very low (1 CFU/sample) limits of detection.

3.5.1.4. Gold standard: When evaluating the validity of a new assay, it is necessary to compare it to a reference method, which is often referred to as "gold standard." For foodborne pathogen detection assays, the reference method is almost always the culture-based method, i.e., cultural enrichment followed by selective-differential plating and confirmation. Complications can arise when the new assay outperforms the reference method. In this case, the new assay might classify a higher proportion of the samples as positive, but the reference method will identify these as false positives because of its poorer sensitivity. This presents a difficult situation for validation because samples containing low numbers of pathogens cannot necessarily be "confirmed" as positive. In addition, because of the possibility of greater sensitivity and specificity of non-cultured based molecular assays, a more ideal method ("a platinum standard") might be considered in the future.

3.5.2. Ruggedness and Credibility

Method durability (ruggedness) is required for reliability in a high throughput testing program. As most tests are performed in several laboratories which are using different personnel and different equipment, it is critical that results obtained under varied environments be comparable. Although laboratory conditions should be consistent, they are rarely identical. Methods should be tolerant of minor variations and must be validated by varying critical test parameters. Methods used by FSIS should have the highest levels

of credibility since the results of laboratory tests can have considerable regulatory (and economic) implications. It is critical that official laboratory test methods have extensive, well-designed validation to achieve defensibility in scientific and legal proceedings.

3.5.3. Workflow: Throughput, Speed, Turnaround

Methods used in a national testing program have specific requirements in terms of the number of sample analyses that need to be performed simultaneously and within a defined timeframe. While related to throughput, the timing of sample processing has important logistical considerations. As many samples are shipped by overnight carrier, assay start times are dictated by the time of sample arrival. To efficiently schedule personnel, the various steps undertaken to complete an assay should fit within reasonable time parameters while also providing results in a timely fashion. As many of the producers operate on a hold-and-test basis, laboratory test turnaround times can have important economic consequences. Perhaps most critical is the time required to obtain negative test result so the particular lots of product can be released into commerce in a timely manner. In this case, improving the speed of screening methods may have substantial positive impact.

3.5.4. Validation

Validation encompasses the entire process by which it is demonstrated that a method meets claimed performance characteristics. Methods that are selected by FSIS for validation must have significant potential to meet the Agency's regulatory need for analytical capacity and should be compatible with Agency laboratory resource demands. Because FSIS laboratories analyze a variety of diverse products types with different microbial loads and compositions, the Agency conducts extensive validations prior to implementing new methods. FSIS laboratories are also accredited to perform within the ISO17025 standard and therefore are required to use validated methods that are fit for purpose.

4. Emerging Microbiological Technologies (Questions 1, 2, 3 and 4)

A variety of technologies are available for incorporation into microbiological testing of foods. Some of these technologies could be used to supplement current FSIS methods with only minor modifications; others would require a completely new way of interpreting positive test results. The Committee reviewed several technologies for potential consideration by FSIS in sampling (pre-analytical sampling and sample processing), microbial detection, and identification. In so doing, the Committee developed performance criteria for evaluation of these technologies.

4.1. Overview of Emerging Technologies

Culture-based methods have been by default the gold-standard given their ease of use, low-cost, established sensitivity, and ability to be widely standardized. In addition, a tremendous amount of historical data exists from the use of culture methods. A major drawback is the time it takes to enrich, screen for, and confirm the presence of pathogens of interest (e.g. 24 - 48 h of cultural enrichment followed by rapid detection using ELISA or PCR, with the potential of another 5 - 8 days for confirmation using conventional biochemical and serological assays).

The most appealing promise of emerging technologies is reducing time to detection without compromising assay validity. In fact, in the early days of PCR, food microbiologists recognized the theoretical potential to replace cultural enrichment with specific nucleic acid enrichment, which could reduce detection time to a matter of hours rather than days. In more recent years, interest has focused on nucleic acid-based assays that can provide rapid detection of DNA sequences (including antibiotic resistance, virulence factors, etc.). Nanotechnology-based methods have the potential for real-time microbiological detection for process control and could be used to detect pathogen harborage in relatively inaccessible sites in processing environments. Portable technologies are particularly appealing because of their potential application to on-site testing.

A comprehensive review of emerging technologies is available and briefly summarized below with representative applications given in Table 3 (Feng, 2007; Emanuel and Fruchey, 2007). A caveat for these methods is that the analytical sensitivity and specificity realized for pure cultures will likely be better than those observed when applied to the detection of the target analyte in a food matrix. Because many of these technologies and methods are still in development and few have been applied to detection of pathogens or indicator organisms in foods, it is premature to assess all their "advantages" and "disadvantages", as requested in the original charge.

TABLE 3. Representative applications of emerging technologies in food pathogen detection

Assay name	Target pathogen (matrix, detection levels reported)	Selected references
real-time PCR (RT-PCR) Format		
SYBR Green I	*E. coli* O157:H7, *L. monocytogenes*, *Salmonella* spp. (fresh produce, 1-10 cells/mL, 1000 cells/mL, 1-10 cells/mL, respectively); *Salmonella* spp. (fresh vegetable rinse water, 1-10 cells/mL); *Staphylococcus aureus* (beef samples, 10 cells)	Alarcon et al., 2006 Bhagwat 2003 Bhagwat 2004
SYBR Green I + IMS (immunomagnetic separation)	*Salmonella* spp. (milk, ground beef, alfalfa sprouts, 1CFU/mL, 25 CFU/25g, 1.5 CFU/25 g, respectively)	Mercanoglu and Griffiths, 2005
5'nuclease (Hydrolysis Probes)	*E. coli* O157:H7 (pure culture, milk, apple juice, beef, and beef enrichment; $10^3 – 10^9$ CFU/mL, $10^4 – 10^9$ CFU/mL, $10^5 – 10^9$ CFU/mL, $10^0 – 10^3$ CFU/mL, respectively); *Staphylococcus aureus* (beef samples, 100 cells)	Alarcon et al., 2006 Hsu et al., 2005

5'nuclease (Hydrolysis Probes) + IMS	*E. coli* O157:H7 (buffer solution and ground beef, $<5x10^2$ cells/mL and 1.3×10^4 cells/g, respectively); Norovirus (Strawberries, 3-7 RT PCR units)	Fu et al., 2005 Park et al., 2008
Molecular Beacon	*E. coli* O157:H7 (skim milk, $10^3 - 10^9$ CFU/mL); *Salmonella* spp. (cantaloupe, mixed-salad, cilantro, and alfalfa sprouts, as few as 4 CFU/25g with enrichment)	Liming et al., 2004 McKillip et al., 2000
Fluorescence Resonance Electron Transfer (FRET)	*L. monocytogenes* (reconstituted nonfat dry milk, $10^3 - 10^4$ CFU/25 mL); *E. coli* O157:H7 (raw ground beef (25g) and raw boneless beef (375g), 10 organisms)	Ellingson et al., 2005 Koo and Jaykus, 2003
Reverse Transcriptase PCR	Detects *Listeria* spp. in 8 h (includes 4 h enrichment). Sensitivity <10 CFU/mL for all *Listeria*, except *L. grayi* - <50 CFU/mL.	not available
Biosensor		

Comment [e1]: Julie to provide

Fiber-optic Biosensors (FOBS) (Evanescent-wave biosensors)	*E. coli* O157:H7 and Shiga-like Toxins (SLTs) (pure SLTs, ~0.5ug/mL, ground beef, 10^5 cells with SLTs); *L. monocytogenes* (Frankfurter sample, 5.4 x 10^7 CFU/mL); *E. coli* O157:H7 (pure culture, 10^3 CFU/mL, ground beef, 1 CFU/mL after 4 h of enrichment); *S. enterica* serovar Typhimurium (spent sprout irrigation water, 50 CFU/g); *L. monocytogenes* (hot dog and bologna after enrichment, 10 – 1000 CFU/g); FRET-based *S.* Typhimurium (homogenized pork, 10^5 CFU/g; *E. coli* O157:H7 (buffer solution, 6.5 x 10^5 CFU/mL); staphylococcal enterotoxin A (hot dogs, potato salad, milk and mushrooms, 10-100 ng/g)	Geng et al., 2004 Geng et al., 2006 Kim et al., 2006 Kim et al., 2007 Ko and Grant, 2006 Kramer and Lim, 2004 Rasooly and Rasooly 1999 Simpson and Lim 2005 Tu et al., 2006
Surface Plasmon Resonance (SPR)	*S.* Typhimurium (10^2 – 10^9 CFU/mL); *E. coli* O157:H7, *S.* Typhimurium, *Y. enterocolitica; L. monocytogenes* (10^5 cells/mL); *S.* Enteritidis *and E. coli* (skim milk, ~25 CFU/mL); *E. coli* O157:H7 (milk, apple juice, ground beef, 10^2 – 10^3 CFU/mL); *L. monocytogenes* (whole cell, 2×10^6 CFU/mL)	Leonard et al., 2004 Nanduri et al., 2007 Oh et al., 2004 Oh et al., 2005 Waswa, 2007

		Waswa et al., 2006
Piezoelectric-Excited Millimeter-sized Cantilever (PEMC) sensors	*E. coli* O157:H7 (buffer solution, 1 cell/mL); *E. coli* O157:H7 (broth and ground beef, 50-100 cells/mL); *E. coli* O157:H7 (10^3 – 10^8 CFU/mL); *E. coli* O157:H7 (ground beef, ~10 cells/mL)	Campbell and Mutharasan, 2007 Campbell et al., 2007 Maraldo and Mutharasan, 2007 Su and Li, 2004
Cell-based Sensors (B cell and Cytotoxicity Assays)	*Listeria* spp (pure cultures); *L. monocytogenes* and *Bacillus cereus* (bacteria culture); with immuno-separation *L. monocytogenes* (hotdogs, bologna, raw beef, chicken, and pork samples; enriched food samples)	Banerjee et al., 2007 Gray and Bhunia, 2005 Shroyer and Bhunia, 2003
Optical Scattering	*Listeria* spp. (1.2 – 1.5 mm colony size, approximately 10^{12}-10^{13} individual bacteria)	Banada et al., 2007 Bayraktar et al., 2006
Multi-analyte Array Biosensor	*S.* Typhimurium and *L. monocytogenes* (“complex samples”); *E. coli* O157:H7 (pure culture and liquid food samples 10^4–10^7CFU/mL)	Radke and Alocilji, 2005

		Taitt et al., 2004
Proteomic Biosensor (reflective interferometry)	Label-free detection of Enteropathogenic *E. coli* (EPEC) in cell cultures	Horner et al., 2006
Microarray		
Oligonucleotide/Amplicon arrays	*Salmonella* spp., *E. coli* (screened for 25 virulence and 23 antimicrobial resistance genes); with IMS *E. coli* O157:H7 (chicken rinsate w/o enrichment 55 CFU/mL); *Campylobacter* spp., *Staphylococcus aureus*, enterotoxin genes, *Listeria* spp. and *Clostridium perfringens* toxin genes	Call et al., 2001 Chen et al., 2005 Sergeev et al., 2004
Suspension Microarray (Luminex/xMAP)	*L. monocytogenes* (broth cultures)	Borucki et al., 2005
Spectroscopy		
Surface-enhanced Raman scattering	*E. coli* (aqueous suspensions, 10^3 CFU/mL); *Listeria* spp. (discrimination between six species)	Sengupta et al., 2006 Goeller et al., 2007 Green et al., 2008

Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF MS)	16S rRNA PCR amplified; various bacteria colonies; *E. coli* and *Bacillus cereus* (bacteria mixture)	Jarman et al., 2000 Madonna et al., 2001 Von Wintzingerode et al., 2002
Intact cell MALDI-TOF MS	*E. coli* (single colony); *E. coli* O157:H7 (bacterial cells)	Bright et al., 2002 Mazzeo et al., 2006
Fourier Transform Infrared (FTIR)	Various (bacterial cocktail of three different species, 10^9 CFU/mL); *E. coli* O157:H7, *Bacillus cereus, Listeria innocua* (apple juice, 10^9 CFU/mL)	Al-Holy et al., 2006 Yu and Irudayaraj, 2006
Others		
BEADS (Biodetection Enabling Analyte Delivery	With integrated IMS/multiplex conventional PCR, *E. coli* O157:H7, *Salmonella* spp., *Shigella* spp., (aqueous solution, 100 cells/organism)	Straub et al., 2005

