RESEARCH R&KM KNOWLEDGE MANAGEMENT

Shiga Toxin-Producing *E. coli* (STEC) Methodology Think Tank

MEETING SUMMARY
Beef Safety Research

On May 20, 2008, at the Doubletree Hotel O'Hare in Chicago, a group of 26 invited experts representing the beef industry, including diagnostic test kit manufacturers, contract laboratories, processors, government and academia, participated in a one-day workshop to discuss laboratory methodology related to the detection and characterization of Shiga toxin-producing Escherichia coli (STEC) in beef products. Emphasis was placed on non-O157 STEC. Considerable scientific uncertainty exists in defining the molecular characteristics associated with pathogenicity and the interpretation of food safety risk associated with this heterogeneous group of organisms. Standardized tests are not available for their detection, or for methodological comparisons. Criteria for assay validation, especially in ground beef products, are warranted.

Discussion Notes

Although the primary sources of human exposure to STEC other than serotype O157 (non-O157) in the United States remains poorly described, available data demonstrate that non-O157 STEC infections are increasingly being diagnosed in the United States. Moreover, available data probably represent a significant amount of undereporting. Unfortunately, the clinical diagnosis and the tracking of possible sources of non-O157 STEC infections is hindered by the lack of sensitive and specific tests capable of identifying pathogenic STEC. Non-O157 STEC are a heterogeneous group of organisms- some strains have the ability to cause disease in humans while other members of this large group of organisms do not appear as significant causes of human illness despite harboring one or more of the variants of the Shiga toxin gene (stx). Considerable scientific uncertainty exists surrounding the exact combination(s) of virulence factors that are required to confer pathogenicity to Shiga toxin-producing *E. coli*. In the absence of additional scientific data, specific guidelines that precisely define STEC of public health significance will not be available. The Health and Human Services Healthy People 2010 food safety objective as it relates to non-O157 STEC is to reduce the prevalence of postdiarrheal hemolytic uremic syndrome (HUS) in children less than 5 years of age. Non-O157 STEC contribute minimally to the overall incidence of this disease. Moreover, the extent that foods of bovine origin specifically contribute to morbidity related to non-O157 illnesses in the United States is unknown. No outbreaks have yet to be traced to beef in the United States. Regardless, given the ecology of the organisms, the beef industry is pro-active and interested in preventing problems from emerging.

To complicate matters further, presently no single, generally accepted method or "Gold Standard" is available for STEC detection. The reason for the lack of standardized detection methodology stems partially from the scientific ambiguity surrounding the exact molecular target(s) of public health significance. The group was advised that recommended culture procedures for the detection of STEC from human clinical specimens will be forthcoming from the Centers for Disease Control and Prevention (CDC). The focus of these recommendations will undoubtedly be on the detection of this group of organisms in human stool specimens. Given differences in sample matrix, organism numbers and background flora, the applicability of these methods to detect STEC in beef products will require additional work.

Despite these critical limitations required for science-based food safety risk assessment, the Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA) will undertake a survey of U.S. beef products to ascertain the prevalence of STEC encoding the eae gene and belonging to the six "O" serogroups most frequently reported

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from human illnesses in the United States (O26, O111, O103, O145, O121, O45). Dr. Pina Fratamico (Eastern Regional Research Center (ERRC), Agricultural Research Service (ARS), USDA) is charged with developing the methodology for this survey. Dr. Fratamico briefly provided an overview of the proposed diagnostic procedure as outlined below:

1. Sample enrichment in broth
2. PCR detection for stx_1 and stx_2 and eae
3. Broth cultures positive for both stx and eae will be subjected to PCR amplification of "O" genes (wzx or wzy) specific for the abovementioned serotypes.
4. Attempts will be made to isolate colonies of stx-positive, eae-positive, *E. coli* of the serogroups indicated by the wzx and wzy PCR reactions. Methods that might be employed include, but are not necessarily limited to, hybridization, immunomagnetic separation, selective agar, and immunoblots.

Dr. Fratamico noted that at this stage, many of the specific details related to laboratory methodology for detection have not been finalized. A large number of factors impact assay sensitivity and limit of detection. Of expressed concern to the group was the magnitude and diversity of background microflora present in the sample. Workshop participants voiced concern about the need to validate the methods to be employed, with specific emphasis on the following:

1. Product to enrichment ratio
2. Type of product to be used (ground beef, trim)
3. The analytical unit (weight) of sample
4. Pre-warming of media, product temperature, and enrichment temperature
5. Type of enrichment media
6. Duration of enrichment
7. Effect of initial inoculum dose on sensitivity.

