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Overview of Performance Standards for Biological Threat Agent Assays for Department of Defense Applications

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History of AOAC INTERNATIONAL Involvement with Biological Threat Agents

There has been a proliferation in the development of biological threat agent detection technologies for use in the field by first responders and private-sec-tor end-users as well as in Department of Defense (DoD) applications in which active combat may be occurring and in other parts of the world. In contrast to the proliferation of detection methodology, there has been a lack of standards defining the required performance of these technologies. Standards are necessary to demonstrate the performance and limitations of the tools, providing confidence in the data to allow appropriate response actions by end-users and responders. In the past, the Department of Homeland Security (DHS), Science and Technology Directorate, funded AOAC to develop standards and perform conformity assessment under three efforts. The first effort began in 2003 to evaluate the performance of lateral flow immunoassay devices used by first responders to screen suspicious powders for *Bacillus anthracis* spores. These devices are colloquially known as “handheld assays” and are frequently referred to as “HHAs.” AOAC formed the Task Force on *Bacillus anthracis* (TFBA), which created a specific set of consensus performance criteria and test protocols (i.e., standards). Five HHA manufacturers submitted their technologies to AOAC so that third-party laboratories could evaluate the tools against the criteria (1).

Based on lessons learned from the 2003 project, DHS funded AOAC in 2006 to facilitate the development of a second round of consensus performance criteria for HHAs (2). AOAC organized a second consensus body, known as the Working Group on Standards for Hand-Held Assays, to revisit the performance criteria developed by TFBA and to develop performance criteria for HHAs that screen suspicious powders for ricin. Like the first effort, AOAC was tasked to evaluate commercially available HHAs against the criteria; however, this second effort was not completed because of a reorganization of DHS at the time. In 2007, in a third effort, DHS refocused AOAC to develop performance criteria for the detection of aerosolized *B. anthracis*, *Yersinia pestis*, and *Francisella tularensis* by PCR assays. AOAC formed the Stakeholder Panel on Agent Detection Assays (SPADA) to organize and oversee this refocused project (3).

As part of the DHS projects, AOAC developed a new standard setting process with standards known as *Standard Method Performance Requirements* (SMPRs). Prior to the DHS project, AOAC’s practice in conformity assessment was always to assign a study director to collect validation data on a candidate method, and then assign an appropriate committee of volunteer experts to judge whether the data demonstrate that the candidate method is adequate to be approved as an AOAC *Official Methods of Analysis*^{SM}. In this model, only general criteria are used to determine acceptability, such as “equivalent to or better than the reference method.” With the DHS project, AOAC began the practice of creating predetermined minimum acceptance criteria for analytical performance of methods that detect biological threat agents. The practice has become routine and has expanded into other analytical method areas (4).

Deputy Under Secretary of The Army (DUSA) Biological Threat Agent Method Standards Project

The DoD Chemical and Biological Defense Program Test & Evaluation (T&E) Executive established a group known as the Test and Evaluation Capabilities and Methodologies Integrated Process Team (TECMIPT). TECMIPT's mission is to provide technical recommendations for T&E strategies, identify T&E capability gaps, and develop community consensus T&E standards. TECMIPT is organized by commodity areas, known as Commodity Area Process Action Teams (CAPATs). There are eight CAPATS: Advanced Threat, Chemical Detection, Biological Surveillance, Modeling and Simulation, Individual Protection, Collective Protection, Decontamination, and Radiological/Nuclear Defense.

The Biological Surveillance CAPAT identified a significant gap and a need to develop performance requirements for biological threat agent assays and, more specifically, a significant need to develop appropriate inclusivity and exclusivity test panels (see Biological Threat Agent SMPR Terms and Concepts for further explanation) for the evaluation of biological threat agent assays. In July 2014, SPA-DA was identified as an asset capable of developing performance requirements for biological threat assays. Subsequently, a project was initiated in November 2014 under the direction of the DUSA Test and Evaluation (DUSA-TE).

Initially, DUSA-TE identified three biological threat agents for SMPR development: *Coxiella Burnetii*, *Staphylococcus enterotoxin B* (SEB), and Venezuelan Equine Encephalitis Virus (VEEV). The following three additional threat agents were added to the list in early 2015: *Bacillus anthracis*, *Francisella tularensis*, and *Yersina pestis*. The list of threat agents was expanded again in 2015 to include *Burkholderia pseudomallei*, *Variola* virus DNA, Botulinum neurotoxins, and *Brucella suis*. Four of the agents identified by DUSA-TE (*Bacillus anthracis*, *Francisella tularensis*, *Yersina pestis*, and *Burkholderia* species) were previous subjects of AOAC SMPRs. However, of these four biological threat agents, only an SMPR for *Bacillus anthracis* was published.