System)		
Flow Cytometry	*E. coli* O157:H7 (with IMS + enrichment, ground beef, 4 cells/g); *E. coli* O157:H7 (milk, apple juice, ground beef, 10^3 cells/mL (milk and apple juice), 10^3 cells/g ground beef); *L. monocytogenes* (with IMS 10^2–10^8 CFU/mL)	Hibi et al., 2006 Seo et al., 1998 Yamaguchi et al., 2003
Immunomagnetic Bead-Immunoliposome (IMB-IL) fluorescence assay	*E. coli* O157:H7 (aqueous matrices: water, apple juice and cider, <1CFU/mL)	DeCory et al., 2005
Phage	*E. coli* 1 – 10^8 CFU/mL in 1.5 -10.3 hrs (pure culture). In lettuce leaf washings – 130 - 10^8 CFU/mL in 2.6-22.4 h	Ripp et al. 2006
PCR+MS	Distinguished 10 bacterial species. LOD – 0.5 genome equivalents/PCR. Human adenovirus screen – 500 samples/day at sensitivity of 100 genome per reaction. Automated system – 1500 PCR reactions/day.	Blyn et al. 2008 Mayr et al. 2005 Sampath et al. 2007
Electrochemiluminescence	ECL-IMS detection (~1 hr) of *E. coli* O157:H7 and *Salmonella* – 10^{2-3}	Yu and Bruno, 1996

(ECL)/luminometer	cells/mL in buffer; 10^3 cells/mL in foods (milk, juices, ground beef and minced chicken and fish).	
	Detection of *C. botulinum* toxin A,B,E and F in foods (milk, apple juice, ground beef, pastry and raw eggs). LOD – 50 – 100 pg/mL.	Rivera et al. 2006
Quantum dots	IMS-quantum dot analysis for *Salmonella* in chicken-carcass wash water – sensitivity: 10^{3-7} CFU/mL.	Trully et al. 2006
	Salmonella and *E. coli* O157:H7 – 10^4 CFU/mL in 2 h (buffer)	Yang and Li, 2005
	Immunostaining of *Listeria monocytogenes*	Yang and Li, 2006

Real-time PCR (RT-PCR) Technology: RT-PCR combines traditional nucleic acid amplification (PCR) with DNA hybridization which occurs while the reaction is progressing. This is accomplished by including a fluorescently-labeled probe in the PCR amplification reactions. In most cases, the probe's fluorescence is quenched in its normal stochiometric conformation. However, if the target DNA is amplified by PCR, the probe will bind specifically during the annealing phase, resulting in a change in conformation which results in the loss of quenching and the occurrence of fluorescence, which is recorded during amplification using a thermocycler with fluorescent detection capabilities. The RT-PCR reaction consists of the sample, primers specific to the target to be amplified, nucleotides, and a polymerase enzyme, which adds nucleotides complementary to the single DNA strand to yield the PCR product or amplicon, and a probe to detect the formation of the PCR product. The method is referred to as "real-time" since PCR detection and confirmation of amplicon identity occur at the same time, in "real-time." The probe may be either nonspecific (e.g. SYBR Green I) or a fluorescently labeled sequence-specific probe (e.g. TaqMan, Molecular Beacon, FRET). When the latter is used, the strength of the fluorescent signal is directly proportional to the amount of initial copy number of the target DNA sequence. RT-PCR assays have the potential to simultaneously identify and quantify the DNA target in a single reaction vial (i.e. closed system).

Although faster and more specific than culture-based methods, PCR platforms require (i) primers specific for the target sequence of interest, ii) stringent amplification conditions, and (iii) optimized DNA extraction to remove PCR inhibitors in foods while simultaneously isolating DNA. Multiple pathogens can be simultaneously detected in a single PCR reaction (multiplex PCR), as detailed in section 5.5. Multianalyte detection for real-time platforms is possible but restricted to no more than four targets due to the limited commercial availability of non-overlapping fluorophores. Finally, incorporating an Internal Amplification Control (IAC) is important in assuring the absence of reaction inhibitors, which may result in false negative results, and as a measure of an analytical method's capacity to remain unaffected by small but deliberate variations in method parameters. Therefore, inclusion of an IAC is an indication of assay reliability. Real-time PCR (and real-time reverse transcriptase PCR) as well as portable real-time thermal cyclers for on-site analysis are commercially available. The fundamentals and application of PCR to food matrices have been reviewed elsewhere (Feng, 2007; McKillip et al., 2004).

DNA Microarrays and SNP Technologies. In contrast to PCR assays which identify one or a limited number of genes, microarrays are used to simultaneously screen for hundreds or even thousands of genes in a high throughput format (Klaenhamer et al., 2007). Often referred to as "Lab-On-A-Chip" technology, probes, including oligonucleotides (<100 bp) or PCR amplicons (100-1000 bp), based on highly specific nucleic acid sequences which may differ by only a single nucleotide (SNP), are attached or printed to a solid support (e.g. polymer, membrane, glass) in a spatially pre-determined order for simultaneous analysis of many different DNA sequences. Theoretically, microarrays can be designed to rapidly detect multiple pathogens, virulence factors, antimicrobial resistance genes and/or any number of targets useful for detection. Nonetheless, while pre-printed oligonucleotide microarrays are commercially available for a limited number of foodborne pathogens (e.g. Affymetrix GeneChip), the technology is not necessarily ready for routine use as applied to the detection of pathogens in foods. Of particular importance is the fact that successful hybridization requires $\geq 10^5$ gene copies which means that some sort of amplification (cultural enrichment or PCR) must precede microarray detection. Hence, microarray

or SNP analysis must be inherently linked to both pre-analytical sample processing and amplification, and is therefore subject to the same considerations required for these methods. In addition, microarray detection requires expensive and sophisticated equipment, and interpretation is tied to complex computer algorithms, neither of which is currently amenable for routine use in pathogen detection in foods or environmental samples.

Spectroscopy Technology: Matrix assisted laser desorption/ionization (MALDI) Time of Flight (TOF) Mass Spectroscopy (MS). Whereas genomics identifies genes, proteomics measures the level of proteins. Proteomics is defined as "…use of quantitative protein –level measurements of genes expression…." (Klaenhammer et al., 2007). Analysis utilizes two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) to separate proteins in the first dimension by their isoelectric point and in the second dimension by their molecular weight. The resultant spot is then excised from the gel, digested into peptides, and analyzed by mass spectroscopy. MALDI-TOF MS "simplifies" the analysis and generates a characteristic spectra or fingerprint for either proteins or nucleic acids. For protein analysis, the starting material ranges from a single colony or liquid culture to a single peptide generated by 2-D gel electrophoresis (Tabatabai, 2004). Analysis is robust and reproducible (e.g. the acquired profile spectra are comparable between different MALDI-TOF instruments). Assays are rapid with minutes needed for sample drying, loading the instrument, and spectra acquisition. Computer software analyzes and compares results against a growing database of ~40,000 protein spectra. MALDI-TOF offers high thru-put analysis, the potential to detect multiple analytes simultaneously. This technology has identified SNPs of *E. coli* (Sauer et al., 2006), can distinguish species of *Campylobacter*, and is being applied to serotype *Salmonella*.

Biosensor Technologies: A biosensor uses biological recognition molecules (i.e. antibodies) to detect and identify a target with high selectivity and sensitivity. The high affinity and avidity of antibodies to their target antigen underlies the specificity of immunosensors. Binding of the antigen to the antibody or cells is measured by light scattering, fiber optic biosensors (FOBS), evanescent wave biosensors, surface plasmon resonance (SPR), and piezoelectric-excited millimeter-sized cantilever (PEMC) sensors. Living cells may also be used to detect the presence of specific pathogens; collagen encapsulated hybridoma cells (Ped2E9) lyse and release alkaline phosphatase, which is colorimetrically detected in pure cultures of *L. monocytogenes* but not *L. innocua* (Banerjee et al., 2008).

Light scattering directs a laser beam on bacterial colonies which scatter light forward into a camera. Unique bacterial byproducts (e.g. extracellular polysaccharides or toxic proteins) generate distinctive images (concentric rings, spokes, and bright central spots), which are analyzed with a computer algorithm. Colonies are viable for further analysis including confirmatory assays. A prototype portable unit facilitates on-site testing.

Optical biosensors achieve detection through optical transduction mechanisms, such as changes in refractive index, absorption, fluorescence, and surface plasmon resonance. Because methods are predominantly antibody-based they are subject to variable sensitivity, especially low level detection, antibody production limitations, and inhibition by high background. Early versions were tested only with pure bacterial cultures but current focus is bacterial detection directly from food.

Fiber-optic biosensors (FOBS) use fiber optic cable with covalently attached antibodies. Target antigen binds to the antibody, which is detected by a secondary antibody conjugated to molecules that, when stimulated, emit fluorescent light measured by a laser detector. Fluorescence is quantitatively related to amount of antigen immobilized on the fiber surface. The RAPTOR™ is a commercially available example used to detect *Salmonella* (Feng, 2007).

Surface plasmon resonance (SPR) sensors use antibodies (or other receptors) immobilized on gold electrode sensing surfaces. Binding of antigens alters resonance frequency generating a signal. Although results generated are in real time (few seconds to minutes) interpretation is difficult in the absence of a strong signal. BIAcore, a commercially available SPR sensor, detected 10^5 *L. monocytogenes* in less than 30 min (Feng, 2007).

Piezoelectric (PZ) biosensors measure resonance frequency changes when the mass of quartz crystals changes in response to the binding of analytes to antibodies immobilized on the crystal surface.

Cell-based sensors use interdigitated microsensor electrodes to measure changes in conductivity seen in cell membranes when eukaryotic cells interact with pathogens. Live bacteria or active cytotoxins that affect the integrity of the membrane alter the conductivity and provide a measurable signal (i.e. impedance of the cells). In the commercial CANARY™ ("cellular analysis and notification of antigen risk and yields") system, antibodies bound to B lymphocytes are engineered to express aquorin, a bioluminescent protein, which emits a light signal in the presence of a specific antigen (Feng, 2007).

4.2. Evaluation of Emerging Technologies Based on Performance Criteria

In response to the FSIS request that NACMCF examine the merits of emerging technologies, the subcommittee evaluated each of the assays listed in Table 4 using the criteria specified in the charge. A few additional criteria, which the Committee believed were important, were added and also considered in the assessment.

The Committee would like to clarify how several criteria were used to assess new and emerging technologies. The Charge requested an assessment of technologies that can be used for enumerating indicator organisms. The Committee decided that the criterion of "scope" of analysis, should include the flexibility of a technology to detect indicators and/or pathogens. The ability to enumerate indicators is addressed under the criterion "quantify". The Charge also requested an assessment of the adaptability of the assay to different sample matrices and/or testing situations; i.e.: food, environmental, clinical, etc. The Committee found it difficult to score this criterion, as few assays can be applied to the direct detection of the agent in the sample matrix without some sort of pre-analytical sample preparation. Therefore, the assay by itself should not be regarded as the sole component in the testing protocol. In fact, the efficiency and performance of any assay is strictly dependant on whether the sample was adequately prepared prior to analysis. For example, an assay used to screen a blood sample for microbial contamination may not be directly applicable to foods unless the food has been previously subjected to a short culture enrichment period to suppress competitive microflora, resuscitate stress-injured pathogens, dilute potential assay

inhibitors, and/or increase the numbers of the target analyte. Similarly, some assays require that the toxin or the DNA content of the target organism in the sample be extracted prior to analysis. Once properly extracted, the target DNA or toxin can be screened using a variety of assays, regardless of whether the original sample was a food, a swab or blood. Because of considerations such as these, the Committee scored the "adaptability" criterion as not applicable (NA) for all assays.