Other significant limitations of the proposed assay that were discussed included the possibility that the PCR screening would identify gene targets that were not necessarily originating from the same (single) organism. Shiga toxin gene may be detected even if it is not part of a bacterial genome. Free DNA or, more likely, phage DNA also encodes Shiga toxins. Unpublished data shared by participants indicate that many of the STEC in bovine fecal samples and beef samples (7%) encode only Shiga toxin, and no other virulence genes. In a similar fashion, organisms are in ground beef that encode eae (7%) and not stx. Only 2% of broths tested were positive for eae and stx both, with less than 1% of samples likely to be generating positive PCR signals from the same cell. Thus, it is expected that the initial screening for these genes will yield large numbers of broth cultures passing the first screen for STEC.

Finally, both of these genes are frequently found in *E. coli* belonging to serogroups other than the 6 serogroups in question. Preliminary evaluation of unpublished data (http://www.fsis.usda.gov/PDF/Non-0157_STEC_Koohmaraie.pdf) indicate that although 26% of ground beef samples test positive for *stx*, only 6% are among the most important serogroups associated with human illness, and less than 2% of isolates contain serogroups of concern that are Shiga toxin-positive. Less than $1/10^{th}$ of 1% of 3668 samples yielded isolates that were among the "top 6", and both *eae* and *stx*-positive.

The isolation of colonies from broth cultures positive by PCR poses additional challenges. Typically, recovery of isolates from PCR-positive broths is poor (<50%). Because of the aforementioned possibility of genes originating from different organisms, it seems as if isolation of individual colonies is critical. Participants were interested in learning how the final diagnosis of positive samples would be determined. What would be the interpretation of lots of beef that were positive by PCR for the abovementioned genetic targets, but from which no isolates could be recovered? Would those samples be considered positive?

The PCR portions of the testing methodology can be performed within hours. However, the initial broth enrichment and the recovery of isolates will extend the procedure to several days (3-5) at minimum. Because of the perishability of the product in question, rapid diagnostic testing is critical. Unfortunately, the proposed procedure is extremely time consuming and extended product holding before test results are known would have detrimental effects to product freshness and shelf-life.

The development of more rapid diagnostic tests is hindered by the lack of consensus of the specific targets that need to be identified and the required detection limit threshold. The latter is being dictated by the analytical sample size, microbiological/immunological sensitivity, and the sampling strategy. Issues surrounding sampling strategy were identified as being of paramount importance, but were intentionally not discussed at this meeting. Pragmatically, development of commercial tests will be driven by the beef industry's needs and the anticipated volume of use. The beef industry's needs will be dictated, in part, by regulatory requirements for testing. Research and development of new technology is an expensive investment. Test manufacturers will weigh the costs

(research and development) and benefits (marketability) prior to developing commercial tests. However, limited commercial test development is likely to proceed until such time that the specific food safety targets are identified and agreed upon.

Regardless of the ultimate methodology adopted for this proposed FSIS study and future sampling strategies, the need for validation of microbiological methods is vital. In the absence of a "Gold Standard" methodology, making guidelines available which describe specific criteria that should be examined prior to accepting a specific test would be valuable to all parties. Guidelines (but not specific requirements) for validation of assays may include suggestions for temperature of product and negative and positive control organisms to be used.

Clearly, it is in the best interest of the beef industry and the public to have multiple tests of comparable sensitivity to have cross-referencing capabilities in assays. Ideally, test validation should be performed by an unbiased third party where possible. Those comparisons of methods that have been published in peer-reviewed scientific literature and are readily available are ideal. Providing guidelines will allow individual companies to assess the validity of novel diagnostic tests and improve on current tests. This is particularly important for small companies that would like to test for specific pathogens but do not have the scientific resources in house to critically evaluate assay validity.

Validation is available through organizations such as Association of Official Analytical Chemists (AOAC, Gaithersburg, MD). AOAC has several validation programs: 1) *PEER-VERIFIED*SM METHODS; 2) the *OFFICIAL METHODS OF ANALYSIS*SM PROGRAM (OMA) and 3) *PERFORMANCE TESTED*SM METHODS administered by the AOAC Research Institute. According to the AOAC website, The *PEER-VERIFIED*SM METHODS is for rapid validation for nonproprietary methods. The *PERFORMANCE TESTED*SM METHODS is for rapid validation of commercial proprietary methods and the OMA is for either commercial or proprietary methods where a high degree of confidence is required. Additional information about these programs can be found on the company's web site (http://www.aoac.org/vmeth/page1.htm). *PERFORMANCE TESTED*SM METHODS are reviewed annually. Any changes to the tested protocol, either in instructions or performance, are evaluated by AOAC and a determination as to whether the entire test needs re-evaluation is made.