Table 4 scored each assay based on the criteria specified in the charge. The scoring system used was:

-	poor
0	unknown or neutral
+	good
++	better

The following are descriptions of criteria used specifically in Table 4, with the abbreviations used in the Table in parentheses.

Specificity (Speci) – The ability of the assay to detect the target specified.

Sensitivity (Sensi) – The analytical sensitivity (LOD) of the assay based on pure culture.

Scope of analyses (Scope) – The capability of the assay to expand to include more targets, which in addition to those mentioned in the Charge could also include viruses, SNPs and indicator organisms. In accordance with the Committee's interpretation of the charge, existing real-time PCR assays, which already can simultaneously detect multiple (3 – 4) targets, scored "poor" (-), due to the limited availability of fluorophores to enable adding more targets. In contrast, DNA microarrays that have the capability to test thousands of targets score very well on scope. Also, assays such as MALDI-TOF or others that require pure cultures for analysis were scored "NA".

Adaptability to other matrices (Adapt) – The ability of the assay to adapt to various matrices and testing situations; i.e.: food, environmental and clinical samples.

Enumeration (Quantify) – The capability of an assay to enumerate the number of bacteria present in the sample or to quantify the target. For many assays, a standard curve using known target number vs. signal strength can be established from which the target levels in the sample can be quantified based on the signal detected.

Data Acquisition and transfer (Data/Tran) - The ease with which the analytical data are collected and whether they can be disseminated electronically.

Speed – The assays were scored on whether they are faster to perform than conventional microbiological methods. The scoring was based solely on the performance of the assay itself and did not consider the time required for culture enrichment or sample preparation. However, procedures inherent to the assay were considered in the scoring. For instance, DNA microarray requires PCR prior to analysis, so PCR is part of the method and requires additional time, hence arrays only scored "good" (+). Biosensors require little or no additional procedures prior to testing and hence were scored "better" (++).

Incorporation into FSIS Methods (xFSIS) – The ease with which the assay can be incorporated into existing procedures for *Salmonella, L. monocytogenes* and *E. coli* O157:H7. All the real-time PCR assays were scored "better" (++) because FSIS is already using some of these assays and should be easy to change to another test. Biosensors were thought to be easily incorporated into existing methods and so were scored "good" (+). Implementation of arrays and other assays whose implementation would be complex logistically, were scored "poor" (-).

Cost and resource efficiency (Afford) – The overall cost of the test including capital equipment, maintenance contracts, training needs and assay costs.

Criteria not included in the charge but, which the Committee decided were worthy of consideration are:

Viability – The capability to determine whether the target is viable or non-viable. As some of these assays will detect the target regardless of the organism's viability; this criterion is important to assess the public health significance of the data.

Simultaneous testing of multiple targets (Target) – This criterion was added to assess the assay's capability to detect various targets simultaneously. For example, real-time PCR that uses SYBR Green scored "poor" (-), as it is based on non-specific intercalation of the dye to double stranded DNA. But, other real-time PCR that use specific probes scored "good" (+). Some biosensors and certainly DNA arrays can accommodate multiple targets, hence scored "better" (++).

Throughput (Through)– This criterion was added to assess whether the assay can be used to screen large numbers of samples. For example, many real-time PCR and biosensors can accommodate multiple samples, so scored "better" (++), but arrays, which can test for multiple targets within 1 sample, but not multiple samples, scored "poor" (-).

Maturity – This criterion evaluated the assay's commercial availability. Unlike the rest of Table 4, a score of "good" (+) is used if the assay is commercially available and "better" (++) if the assay has been evaluated and validated for use in food testing.

TABLE 4. Evaluation of emerging technologies for analysis of pathogens in foods[a]

Format	Assay name	Speci[b]	Sensi c	Scope[d]	Adapt[e]	Quantify[f]	Viability[g]	Data/Tran[h]	Speed[i]	xFSIS[j]	Target[k]	Afford[l]	Through[m]	Maturity[n]
RT-PCR	SYBR Green I	+	++	-	NA	+	-	+	+	++	-	+	++	++
	SYBR Green-IMS	+	++	-	NA	+	+/-	+	+	++	-	+	++	++
	5' Nuclease	++	++	-	NA	+	-	+	+	++	+	+	++	++
	5'Nuclease-IMS	++	++	-	NA	+	+/-	+	+	++	+	+	++	++
	Molecular beacon	++	++	-	NA	+	-	+	+	++	+	+	++	++
	FRET	++	++	-	NA	+	-	+	+	++	+	+	++	++
	Rev trans PCR	++	+	-	NA	+	+	+	+	++	+	+	++	+
Biosensors	Fiber optic	++	++	+	NA	+	+	+	++	+	++	+	++	+
	Surf. plasm. res.	++	++	+	NA	+	+	+	++	++	++	+	++	+
	Piezoelect. PEMC	++	++	0	NA	+	+	+	++	+	0/+	+	++	0

	Cell-based sensor	+	+	-/0	NA	+	+	+	++	+	-/0	++	+	+
	Optical scattering	0/+	+	-	NA	+/-	++	+	+/-	+	0	++	-	0
	MAAB	++	++	++	NA	+	+	+	+	+	++	+	+	
	Proteomic	++	+	0	NA	0	+	+	0	-	-	0	0	
Microarray	Amplicon arrays	++	++	++	NA	-	-	0	+	-	++	-	-	0
	Oligo arrays	++	++	++	NA	-	-	0	+	-	++	-	-	+
	Suspension array	++	++	++	NA	-	-	0	+	-	++	-	-	0
Spectro-	Surface enh.	++	0	0	NA	0/+	-	0	++	-	++	-	-	0
scopy	Raman													
	MALDI-TOF MS	0/+	0	NA	NA	-	-	+	+	-	-	-	-	0
	FTIR	0	0	-/0	NA	-	-	+	+	-	-	-	-	0
Other	Flow cytometry	0/+	+	+	NA	++	+/-	+	+	+	+	-	-	+
	Immuno-liposomes	++	+	+	NA	0	+	+	+	+	-	++	0	0
	phage	+		-	NA	+	+	0	+	+	-	0	+	0

PCR + Mass Spect	++	++	0	NA	0	+	+	++	+	++	0	++	+
Luminometer/ECL	++	++	++	NA	0	+	+	+	+	++	++	+	++
Quantum dots	+	+	+	NA	+	++	+	+	+	+	++	+	+
IMS													

[a] The scoring system was:

- - poor
- 0 unknown or neutral
- + good
- ++ better
- NA not applicable

[b] Specificity (Speci.) – scored as the ability of the assay to detect the target specified.

c Sensitivity (Sensi.) – scored as limit of detection (LOD); however, since the LOD for an assay can vary greatly depending on food matrix, it was scored here based on pure cultures only.

[d] Scope of analyses (Scope) – capability of the assays to expand to include more targets, which in addition to those mentioned in the charge could also include viruses, SNPs and indicator organisms. In accordance with our interpretation of the charge, existing real-time PCR assays, which already can simultaneously detect multiple (3 – 4) targets, but yet, scored poor (-), due to the limited availability of fluorophores to enable adding more targets. In contrast, DNA microarrays that have the capability to test thousands of targets score very well on scope. Also, assays such as MALDI-TOF or others that requires pure cultures for analysis were scored as NA.

[e] Adaptability to other matrices (Adapt.) – ability of the assay to adapt to various matrices and testing situations; i.e.: food, environmental and clinical samples.

[f] Enumeration (Quantify) – the capability of an assay to provide enumerate the number of bacteria present in the sample or to quantify the target. For many assays, a standard curve using known target number vs signal strength can be established from which, the target levels in the sample can be quantified based on the signal detected.

[g] Viability – the capability to determine whether the target is viable or non-viable. As some of these assays will detect the target regardless of the organism's viability, this criterion is important to assess the public health significance of the data.

[h] Data acquisition and transfer (Data/Tran.) – the ease with which the analytical data are collected and whether they can be disseminated electronically.

[i] Speed – scored on whether the assays are faster to perform than conventional microbiological methods. The scoring was based solely on the performance of the assay itself and did not consider the time required for culture enrichment or sample preparation. However, we did take into account procedures that are part of the assay. For instance, DNA microarray requires PCR prior to analysis, so PCR is part of the method and requires additional time, hence arrays only scored good (+). Whereas biosensors, which require little or no additional procedures prior to testing were scored as better (++).

[j] Incorporation into FSIS Methods (xFSIS) – The 3 pathogens currently tested by FSIS are *Salmonella, Listeria monocytogenes* and *E. coli* O157:H7, so we evaluated as to how easily can the assays be incorporated into existing procedures. All the real-time PCR scored better (++) because FSIS is already using some of these assays and will be easy to change to another test. The Committee also felt that biosensors can be easily incorporated into existing methods and were scored good (+). But implementation of arrays and other assays may be complex logistically, hence scored poor (-).

[k] Simultaneous testing of multiple targets (Target) – criterion added to assess the assay's capability to detect various targets simultaneously. For example, real-time PCR that uses SYBR Green scored poor (-), as it is based on intercalation of dye to double stranded DNA without differentiation. But, other real-time PCR that use specific probes scored good (+). Some biosensors and certainly DNA arrays can accommodate multiple targets, hence scored better (++).

[l] Cost and resource efficiency (Afford.) – scored not on a per test basis, but rather as overall cost of the test, including capital equipment, maintenance contracts, training needs and assay costs.

[m] Through put (Through) – criterion added to assess whether the assay can be used to screen large numbers of samples. For example, many real-time PCR and biosensors can accommodate multiple samples, so scored better (++), but arrays, which can test for multiple targets within 1 sample, but not multiple samples, scored poor (-).

[n] Maturity – assay's commercial availability. Unlike the rest of Table 4, a score of good (+) is used if the assay is commercially available and better (++) if the assay has been evaluated and validated for use in food testing.

4.3. Sampling and Pre-Analytical Sample Processing Technologies

A variety of techniques are available for sampling and pre-analytical processing of foods and environmental samples. Integration of these techniques with new technologies is essential for enhancing FSIS' analytical capabilities.

4.3.1. Sampling and Pre-Analytical Sampling Considerations

Optimal strategies for collecting, transporting, and preparing test specimens are critical to the quality and interpretation of pathogen detection results. At a very basic level, sampling may be categorized as either "destructive" or "non-destructive". In destructive sampling, such as excision sampling of carcasses, a specific weight of product is collected and tested by the laboratory as a sample test portion measured in grams. The destructive sampling-testing approach offers the advantage of near-100% recovery of the target pathogen from the sample as well as the potential for detection of the pathogen if internalized within the product. Excision is generally considered to be the sampling method that yields the highest recovery of pathogenic and indicator bacteria (Palumbo et al., 1999; Sharpe et al., 1996). However, recent comparisons between swab and excision sampling showed no significant differences (Gill and Jones, 2000).

Non-destructive sampling, such as the whole-bird rinse technique used for chicken carcass sampling, or the sponge technique used to collect samples from turkey, cattle, and hog carcasses, employs an indirect means of collecting the pathogen from the surface of the product to be tested. A non-destructive sampling approach is warranted where the focus is detection of contaminants which do not penetrate below the surface of the product. Such an approach may be advantageous when it is desirable to sample large surface area of the product (e.g., to increase sensitivity and/or potential detection of heterogeneously distributed contamination), or where the entire product or sampled surface cannot be submitted to the laboratory due to its size. For any indirect sampling approach, recovery from the product and, in some cases, the test portion, may be significantly less than 100%.

The appropriate sampling method depends on the purpose of the test. For example, the optimal sampling location and method might differ if one was trying to determine the prevalence of an organism in live animals (e.g., rectal, fecal, cecal, hide, feather, pen samples), vs. its prevalence in market samples (e.g., whole birds or cuts of meat), vs. evaluation of the efficacy of a candidate control strategy (e.g., in-process sampling of carcasses or equipment). The sampling location and method may also be influenced by the type of information wished to be gained. For example, there may be specific locations on a carcass where contaminants are concentrated. Carcass mapping studies have been carried out to predict the areas with concentrated contamination levels (Sofos et al., 1999; Gill et al., 2005, 2007). Another consideration in choosing the sampling location and method is minimizing the degree of disruption to the production process, and the cost of product lost to sampling. These considerations have led to comparisons of the efficacy of excision and sponge sampling (Gill and Jones, 2000; Ware et al., 1999; Hutchison et al., 2005; Palumbo et al., 1999). Recovered pathogen subtypes may vary with the sampling location and protocol (Simmons et al., 2008).

Finally, suitability of the sample for the specific detection method being used ("fit for purpose") must be considered. For example, if sampling previously cleaned or disinfected surfaces, there may be a need to neutralize or remove residual antimicrobials or compounds that may interfere

with the detection system. There may also be a need to dislodge attached microorganisms from the sampled portion or site, for example, as might occur during optimization of the recovery of *Salmonella* and *Campylobacter* imbedded in feather follicles or *E. coli* O157:H7 encapsulated in beef fat.

4.3.2. Novel or Emerging Sample Collection Methods.

Much more research has been conducted on detection technologies than on sampling methods. Some of the relatively few examples of novel or emerging sampling technologies include the Microbial-vac system (http://www.m-vac.com), the sampling of beef trim combo purge (Dorsa and Siragusa, 1998), and thin surface sampling of trim (Kiermeier et al., 2007). The package rinse method for *L. monocytogenes* (Luchansky et al., 2002) has been evaluated and was found to be superior to several other product sampling methods. Tissue paper wipes have been found to be a good alternative to sponges or swabs for environmental monitoring (Vorst, Todd and Ryser, 2004). Other novel or emerging sampling ideas include sampling of rinsate from spray cabinets in slaughter facilities and turkey wing tip sampling.

4.3.3. Pre-Analytical Sample Processing

There are factors aside from assay validity that can also impact the performance of a test method for pathogens. One of these is volume considerations. For example, while most nucleic acid amplification methods and biosensor approaches are theoretically able to detect a single target molecule (or cell) per sample the volume amplified utilized in these assays is very small (<10 µl). Clearly, it is not feasible to screen the entire sample volume in such a test method, so if intermittent and/or low levels of contamination are present, they are likely to be missed. An additional consideration is the fact that food samples frequently contain relatively high levels of non-pathogenic bacterial flora and/or food components which can inhibit the assay or otherwise raise the lower limit of detection. Furthermore, most rapid detection methods require the sample as a liquid but most foods are not liquid. These and other important issues that might otherwise influence assay performance are described in detail by Feng (2001). These also provide the basis for the recent increased interest for the use of novel pre-analytical sample preparation technologies, most of which are intended to reduce sample volumes, remove matrix-associated inhibitors, yet simultaneously result in recovery of most (if not all) of the target pathogen.

4.3.4. Novel Approaches to Sample Preparation

Cultural enrichment could be considered the first form of pre-analytical sample processing in that this process is intended to suppress the growth of competitive microflora, dilute food-associated inhibitors, and increase the numbers of the target organism. Recent studies have focused on the refinement of enrichment media resulting in faster multiplication of the target pathogen. For example, enrichment in non-selective broth can be done with the addition of bacteriophages which eliminate certain competitive or interfering microflora (Stave et al., 2006). Enrichment times have also been shortened by enriching in a non-selective broth followed by immunomagnetic separation, which will provide both amplification and concentration in a single test protocol. This is the current approach being used in some *E. coli* O157:H7 testing protocols as applied to foods (Arthur et al., 2005).

Theoretically, improvements in how samples are collected and shipped to the testing laboratory could enhance the speed, sensitivity and selectivity of a pathogen assay. One option might be to

prepare and place the sample into the enrichment medium immediately after sample collection, then ship the inoculated medium to the detection laboratory in a temperature-controlled incubation chamber, i.e., enriching the sample en route. At this time, there do not appear to be any practical methods to achieve this, at least using U.S. commercial overnight carriers. Another option might be to lyse the bacterial cells and stabilize the nucleic acids in a transport medium prior to shipping, preventing the laboratory from having to undertake time-consuming nucleic acid extraction steps. A commercially available method for preparing vaginal swabs or urine samples for the detection of *Chlamydia trachomatis* is based on this principle (www.fda.gov/cber/510klabel/k043072lb.pdf). Similar systems for foodborne pathogens could be developed.

Over the last decade, there has been recognition of the need for pre-analytical sample processing prior to the application of rapid and emerging test methods. This is based on the supposition that ultimately the limit of detection for a test could be improved if the pathogen(s) were separated and concentrated from the matrix prior to detection. The general principles applied to pathogen concentration have been reviewed elsewhere (Stevens and Jaykus, 2005; Tsai et al., 2006; Fukushima et al., 2007) and some of these approaches are detailed in Table 5.

To date, almost all of the methods outlined in Table 5 have only been applied after a prior cultural enrichment step. None of the sample preparation approaches described in Table 5 are ideal and the choice of method depends on the purpose of the analysis. For example, some sample preparation methods will concentrate and purify the entire bacterial population, while others are specific for one or more pathogens; some will result in recovery of viable cells, others will kill the target cell but maintain the integrity of the target molecule. No pre-analytical sample processing method recovers 100% of the target from a complex sample matrix, and the efficiency of concentration and purification can be matrix-dependent. Further, not all methods are applicable to all types of food products. Many of the sample preparation methods are cumbersome, require specialized equipment or training, and are not adaptable to the routine processing of large numbers of samples. The volume that can be processed in sample preparation is also method-dependent. Sometimes the complexity of matrices requires the use of multiple sample preparation methods in sequence. Taken together, it is clear that the field of pre-analytical sample preparation is fertile ground for future research that is needed to maximize the potential benefits of emerging methods.

TABLE 5. Partial listing of microbiological sample preparation approaches[a]

Method	Principle/application	Advantages/efficacy	Comments	References
Ion exchange resins	Cationic exchange resins bind bacteria by ion exchange; release of bacteria from resin accomplished by pH manipulation	Rapid; relatively inexpensive; broadly inclusive	Not practical for large sample numbers; sample pre-treatment to remove debris recommended; pH manipulations needed for desorption; destroys cell viability	Jacobsen and Rasmussen, 1992
Metal hydroxides	Hydroxides of zirconium, titaneous, or hydroxyapatite adsorb and "flocculate" bacteria; Used in conjunction with centrifugation	Rapid; inexpensive; simple; broadly inclusive; amenable to large sample sizes	Not practical for large sample numbers; sample pre-treatment to remove debris required; appears to work best on less complex sample matrices	Berry and Siragusa, 1997 Cullison and Jaykus, 2002 Lucore et al., 2000

Aqueous two-phase partitioning	Cells partition in one of two immiscible liquid phases (PEG and dextrans) based on charge	Rapid; inexpensive; simple; broadly inclusive	Not practical for large sample numbers; partitioning frequently incomplete; composition of the phases may impact cell viability; fat interferes with separation	Lantz et al., 1994a Lantz et al., 1994[a] Magnusson and Johanssoon, 1977
Affinity separation	Immobilization of molecules (lectins) with high affinity for bacteria to a solid support such as agarose beads, affinity columns, or magnetic particles	Rapid; simple; specificity unknown	Not practical for large sample numbers; expensive; sample pre-treatment to remove debris recommended; release of bound cells may be inefficient; best applied to small sample volumes	Patchett et al., 1991 Payne et al., 1992
Simple centrifugation	Low speed (<1,000 x g) sediments debris	Rapid; inexpensive; simple; broadly	Not practical for large sample numbers; bacteria	Wang et al., 1997

		inclusive; amenable to large sample sizes	adhere to and sediment with matrix components; best if preceded by an elution step	
	High speed (>8,000 x g) sediments bacteria Used with or without coagulation or flocculation			
Differential centrifugation	Low speed centrifugation followed by high speed centrifugation; Used with or without coagulation or flocculation	Rapid; inexpensive; simple; broadly inclusive; amenable to large sample sizes	Not practical for large sample numbers; Bacteria adhere to and sediment with matrix components; few products available to promote desorption without destroying cell viability	Meyer et al., 1991; Neiderhauser et al., 1992;
Density gradient centrifugation	Cell separation by centrifugation within a density gradient; Requires	Can be designed to separate very distinct species from one another	Not practical for large sample numbers; expensive; difficult to	Lindqvist, 1997

	use of chemical additives to establish a gradient		perform; osmotic strength of gradient destroys cell viability; fat entraps bacteria at interfaces	
Crude filtration	Cheesecloth; Filter paper; Filter homogenizer bags	Rapid; inexpensive; simple; broadly inclusive; amenable to large sample sizes	May not be practical for large sample numbers; high particulate foods clog filters; bacterial cells can absorb to the filter or retentate	Fernandez-Astorga et al., 1996; Uyttendaele et al., 2000;
Electro-positive / negative filtration	Bacteria tend to have a net negative charge, so electropositive filters often used; sample prefiltration to remove debris frequently required	Rapid; inexpensive; simple; broadly inclusive	Not practical for large volumes and sample numbers; Filters clog rapidly even if samples are pre-filtered; desorption of bacteria from filters	Thomas, 1988

			frequently inefficient	
Immunoseparation	Immobilization of antibodies to a solid support such as polystyrene beads or magnetic particles	Rapid; simple; highly specific; standard method for some foods	Not practical for large sample numbers; Expensive; Sample pre-treatment to remove debris recommended; many formats available; best applied to small sample volumes although recirculating IMS is available for larger volumes	Fu et al.,2005; Islam et al., 2006; Nou et al., 2006; Tsai et al., 2006; Uyttendaele et al., 2000; Warren et al. 2007
Nucleic Acid Extraction	Purification of DNA or RNA template	Removes matrix-associated inhibitors and concentrates template; matrix and method-	Not practical for large volumes or sample numbers; many commercial kits available,	Sair et al., 2004

		dependent efficacy	some with matrix specificity; automation available but expensive; destroys cell viability	
Novel Methods	Phage-based--synthetic phage ligand to capture target bacteria	Less susceptible to cross-reactivity; reagent stability	Very new technologies with limited history of performance	Kretzer et al. 2008
	Magnetic nanoparticles--Ultra small magnetic particles to which target-specific-ligands are conjugated	Nanoparticles have higher capture efficiency than microbeads		Varshney et al., 2005

[a] Adapted from Stevens and Jaykus, 2005

5. Considerations when Choosing Emerging Technologies/Methods (Questions 1 & 2)

Some of the advantages of emerging technologies are a reduced time to detection, a high degree of sensitivity and specificity, and a low limit of detection. If robust and dependable pre-analytical sample preparation methods were available, one could even envision completely bypassing cultural enrichment. While this is theoretically possible, there are many other considerations which must be taken into account before adopting emerging technologies, whether preceded by cultural enrichment or not. These are discussed below.

5.1. Potential for rapid, on-site analysis

Rapid, or ideally real-time, screening methods that might be suitable for on-site and in-plant use (e.g., biosensors) would be particularly valuable. Such methods offer the opportunity to screen samples prior to shipment to the laboratory, thus saving resources and decreasing the time required to hold a product. However, these methods must be held to high performance standards and accountability to minimize false positive and negative results, and they must be appropriately validated before use.

Cultural enrichment is the universal starting point for most pathogen detection assays. The manipulation of cultures enriched for pathogens within or even adjacent to a food processing facility requires strict precautions to prevent cross-contamination. For on-site analysis to become widely practical, either enriched pathogen cultures would need to be self-contained, leak-proof, and disposable, or cultural enrichment steps themselves would need to be eliminated. Self-contained pathogen assays are currently available, but only for a very few applications, e.g., an assay for the detection of *Listeria* spp.(AOAC International, 2006).

5.2. Discrimination between Viable and Non-Viable Cells

An inherent advantage of culture-based methods is the detection of viable cells capable of causing illness. Culture-based methods are considered to be the "gold-standard" and are critical in helping the Agency to meet its mandate of assuring the safety of meat, poultry and egg products. However, many of the newer tests target the pathogen's nucleic acids, which may be detected long after cell death (days to weeks). This means that nucleic acid amplification methods cannot always differentiate living from dead cells. For foods, this is especially important due to commonly used food processing or preservation methods which are intended to inhibit or inactivate pathogens.