Screening product by microbiological testing may identify occasional lots of beef that are contaminated with *E. coli* O157, but as a whole this strategy by itself is inadequate to ensure complete freedom from the organism in processed lots of beef. Instead, product screening has had significant *indirect* impact on public health. Baseline prevalence screening for *E. coli* O157 has driven changes in post-harvest intervention technology to reduce levels of contamination to less and less frequent sporadic events. Based on what is known about non-O157 STEC, it is predicted that the current interventions targeted at *E. coli* O157 are equally effective at reducing non-O157 STEC. Thus, it is unclear what additional interventions that are not already in place, may be suggested to producers to further combat non-O157 STEC if they are detected in product.

Testing by USDA for O157:H7 in imported ground beef has recently increased. Testing for non-O157 may have important food safety concerns in some products. For example, in imported beef derived from animals originating from areas where *E. coli* O157 is uncommon or absent, then it would be more logical to screen for non-O157 *E. coli* to determine the likelihood of contamination with unwanted organisms.

Additional discussion concerning the methodology limit for detection of generic *E. coli*, *E. coli* O157 and other non-O157 STEC centered on the food safety objectives, and subsequent public health impacts, the testing procedure is trying to achieve. The beef industry strives to provide a wholesome and safe product to customers. Generic *E. coli* testing provides processors with an indication of process control. However, these numbers do not correlate with the incidence of *E. coli* O157- probably because *E. coli* O157 contamination is sporadic and the fact that sensitivity of detection changes dependant upon the level of background flora.

A risk-based minimal detection limit, one that allows assay results to be translated to management interventions to reduce adverse human health impacts, would best serve the public. Given the gaps in knowledge of *E. coli* O157 and other non-O157 STEC, these criteria are not available. Currently, the limit of *E. coli* O157 detection has been based on the analytical capacity (65 g) at the time of assay development. The current test for *E. coli* O157 is performed on five, 65 g sub-samples, for a total of 325 g of meat. Highly selective media are available for the detection of *E. coli* O157. The reported limit of detection for this assay is 0.23 colony forming units (cfu) /g in a 25 g sample of 75/25 (lean/fat) ground beef. Below this level, as may be the case in naturally contaminated samples, the sensitivity of detection is decreased. The sensitivity of detection of non-O157 in ground beef using the proposed FSIS method, or any method for that matter, is unknown.

Selective media is not available for all non-O157 serogroups. Nevertheless, since some non-O157 strains are equally hazardous as some O157 strains, it is desirable to have detection methods as equally sensitive for the detection of this group of organisms as is currently available for *E. coli* O157. This will require additional research and considerable assay validation. Given the heterogeneity of isolates belonging to the non-O157 group of organisms, the idea of focusing attention to the next most important serogroup of non-O157 (O111) was considered as an alternative to trying to tackle all serogroups at once.

Critical Gaps in Knowledge & Resources

1. Specific molecular characteristic associated with pathogenic STEC is needed for:
    a. Developing standardized, rapid, and specific diagnostic tests
    b. Determining the food safety risks associated with beef contaminated with non-O157 STEC.
2. Better description of non-O157 STEC in cattle and beef.
3. Clearer understanding of the role of beef as a vehicle of human non-O157 infections.
4. Funding to support research within ARS as well as in other programs with research funding.

Meeting Recommendations

1. To assist in the standardization of assays, it was decided that access to a collection of pre-determined isolates (O157 and non-O157 strains), with rationale for inclusion in the group, would be helpful for the assay validation. All interested parties should have access to the strain collection.
2. Evidence of the beef industry's proactive approach to prevent non-O157 STEC from becoming a public health concern should be made available.
3. Industry partners have a wealth of information concerning the practical considerations of pathogen detection in beef products. Numerous individuals and companies should be asked for suggestions to complement the government's molecular approach in assay development.
4. Research funding agencies such as USDA, ARS and NRI should be asked to prioritize research in this area. For example, despite this novel, monumental task facing Dr. Fratamico's laboratory, no additional funding has been made available for her research. Likewise, *E. coli* O157 in beef or cattle has not been a research priority for USDA's NRI Food Safety Program (32.0) since 2006.

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