The use of nucleic acid amplification methods in pathogen screening is easily defensible if followed by culture-based confirmation. However, if the elimination of cultural enrichment is an eventual goal, the "live-dead" dilemma will need to be resolved. Recently, the DNA intercalating agents ethidium monoazide (EMA) and propidium monoazide (PMA) have been used in conjunction with quantitative PCR for the selective detection of live cells of foodborne pathogens (Nogva et al., 2003; Nocker et al., 2006a; Wang and Levin, 2006; Rudi et al., 2005a and b). At the time of this writing, none of these methods has been commercialized and it is still unclear as to whether the approach will be suitable for widespread application for viability discrimination for the detection of pathogens in foods.

Though not a viability issue per se, a positive result with a toxin gene-specific PCR assay indicates that those gene sequences are present in the target organism, and that the cells are potentially toxigenic. It does not, however, assure that the gene is actually expressed or that the toxin, if produced, is functional (Feng, 2007).

5.3. The need for a viable isolate

Related to the viability issue is the need for a live culture in order to further characterize the strain by phenotypic and/or genotypic methods (see Section 2). Many of the newer detection platforms are based on the detection of one or more genes or antigens that are present in the microbial target. Such molecular targets might be species- or serotype- specific, associated with virulence located on plasmids, cell surface components, or associated with biochemical abilities. Because of the sensitivity and discriminatory power of some of these methods, especially the genetically-based ones, it is no longer essential that viable isolates be generated for testing purposes. However, when a pure culture is not available for further testing, subsequent confirmation or subtyping cannot be performed. Even though this situation may not be an important factor for tests that target a single gene, consider cases in which tests rely on results from multiple genetic targets. The interpretation of these results can have serious shortcomings because the result might indicate a positive test for all the required markers that would ordinarily identify the designated pathogen. However, in a non-clonal culture, the individual positive test results might have been generated by genes present in different cells, with no one cell having the required genotype to give a confirmed positive result. In this case, further analyses on purified strains would fail to confirm the presence of the pathogen. While such a test might be appropriate for screening purposes, especially when time is of the essence, one may anticipate a higher level of false positives under these circumstances.

Strain isolates can be readily archived and stored for years. Although nucleic acid extracts also can be archived, the stability of the material may be in question. Since the material would undoubtedly consist of a mixture of nucleic acid moities, differential degradation would increase uncertainty that the identical material is being tested upon subsequent analysis. An additional difficulty is that the same material (that is, a pure culture of an isolated pathogen) would not be available in a legal dispute. Such inconsistencies, understandable from a scientific standpoint, could lead to substantial difficulties in a legal context.

With newer technologies often comes faster, more specific and sensitive assays, but the complexities of testing foods remain. Cross-contamination with positive controls or other sources and the potential for antibody cross-reactivity or non-specific binding linger as issues to be addressed. Furthermore, matrix-associated inhibitors can impact assay performance. In short, having an isolate for confirmation remains the definitive proof of contamination.

5.4. Qualitative versus Quantitative Results

Most foodborne pathogen detection methods are qualitative and yield positive or negative results (see Section 3). However, determining the number of pathogenic cells in a sample can provide important information for process control, risk assessment, and support of regulatory decision-making. With the introduction of quantitative real-time PCR (qPCR) techniques, direct estimation of pathogen load, is becoming a practical reality. The basis for such quantification is that the fluorescent signal generated by the amplification reaction is proportional to the concentration of

DNA in the sample. Hence, by incorporating standards in real-time PCR assays, it is possible to estimate the absolute or relative amounts of nucleic acid target, which indirectly estimates the number of microorganisms present in the sample (Ronner and Lindmark, 2007; Takahashi et al., 2006). While qPCR has promise, issues related to viability, the requirement for enrichment, the effects of matrix-associated inhibition and subsequent target recovery continue to affect accuracy.

5.5. Multianalyte Considerations

Within a testing program designed to screen foods for the presence of specific pathogens, single-target assays meet a critical need. However, in surveillance situations, the process control setting, and outbreak investigations, multianalyte analysis (sometimes called multiplexing), in which two or more targets are measured simultaneously in a single assay, offers an increase in test throughput, work simplification (i.e. fewer assay tubes, fewer pipeting operations, etc.) and possibly a reduction in the overall cost per test. Many of the newer technologies e.g. real-time PCR, biosensors, genotype and phenotype microarrays, offer the potential to detect several genes, species, or toxins simultaneously.

Obstacles do exist that might preclude the routine implementation of multianalyte assays. These include the possibility of cross-reactions and difficulties in optimizing the assay as applied to the individual analytes or the wide variety of sample matrices. Also, with the addition of multiple targets comes the possibility of quality control failure for one analyte that could jeopardize the validity of an entire run. If an assay needs to be repeated, any savings of cost or analyst time could be lost. The difficulties with non-clonal cultures have been discussed above (Section 5.3).

5.6. Fit for Purpose

The selection of new methods must always be made with the consideration that it must be appropriate for intended use. Presently, it appears that the emerging pathogen detection methods under development will be most appropriate for screening purposes. Due to complications described above, methods used for regulatory decision-making will most likely need to remain based on standard cultural procedures, at least in the near term.

6. Review of Technologies/Methodologies to Meet Public Health Goals (Question 6)

As stated in the rationale for addressing the charge (Section 1), NACMCF determined during its deliberations that the recommendation of any new technology for use by FSIS must be presented in an appropriate context to have applicable meaning and utility. Microbiological testing objectives and resulting test criteria of any proposed new technology/method should clearly support the FSIS testing objectives outlined in the FSIS Strategic Plan (www.fsis.usda.gov/PDF/Strategic_Plan_2008-2013.pdf). The broad elements of testing itself must be addressed in the submitted proposal, including statistical and sampling requirements, sample collection and transportation, laboratory analysis and reporting, database generation and statistical analyses. The proposal should also address the degree of validation required for adoption and use of the method within the agency (e.g., interim, no validation/emergency use only, single lab validation, full collaborative validation). This section describes a process for FSIS to consider before adopting a new method for an intended programmatic purpose, within the context of the public health focus. It was the intent of the Committee to describe the process in broad, rather than prescriptive, terms to allow FSIS flexibility to develop their own policy and protocols.

6.1. Overview of the Proposed System for Evaluating New Technologies/Methods

Because laboratory methodologies for regulatory use do not exist in an analytical vacuum, it is necessary to consider external factors when evaluating the appropriateness of technologies. A model for method evaluation could consist of a holistic approach such as:

1. Indicate which FSIS public health strategic goal/objective the method attempts to addresses
2. Describe what sampling plans can be implemented and resulting statistical consequences
3. Analyze the performance and capabilities of candidate method(s)
4. Establish reporting requirements

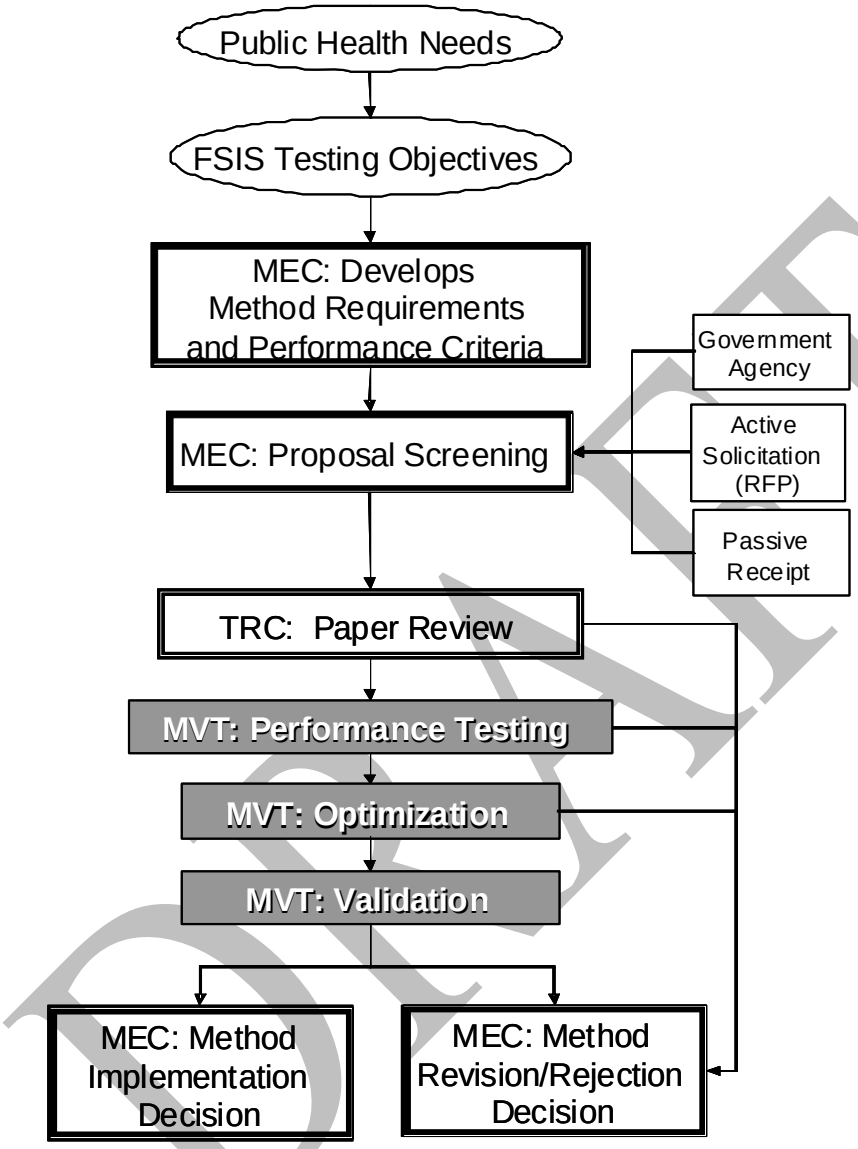
The development of microbiological methods for the analysis of food to detect and enumerate bacterial pathogens is a complicated and costly process. Presently, FSIS does not have the mandate or the resources to conduct methods research in-house and therefore, must rely on a variety of resources from outside the Agency. This leveraging could include other governmental agencies (such as ARS, FDA and CDC), companies that carry out methods development research (especially methods for industry use) and academic researchers who may have innovative ideas needing further development before they can be adopted for regulatory use. For methods developed by these diverse groups to receive a fair, timely and technically appropriate review and evaluation, FSIS should consider adopting a comprehensive system for ongoing evaluation, selection, optimization, validation and implementation of new microbiological testing technologies/methodologies to meet public health goals. This recommended "idealized" system should include the staff, facilities and organizational structure necessary for successful implementation of appropriate new technologies that will allow the agency to meet its public health goals. Descriptive text and a schematic diagram (Figure 1) of the proposed system for evaluating new technologies and methods follows.

Figure 1: Proposed System for Evaluating New Technologies/Methods

The proposed system includes a Method Evaluation Committee (MEC), a Technical Review Committee (TRC) and a Method Validation Team (MVT). The MEC is a standing committee composed of subject matter experts that identify, define, and develop testing objectives/needs, proposal requirements and performance criteria. The TRC is an *ad hoc* committee of technical experts that conducts technical reviews of proposals for new technologies/methods referred by the MEC. The MVT is a committee of laboratory and other experts responsible for performance testing, optimization and validation of those methods that have been selected by the TRC for further testing.

1

Proposed System for Evaluating New Technologies/Methods



6.2. Method Evaluation Committee (MEC)

The MEC is envisioned as a standing committee that will organize and coordinate the solicitation, receipt and initial categorization and screening of proposed methods. The MEC should be composed of subject matter experts from multiple program areas within FSIS with input from academia, industry, and other stakeholders as needed. The MEC should include experts who have responsibility for policy development, data analysis, public health and contracting.

To ensure that FSIS has the opportunity to consider and evaluate all appropriate new methods and technologies for use in its laboratories, the MEC should serve as the point of contact for method submissions coming into the FSIS. The MEC would receive method descriptions and other testing proposals and review them for their applicability in supporting the Agency's public health objectives. If these methods appear to be able to fulfill an FSIS testing need, they will be forwarded to a specially constituted TRC.

The MEC will also work closely with senior management to identify, define and develop FSIS' testing objectives/needs and develop proposal requirements and performance criteria including, checklists/guidelines to determine if proposed methods should proceed to the technical review stage. If at any point in the process, the proposal fails to meet the established criteria the MEC may choose to generate a report detailing the method's failings and notify the submitter.

6.2.1 Develop Proposal Requirements and Performance Criteria

A standardized evaluation protocol should be developed and applied to any proposal for new or revised technologies/methods for use in FSIS laboratories. Once the testing objectives have been defined by FSIS, the MEC should construct checklists for a) proposal format requirements and b) method performance criteria that an analytical method must possess to be considered for use in FSIS laboratories.

6.2.1.1. Proposal Format Requirements

For a technology/method to be considered by FSIS, a formal written proposal must be submitted to FSIS that meets the proposal format requirements. The written submission must be organized for easy review, with all logically related materials sorted into appropriate sections and all pages numbered. FSIS should develop a standardized form and make it available on its web site. At a minimum, the written report must include the following information: (FERN-ADM.0003.00 – ref)

- Contact Information. All technologies/methods submitted to FSIS for consideration must include an address, phone number, and e-mail address for the point of contact (POC). The POC should be able to answer detailed questions concerning the development and application of the submitted technology/method.
- Date Submitted.
- Background. A summary of the test principle and the target agent must be included, as well as the matrices to which the test system/method can be applied. The nature of the

method, either qualitative or quantitative, should be identified. The background should also include justification and reasons for either an initial submission or substitution of the method for an existing FSIS method.

- Safety Precautions. A description of any biological, chemical, or radiological hazards associated with the method must be included along with any special instructions for disposal of hazardous materials.
- Sample Collection. Instructions for the collection, handling and storage of the test samples, including criteria for sample rejection, must be included.
- Sample Preparation. A description of the special procedures that are used to prepare a sample for analysis must be submitted.
- Reagents. Critical reagents required to complete the submitted test/method must be identified, including the source (commercial or governmental), storage requirements, and any regulatory stipulations for purchase and utilization. Suitable reagent substitutions should be provided, as applicable.
- Reagent Preparation. Procedures for the preparation of the submitted test/method reagents must be clearly delineated.
- Equipment, supplies, and analytical instrumentation. Sources (commercial or governmental) and regulatory requirements for instrumentation and supplies needed to complete the test/method must be identified. Suitable equipment substitutions should be provided, as applicable.
- Equipment operation. Instructions for operation of equipment necessary to complete the submitted test/method must be included. These may include manufacturer instructions, identification of variable parameters, etc.
- Laboratory Protocol. Clear and concise step-by-step instructions of the test method must be given for rapid implementation in another laboratory.
- Data analysis. Raw data, statistical methods and a summary of data analysis must be included. Results from multiple laboratories should be included, if available.
- Quality assurance. Procedures and controls for reagents and instrumentation must be included.
- Method performance. Reportable range, sensitivity, specificity, accuracy, precision, linearity, throughput, and sample process time must be determined on test matrices as well as standards.
- Limitations and interferences. Concerns related to analytes and matrices.
- References. Documentation used to support the development, testing and validation of the submitted test/method must be included.

These generic proposal format requirements may be modified by the MEC as needed to address specific needs within the FSIS.

6.2.1.2. Method Performance Criteria

To evaluate new technologies/methods, FSIS should develop specific method performance criteria based on practical considerations for the intended use. Information must be provided in order to evaluate the amount of optimization/validation that has been done and to determine if the method will be suitable for its intended use by FSIS. These validations may be done by the submitter with appropriate data submitted for review by the TRC or may be done internally by FSIS.

Administration information and data needed to evaluate method performance should include:

1. **Need for Method:**
   - Has the need for a new method been clearly defined?
   - Does the method address a specific FSIS public health objective?

2. **Method Background:**
   - Was there sufficient summary of the test principle and the target agent?
   - Was there inclusion of matrices to which the test/method can be applied?
   - Was the qualitative or quantitative nature of the method identified?

3. **Safety Precautions:**
   - Was a description of any biological, chemical, or radiological hazards associated with the method included?
   - Were there instructions for the disposal of hazardous materials included?

4. **Sample Collection and Sample Prep:**
   - Were there instruction for the collection, handling, and storage of test samples, including criteria for sample rejection included?
   - Was there a description of the special procedures that are used to prepare a sample for analysis?

5. **Reagents:**
   - Were any critical reagents required to complete the submitted test/method identified?
   - Did the submitter identify sources, storage requirements, and any regulatory stipulations for purchase, utilization and disposal of reagents?
   - Were suitable reagent substitutions provided, if applicable.
   - Were procedures for the preparation of reagents clearly lineated?

6. **Equipment, Supplies, and Instrumentation:**
   - Did the submitter identify sources and regulatory requirements for instrumentation and supplies needed to complete the submitted method?
   - Were suitable equipment substitutions provided, if applicable?
   - Were sufficient instructions for operation of equipment provided?

7. **Quality Assurance Procedures:**
   - Were quality assurance procedures and controls for reagents and instrumentation included?

8. **Method Performance:**
   - Was information provided on method performance, including methods used to determine the following parameters?

Sensitivity
Specificity

Accuracy
Precision (Includes repeatability and reproducibility)
Linearity
Measurement Uncertainty
Ruggedness
Matrix Effects
Through-put
Sample Process Time

- Were known limitations and interferences reported?
- Were each step-by-step procedures for the method provided?

9. Biosafety/Biosecurity:

- Was information given on the level of required laboratory biosafety or biosecurity?

10. Clarity:

- Was the submitted method sufficiently understandable or clear for rapid assimilation and use in another laboratory?

11. Laboratory Validation/Optimization:

- Were multiple strains of the target organism used (inclusivity)?
- Were strains of non-target organisms used (exclusivity)?
- Were a number of foods and/or food types used?
- What was the analyte level/matrix (inoculated/uninoculated)?
- Were appropriate replicates per food at each level tested?
- Were inoculated samples aged prior to testing?
- Were additional competitor strains present?
- Was the method compared to the FSIS recognized method(s)?
- Was a multiple laboratory collaborative study conducted?

12. Final Review Recommendations by MEC:

After the TRC review, the MEC may recommend:

- Approved/Accepted for FSIS implementation as submitted (Sufficient laboratory review and validation done by submitter)
- Not appropriate for current FSIS stated objectives, but recommend FSIS use this information to inform future objectives
- Not approved (provide a brief summary of deficiencies that need correction before acceptance or resubmission)

FSIS will determine the specific acceptable numbers based upon the intended use of the method within the program.

Any other supporting documents and/or publications needed for a review and understanding of the new technology/method should also be included. All raw data should be available for review if necessary. These may include:

1. Worksheets/notebooks.
2. Identification of all matrices and analytes tested.
3. A unique identifier for all standards, controls, or test portions analyzed.
4. Organism inoculation levels and protocols.
5. Test portion weights, volumes, etc.
6. Identification of all critical standards, reagents, and instrumentation used during analysis.
7. Instrumental readouts.

These criteria may be modified by USDA/FSIS depending upon the specific objectives and need for the new method.

6.2.1.3. Receipt of Proposals: Active, Passive, Government

Once proposal requirements have been developed, a request for proposals may be issued to advertise FSIS requirements and generate interest. New technologies/methods can be submitted to USDA/FSIS as either a direct response to a call for proposals by USDA/FSIS (active) or by another Government Agency, academia, or industry submitting a new or revised method to USDA/FSIS without a formal request for proposals (passive).
Regardless of how the technology/method is submitted, the MEC will then conduct a non-technical review of the proposals and determine which ones generally meet the testing objectives defined by the FSIS. At this point, the MEC will pass onto the TRC those proposals that appear to satisfy the overall testing objectives.
A general scheme for submitting method proposals:

1. USDA/FSIS will put out a formal call for proposals in the Federal Register (active only)
2. The FSIS will collect all proposals and submit to the MEC for consideration (active or passive).
3. The FSIS will determine if USDA/FSIS has a need for the proposed technology/method and if there is merit for a full evaluation of the method (passive only).
4. The MEC will review proposals, obtain appropriate documentation and prioritize the submitted proposals (active or passive).
5. The MEC in consultation with appropriate USDA/FSIS personnel will identify potential technical reviewers for the proposals (active or passive).
6. Methods will be evaluated by the TRC using established criteria and recommendations will be made to the MEC (active or passive).

6.3. Technical Review Committee (TRC)

The TRC is an *ad hoc* committee constituted to conduct technical reviews of proposals for new technologies/methods referred by the MEC. The TRC will be composed of technical experts from within and outside FSIS and will be constituted under the direction of the MEC to assure that the committee's composition contains the expertise required to perform the technical review. Thus, the TRC membership is not constant but changes to accommodate changes in technical expertise

needs. Where disparate proposals are being considered more than one TRC may be required at any given time.

The TRC will undertake a technical proposal review of the method, its claims and supporting data. The method will be evaluated and rated with respect to, but not limited by, the following parameters: sampling requirements, method sensitivity (CFU/sample), pure culture requirement, false positive and negative rates, ruggedness, throughput, workflow, turnaround time, credibility, cost, flexibility, data integration, quantitative and qualitative capabilities, and portability.

The TRC's review will objectively evaluate the proposals against the checklist criteria that were constructed to assure that methods would allow the FSIS to meet its testing objectives. If any of the proposed methods appear to be more appropriate for an alternative testing objective, they will be referred back to the MEC to determine if the testing objectives should be redefined.

6.3.1. Proposal Review

Following an initial proposal screening by the MEC, a TRC will be established to conduct a review of the documentation submitted for the proposed technology/method. The make-up of the TRC will be dependent upon the intended use of the method, the type of method, and the degree to which it has previously been validated. The TRC will consist of reviewers from FSIS, other Federal agencies such as CDC and FDA, academia, and any other expert reviewers called in by FSIS as needed. As long as the data are submitted according to the submission requirements provided previously, this review will be conducted by a process similar to a journal review. Each panel member will review the submitted documents based upon the generic criteria established by the MEC using the checklist provided as well as any additional criteria specific to that method (to be supplied by FSIS). Following the individual reviews, the panel will discuss the overall review via teleconference or a face-to-face meeting in order to provide FSIS with a consensus technical review recommendation. The proposed method can be accepted for immediate use; accepted to proceed to the next step; rejected; or recommendations made for revisions to the submitted documentation. This technical proposal review will be completed in a timely manner, within no more than one month from the time of submittal.
Following the TRC technical paper review, the proposal will be referred to the MVT with any necessary comments sent to the MEC. Once the MVT has completed laboratory evaluation, a complete report with recommendations will be sent to the MEC.

6.3.2. Laboratory Data Review

Submitted data will be reviewed by the TRC. The performance of top-rated methods under close-to-real-world conditions will be assessed by the TRC. As a rule, the data should be sufficient to evaluate the performance of methods. If the data are determined to be insufficient, then additional laboratory validation may be requested. If multiple promising new technologies/methods have been identified, FSIS may invite the various method proponents to test a panel of coded samples, similar to the AOAC review process (http://www.aoac.org/vmeth/oma_program.htm). In emergency situations where rapid response is necessary, FSIS may use alternate mechanisms to select new technologies/methodologies that are transparent and defensible. The TRC would statistically evaluate the test results and determine overall method performance. Those methods

meeting the minimum requirements may be selected by FSIS for further evaluation in its own laboratories with actual samples. FSIS should have mechanisms for recovering the costs of method evaluation.

6.4. Method Validation Team (MVT)

The MVT is charged with conducting (i) performance testing, (ii) optimization and (iii) validation of the proposed method(s). Here, the FSIS has considerable discretion in how these tasks will be conducted. Where disparate proposals are being considered more than one MVT may be required at any given time.

Following the technical proposal review of a method, FSIS may determine that, based upon the submission of the data outlined in 5.4, enough data are provided to validate the method for its intended use without further laboratory review. However, if insufficient data are provided by the submitter, FSIS may request additional data and/or conduct an internal laboratory review. In-house testing of the method using appropriate matrices and organisms would determine if the technology/method is repeatable and meets the needs of FSIS. Specific criteria for the laboratory review will need to be developed by FSIS based upon the nature of the technology/method and its intended use. At this level of the review process, the submitter may be asked to provide necessary training, test kits, reagents and labor required to evaluate the technology/method in an FSIS laboratory. If sufficient multiple laboratory testing and method optimization/validation has already been done, the method may be recommended for acceptance and implementation as submitted.

Methods that have successfully passed the review process conducted by the TRC are those that show considerable promise for meeting the FSIS needs as defined in the method requirements and performance criteria. However, as it is quite unusual for laboratory testing methods to be "off-the-shelf" ready for use in a regulatory laboratory, the FSIS must collect data as to actual method performance under "real-world" conditions.

6.4.1. Performance Testing

Method performance testing should be conducted under controlled circumstances to prevent undue outside influence on the test results. The MVT would supervise performance testing and analyze data to determine if any or all of the methods meet the FSIS performance goals for the testing requirements. If multiple methods are to be considered, a number of approaches to method comparison might be taken, both within the FSIS' own laboratories and externally. One scenario might include a parallel comparison of methods conducted under the actual conditions used by the FSIS. Appropriate blinded samples representative of actual FSIS samples should be provided. The details of the actual testing protocol depend on the FSIS' goals for the method but the testing design could resemble that used by AOAC for performance tested methods.

6.4.2. Method Optimization/Validation

The evaluation of new technologies can be divided into three phases: 1) Selection; 2) Optimization and 3) Validation. These phases become increasingly more expensive as a method moves from selection to optimization to validation. In addition, adaptation of inappropriate methods would be

both expensive and potentially harmful to public health. Therefore, it is critical that appropriate *objective* processes be put in place to ensure that only optimized and validated methods that meet the performance and convenience criteria set by FSIS and that maximize public health go forward and are adopted.

Prior to adoption of a new microbiological testing technology, FSIS should first subject ALL potential new methods to Phase 1 – Selection. This phase includes reviewing: inputs from the Method Evaluation and Technical Review Committees ; Public Health Goals ; FSIS Microbiological Testing Objectives; the Criteria Checklist; and relevant paper and laboratory reviews. The two general types of criteria to consider when reviewing and evaluating new microbiological testing technologies are: 1) performance (efficacy) and 2) convenience (efficiency). These criteria should be evaluated in the context of FSIS's regulatory and public health objectives. To help in selection of new methods, FSIS should first prioritize and weight performance and convenience criteria using an objective mathematical formula developed and updated as needed by the Method Evaluation and Technical Review Committees.

6.4.2.1. Optimization

New methods that show the most promise of meeting the performance and convenience criteria and contributing to public health should proceed to Phase 2 – Optimization. After a new technology has been reviewed and selected by the TRC, it should be handed over to the MVT for Phase 2 – Optimization. Optimization is defined as the procedure or procedures used to make a system or design as effective or functional as possible. While a method's performance might be satisfactory for FSIS applications, it might not be totally suitable for implementation into the regulatory environment of the FSIS's own testing laboratories. The MVT will make appropriate adjusts to the method so that it will be compatible with normal laboratory operations. Such variables that might be considered may be sample volumes, incubation durations and incubation temperatures.

Given the many and often competing criteria that must be considered before adopting a new technology (accuracy, precision, sensitivity, specificity, reproducibility, speed, cost, etc.,) it will typically not be feasible to achieve maximum values for each criterion. The Committee recommends that the MEC take advantage of "optimization" computer software to aid in the optimization process. Prioritization, weighting and use of computer software will help ensure that the selected method will be truly optimized for its intended purpose. The optimization phase can be conducted at either FSIS or ARS laboratories.

6.4.2.2. Validation

If and when a new method has been optimized it is then necessary to subject it to Phase 3 – Validation. Method validation is defined as the process of verifying that a method is fit for purpose. The process of validation ensures that a new method meets the defined performance and convenience criteria when analyzing multiple samples of every type that FSIS analyzes. Once the method has been shown to perform adequately under FSIS regulatory laboratory conditions, a final method validation will be conducted by the MVT to assure that regulatory results will be supported by the appropriate scientific testing underpinnings. Thus the methods will be appropriate for regulatory use and supportable in legal proceedings.

In order for this critical phase to be performed correctly the Committee strongly recommends that these validation studies be conducted at FSIS laboratories by scientists that are familiar with FSIS's samples and testing needs and are specifically dedicated to new method validation. Personnel working in method validation at FSIS should include experts in microbiology, molecular biology and statistics. If such personnel are not currently available at FSIS for this purpose, then the Committee strongly encourages FSIS to recruit such personnel and organize them into an effective method validation team under appropriate leadership within FSIS.

Following the optimization and validation of the technology/method, the MVT will make specific recommendations on the acceptability and appropriateness of the method for use by FSIS to the MEC. Based upon these recommendations and those of the TRC, the MEC will provide a report to the proposal submitter.

7. Barriers and Research Gaps (Question 6)

The Committee identified barriers and research gaps which should be addressed as FSIS adopts new technologies to enhance public health.

1. There are three major barriers that need to be addressed as part of making newer and promising technologies an effective reality:
   - inadequate in-house methods development and validation capabilities at FSIS;
   - insufficient application and transparency of statistically-based sampling and analysis plans; and
   - limited data and methods harmonization and sharing across Federal agencies.

2. As the Committee evaluated technologies applicable for laboratory testing, it became apparent that portable user-friendly instrumentation for in-plant testing offered the potential for "real-time" monitoring of process control and pathogen detection. Although advanced on the spot detection methods are not ready for prime time, reduction in cultural enrichment time could be pursued now. At a minimum, research should be pursued to incorporate enrichment or DNA extraction of samples during transport and to develop shortened enrichment protocols to reduce analysis time.

3. The major barrier to the implementation of real-time detection methods is the need for pre-analytical sample preparation to compensate for (i) matrix-associated residual compounds which impact assay sensitivity, specificity, and limit of detection; and (ii) the need to test large sample sizes to account for uneven distribution and low levels of pathogen contamination. Methods to concentrate and purify the target agent(s) from the matrix, prior to detection are critical for achieving representative recovery and true real-time detection. This problem is not unique to food and environmental samples and continues to be a major impediment for the application of biotechnological methods in general.

4. An enrichment-related problem is the biased selection of strains that flourish in conventional media. The strain that predominates in current enrichment methods may not be the strain that is predominant in the natural setting. This barrier results in the potential for over-representation of one or more strains which may or may not be of public health

importance. For example, research is needed to understand the competitive dynamics between *Salmonella* serotypes in various enrichment environments.

5. A barrier to the regulatory adoption of enrichment-independent or non-culture-based detection methods is the need to confirm that the agent is viable and/or infectious. Although there are candidate methods (e.g. reverse transcriptase, fluorescent activated cell sorting) that can detect organisms without growth or enrichment, none of these methods has been validated to unequivocally confirm viability, as well as to provide other important public health information, e.g., strain subtyping and virulence. For regulatory action, however, it is beneficial to have a physical isolate to compare different isolates as well as demonstrate that an adulterant was indeed present. Development of a non-culture-based technology to reliably differentiate viable and non-viable agents is a research gap.

6. There are alternatives for molecular subtyping which may perform better than PFGE. To implement these technologies, they must be thoroughly evaluated and standardized. Development of alternative molecular subtyping methods is a research gap. A barrier to implementation is the necessary protocol standardization across agencies. Only then can such data be meaningfully interpreted for epidemiological purposes.

7. Every new detection method has its own set of strengths and weaknesses (see Table 4). The "ideal" method might include the following characteristics: rapid or real-time detection at a high degree of sensitivity and specificity; low limit of detection; simplicity and ease of use; cost efficacy; high throughput and reliability; the ability for multianalyte detection; adaptability to a wide variety of sample matrices; discrimination between viable and inactivated cells; production of enumerative data; portability; and simultaneous isolate characterization and subtyping. The absence of ideal methods that adequately fulfill all of these criteria is a formidable research gap.

8. Recommendations

1. NACMCF recommends that FSIS continue to clearly articulate measurable public health goals and microbiological testing objectives and integrate new technologies to achieve these goals and objectives.

2. To meet public health goals and FSIS' microbiological testing objectives, appropriate statistically-based sampling and analysis plans must be developed. The plans should address the required sample size to achieve statistical power, the frequency and process of sample collection in the field and in laboratories, microbiological criteria, and the final statistical analysis. Given the importance of statistical considerations and the fact that this Committee was specifically directed to not address statistical issues, the Committee recommends that NACMCF be charged to look at the statistical considerations as they relate to microbiological testing.

3. Diverse methods are used to collect data by multiple agencies. There is a need to harmonize methodologies and share data among agencies and other partners (industry,

academic) in the interest of improving public health. The Committee recommends continued collaboration between USDA, FDA, CDC, Federal agencies, state health departments, and relevant national and international entities. In addition, representatives from the scientific community (public health and epidemiology, veterinary and human medicine, agriculture and food science, among others) can help bring technologies to fruition in a timely manner.

4. The Committee is concerned that FSIS has no clearly defined mandate and limited infrastructure for method development and validation activities to support its public health regulatory program. This Committee disagrees with the current interpretation that methods development constitutes a research activity and therefore falls outside the FSIS mandate. Consequently, this Committee recommends that FSIS immediately assess the resource needs to conduct methods development and validation and seek funding for this effort, including in-house staff, facilities, equipment, and organizational structure necessary for successful implementation of appropriate technologies that will allow the Agency to meet its public health goals.

5. The creation of new testing methods that apply new technologies is a multi-disciplinary and resource intensive process. Stringent prerequisites must be met to take full advantage of state-of-the-art advancements in science and technology and the translation to the testing laboratory. To introduce, enhance, and maintain scientific expertise in methods development and implementation and/or to develop methods that address public health goals and microbiological testing objectives, the Committee recommends that FSIS devote resources to strengthen its laboratory research capabilities. For example, FSIS should:
   - initiate formal inter-governmental personnel agreements (IPA);
   - expand the FSIS Fellows program;
   - promote further collaboration with academia and the private and Federal sectors, through the USDA/ARS-FSIS liaison;
   - contract directly with appropriate private companies and academia through the Federal government's open and competitive process;
   - award cooperative agreement-type grants, administered through CSREES, either to principal investigators or Centers of Excellence (*e.g.*, academic or academic/industry consortia); and
   - negotiate Cooperative Research and Development Agreements (CRADA) between FSIS and commercial method developers.

6. The Committee recommends that FSIS adopt a systematic process to identify and evaluate new technologies that address FSIS' public health goals and microbiological testing objectives as discussed in Section 6. All methods should be evaluated against a set of previously established performance and efficiency criteria.

7. Safety cannot be tested into a food product, but must be built into prerequisite programs and HACCP systems by the food industry. Food processors can utilize new technologies/methods to enhance their food safety systems. Therefore, the Committee recommends that FSIS establish a mechanism for sharing new detection technologies with the food industry as they are validated and adopted by FSIS. The Committee further recommends the reciprocal

exchange of data and ideas between industry and regulators, which can lead to the application of improved methods that can enhance public health.

8. Some current and emerging detection platforms are quite good, provided the test analyte is stable, free of inhibitors, and present in adequate concentration in a sample of low volume. This situation is seldom the case for food and environmental samples (Research Gap No. 2) and in the opinion of the Committee, this is the ultimate limitation to the practical application of emerging technologies. Therefore NACMCF recommends broad-based multi-disciplinary research efforts that integrate pre-analytical sample processing technologies with advanced detection technologies to yield new methods that are adaptable to a wide variety of sample matrices. This recommendation could be achieved with a presidentially-directed task force with broad expertise to plan and implement a collection-to-detection initiative to:
    - Engage all relevant constituencies (*e.g.,* food, water, environmental, biological and chemical preparedness);
    - Identify high priority agents and/or matrices;
    - Identify relevant disciplines and experts for participation in the initiative (*e.g.,* microbiologists, food technologists, chemists, engineers, physicists, statisticians);
    - Develop a coordinated Federally funded initiative in pre-analytical sample processing with direct linkage to emerging detection platforms (*e.g.,* perhaps a centrally managed industry/academic/government consortium may be the ideal mechanism); and
    - Work within the mission of the initiative (or consortium) to develop relatively simple, inexpensive, and rapid pre-analytical sample processing methods that can be commercialized in the near term (3-5 years) and in an environment flexible enough to respond rapidly to both known and unknown agents or unexpected events.

9. Under certain circumstances, FSIS should consider accepting results based on stringently validated new technologies in the absence of cultured isolates. For agents that cannot be cultured, the agency should lay the groundwork to allow decision-making to occur in the absence of a viable isolate, with the ultimate goal of acceptance of these new detection and typing methods as equivalent to cultural methods. The Committee recommends that these issues and their ramifications be carefully considered before adoption of new technologies.

10. Enumeration of foodborne pathogens and indicator organisms using real-time molecular methods would accelerate the evaluation of control strategies and provide quantitative data to support risk assessment. Therefore, the Committee recommends that such new technologies be given priority for adoption by FSIS.

11. Microarray and/or SNP analyses, while promising for genotyping and subtyping applications, are not yet practical for detection although they are relevant for molecular epidemiological purposes. The Committee recognizes the importance of this issue and therefore recommends that evaluating new genotyping and subtyping technologies should be a potential future NACMCF charge.

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10. APPENDICES

10.1. Glossary of terms

The definitions below apply to this document.

Term	Definition
Accuracy	The closeness of agreement between a measured value and the accepted "true" or reference value.
Adaptability	The applicability of an assay to various matrices and testing situations; ie: food, environmental and clinical samples.
Amplification	A step or procedure that either increases the quantity of the analyte or enhances the signal resulting from the analyte's presence.

Analyte	The specific organism or chemical substance sought or determined in a sample.
Assay	The specific analytical component of a method that is used to detect a specific analyte.
Clone	A strain or group of strains descended asexually from a single ancestral cell (source strain) that has identical or similar phenotypes or genotypes as identified by a specific strain typing method.
Confirmation	The unambiguous substantiation of an analyte's presence by comparison to a standard or reference culture.
Detection	The act of discovering or determining the presence of a specific microorganism in a sample. Note that this may apply to the detection of nonviable cells by a non-culture-based method.
Epidemic	One or more outbreaks caused by an epidemic clone that survives and spreads over a long period of time.
False Negative	A test result that wrongly determines that an analyte is absent.
False Negative Rate	The ratio of false negatives found divided by true positives present, expressed as a percentage.
False Positive	A test result that wrongly determines that an analyte is present.
False Positive Rate	The ratio of false positives found divided by the number of true negatives present, expressed as a percentage.
Fluorophore	A tag or marker that generates a fluorescent signal.
Format	The material form or layout of a platform.
Generalizability	The ability to apply inferences drawn from a sample to the population from which the sample is drawn.
Genotyping	Testing to determine the complete genetic constitution of an organism or group, as determined by the specific combination and location of the genes on the chromosomes.
Gold Standard	A reference method, to which candidate procedures are compared.
Identification	The process of determining that a viable microbial isolate belongs to one of the established, named taxa.
Indicator Organism	A non-pathogenic microorganism that may be naturally present in food or water, which is used to indicate a state or condition suggesting the presence of a pathogenic microorganism.
Isolate	A population of microbial cells in pure culture derived from a single colony on an isolation plate.
Limit of Detection	The lowest amount of analyte that can be reliably observed or found in the sample matrix by the method used. Limit of detection is matrix- and analyte-dependent
Matrix	The substrate of a test sample.
Method	A body of pre-analytical and analytical procedures and techniques for performing an activity (e.g., sampling, analysis, quantification), systematically presented in the order they are to be executed.
Nanotechnology	A field that focuses on control of matter on an atomic and molecular scale.
New Technology	A technology that has not existed previously, or that is being applied in a novel way.
Outbreak	An acute appearance of a cluster of an illness that occurs in numbers in excess

	of what is expected for that time and place. In the case of a foodborne outbreak, the source is often a specific food vehicle that contains one specific outbreak clone.
Platform	The physical surface or structure to which a technology or technologies is/are applied.
Precision	The closeness of agreement between independent test results obtained under stipulated conditions.
PR-HACCP	An adaptation of HACCP intended to achieve reduction of the incidence of a particular pathogen in food.
Pyrosequencing	A DNA sequencing technique in which complementary strands are synthesized and nucleotide sequences are determined by the pyrophospate released during the addition of the nucleotide base.
Quality Assurance	Those systematic activities, defined by management, that are done outside of the actual analysis to provide confidence that the analysis will satisfy given requirements for quality. Examples of these activities include training, audit and review.
Quality Control	Those activities that are performed during the analysis to fulfill the requirements for assuring quality. Examples include control charting, blank determinations, spiked samples, repeat determinations and blind samples.
Recovery	The amount of analyte quantified by the analytical method, expressed as a percentage of the amount known to be present in the sample.
Repeatability	The measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
Reproducibility	The measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
Ruggedness	The ability of an analytical procedure to resist changes in results when subjected to minor changes in environmental and procedural variables, laboratories, personnel, etc.
Sample	Any material brought into the laboratory for analysis.
Sample Preparation or Processing	The process of obtaining a representative test portion from the sample which includes selecting a sub-sample(s) and in-laboratory processing (e.g., mixing, reducing, coring, quartering, blending, and grinding).
Sampling	A procedure whereby a part of a substance, material or product is taken to be used for testing or calibration as a representative sample of the whole. In some cases, such as forensic analysis, the sample may not be representative but is determined by availability. The term refers both to the statistical methods used to determine which and how many samples to test in order to represent a larger amount of product, and to the technical methods used to collect, preserve and process that sample for microbiological testing.
Screening Method	A method designed to detect the presence of an analyte in a sample at or above some specified concentration (target level). Screening-method results are usually reported as yes/no values.
Selectivity	The extent to which the analytical method can determine particular analyte(s)

	in a complex mixture without interference from the other components in the mixture. The probability that the method will classify a test sample as negative, given that a test sample is a known negative. A method that is perfectly selective for an analyte or a group of analytes is said to be specific.
Sensitivity	The probability that the method will classify a test sample as positive given that a test sample is a known positive. Analytical Sensitivity, also known as Limit of Detection (LOD), represents the smallest amount of an analyte in a sample that can be accurately measured by a platform or assay. Diagnostic Sensitivity is the probability of detecting an analytical target (i.e., pathogen, toxin) in a sample from a population of samples (i.e., a production lot) which is contaminated.
Specificity	A performance characteristic that judges the ability of a laboratory test method to exclude non-target analytes in chosen matrices, whereby the method will classify a test sample as negative, given that the test sample is a known negative. Analytical Specificity is defined as the ability of an assay to exclusively identify a target rather than other similar analytes in a sample. Diagnostic Specificity is defined as the probability that the sample tests negative when the pathogen is absent from the sampled population.
Strain	An isolate or group of isolates exhibiting phenotypic and/or genotypic traits that are distinctive from those of other isolates.
Subtype	A specific pattern, or set of marker scores, displayed by a strain upon application of a particular typing system.
Technology	A capability given by the practical application of knowledge, specifically, the method and material used to attain a microbiological testing objective .
Test	A technical operation that consists of the determination of one or more characteristics or the performance of a given product, material, equipment, organism, physical phenomenon, process or service according to a specified procedure.
Test Method	Specified technical procedure for performing a test.
Test Portion	The actual material weighed or measured for the analysis
Test Sample	Material prepared from the laboratory sample and from which test portions will be taken.
Throughput	The volume of samples that an assay can process.
Validation	Establishment, by systematic laboratory studies, that the performance characteristics of the method meet the specifications related to the intended use of the analytical results.
Validity	Validity is a measure of the ability of the test to do what it is intended to do under specific conditions of use, i.e., to detect the organism(s) of interest if it is present, and not to detect it if it is absent. The two major measures of validity are sensitivity and specificity.
Verification	Confirmation, through the provision of objective evidence, that specified requirements have been fulfilled.
Viability	Ability of an organism to multiply in culture or in a matrix.

1

10.2. Details about FSIS Testing Protocols. FSIS method protocols currently report foodborne pathogens using the following criteria:

Salmonella spp.

- Non-Typhi/Paratyphi *Salmonella* strains are not necessarily detected (*i.e.*, the MLG 4.x method does not provide sensitive detection of *Salmonella* strains that are not typically harbored by food animals or non-*S. enterica* species that are not implicated in human foodborne illness)
- Atypical hydrogen sulfide-negative strains are detected and identified.
- Traditional biochemical and serological definitions are applied
- Genetic criteria are currently not applied

Listeria monocytogenes

- ∃-hemolytic *L. monocytogenes* strains are detected (*i.e.*, non-hemolytic strains are not detected but are rare and generally regarded as having attenuated virulence potential).
- Genetic criteria, serology and virulence capability testing is currently not applied in FSIS methodology.

E. coli O157:H7

- Isolates that are biochemically confirmed as "*E. coli*", serologically or genetically positive for "O157", and positive for either of the following criteria are reported by FSIS as "*E. coli* O157:H7":
  - genetically confirmed as "H7", or
  - serologically confirmed for Shiga Toxin production, or harbor a gene sequence associated with Shiga Toxin capability.

TABLE B-1. *PR-HACCP* Salmonella *carcass testing conducted by FSIS laboratories*

Carcass	Sampling method and test portion	Category 1- Maximum *Sal*+ samples	Category 2- Maximum *Sal*+ samples
Heifer/Steer	3-site sponge 300 cm^2 total or 60cm^2 excision	0 of 82	1 of 82
Cow/Bull	3-site sponge 300 cm^2 total or 60cm^2 excision	1 of 58	2 of 58
Market hog	3-site sponge 300 cm^2 total or 60cm^2 excision	3 of 55	6 of 55
Chicken	Whole carcass 400 ml rinse with 30 ml tested	6 of 51	12 of 51
Young turkey[a]	2-site sponge 100 cm^2 total	7 of 56	13 of 56
Goose[a]	2-site sponge 100 cm^2 total	5 of 54	9 of 54

[a] New for 2006

Table B-2. PR-HACCP Raw Ground Product *Salmonella* Testing

Commodity	Test portion	Category 1- Maximum *Salmonella*+ samples allowed	Category 2- Maximum *Salmonella*+ samples allowed
Raw Ground Chicken	25-g	13 of 53	26 of 53
Raw Ground Turkey	25-g	15 of 53	29 of 53
Raw Ground Beef	25-g	3 of 53	5 of 53

Table B-3. "Zero Tolerance" Verification Testing Conducted by FSIS Laboratories for Domestic and Imported Products

Commodity	Pathogen	Test Portion
Raw ground beef	*E. coli* O157:H7	Five individually analyzed 65-g portions (*i.e.*, 325 g total)
Raw ground beef components	*E. coli* O157:H7	Five individually analyzed 65-g portions (*i.e.*, 325 g total)
Ready-To-Eat (RTE) products (all except commercially sterile products)	*Listeria monocytogenes*	25 g
Ready-To-Eat (RTE) products (all except commercially sterile products)	*Salmonella* spp.	325 g
Certain RTE (i.e., Dried/semi-dried Fermented sausages and cooked meat patties)	*E. coli* O157:H7	Five individually analyzed 65-g portions (*i.e.*, 325 g total)
Food contact surfaces (FCS) in RTE establishments	*Listeria monocytogenes*	Sponge sample representing variably-sized surface area.