A primary goal of the DUSA-TE project was to create SMPRs for the complete set of 10 biological threat agents. Technology, especially PCR methodology, has advanced significantly in recent years. Method evaluators now have a broader body of knowledge to draw from regarding method evaluation. Therefore, DUSA-TE tasked SPADA to review the four previously developed SMPRs with the goal of updating them based on knowledge gained.

Another primary goal of the DUSA-TE project was to consider all the performance requirements in terms of DoD applications. Previous projects were funded by and focused on DHS scenarios, which included domestic (within the United States) venues such as mail sorting systems, mass transit sites, public spaces, sports stadiums, etc. DoD applications shifted the focus from domestic to international sites and added combat and hostile-site venues. This application necessitated adding potential combat-related interferents, such as explosive powders, to the environmental factors study.

A secondary goal of the project was to reconsider whether the number of strains and species identified for evaluation could be streamlined based on years of experience of real-life evaluations carried out since 2005.

The DUSA-TE project of developing, adopting, and publishing 10 SMPRs was completed in October 2017. Another project reconsidered the environmental organisms specified in the environmental factors study, and it was added in October 2016 and completed in March 2017.

SPADA

All biological threat agent method SMPRs are developed under the supervision of SPADA. SPADA is a voluntary consensus standards body of volunteers that is organized, managed, and administered by AOAC. It is constructed to represent the interests of various federal, state, and local governments, first responders, public health institutions, and industry. Federal agencies interested in monitoring biological threat agents include the DHS, DoD, Federal Bureau of Investigation (FBI), Centers for Disease Control and Prevention (CDC), and the U.S. Environmental Protection Agency. SPADA's mission is to establish method performance requirements through SMPRs. When active, SPADA meets two to four times per year to deliberate on the different methodological needs of the biological threat agent detection community.

To accomplish its work, SPADA is organized into a series of working groups that develop SMPRs and carry out other technical activities. Twelve working groups were commissioned for the DUSA-TE project. One working group was created for each of the 10 DUSA-TE identified biological threat agents, plus additional working groups were created to consider potential interferents and to review the environmental organisms specified in the environmental factors study.

Working groups performed the challenging task of translating the methodological needs of the biological threat agent detection community into concise SMPRs. Each working group is chaired by a subject matter expert and consists of 10 to 20 other subject matter experts. The working groups are ad hoc and adjourn sine die when their missions are completed. The mission of most working groups is to develop the necessary standards using the best available science while balancing the need to keep testing against the standards practical. The primary tasks of most working groups are to determine the method performance requirements and identify inclusivity and exclusivity species and/or strains. Ancillary working groups are sometimes organized to address additional issues in support of the main mission of developing SMPRs.

Draft SMPRs (and other recommendations) are presented to the entire assembly of SPADA for review during formal SPADA meetings. All SPADA members are permitted to comment on the draft standards and recommendations. However, a subset of 20 to 22 members are designated by AOAC as voting members. Voting members are carefully selected by AOAC to ensure a balance of stakeholder perspectives. The adoption of SMPRs and/or other actions requires a super-majority of greater than 66% of the designated voting members.

Biological Threat Agent SMPR Terms and Concepts

A standard set of terms and performance parameters were developed for biological threat agent method SMPRs and published in 2011 (5). This standardized approach has worked well to maintain consistency from one SMPR to the next. These terms and parameters are as follows.

(**a**) *Probability of detection (POD).*—POD is the proportion of positive analytical outcomes for a qualitative method for a given matrix at a given agent level or concentration. POD is concentration-dependent (6).

(**b**) *Acceptable minimum detection level (AMDL).*—AMDL is the predetermined minimum level of a biological threat agent that must be detected by a candidate method with an estimated 5% lower confidence limit on the POD of 0.95 or higher.

(**c**) *POD in the absence of analyte.*—This is designated as $POD_{(0)}$ and is the rate of positive results in a population of known negative test portions. $APOD_{(0)}$ can be calculated from both the single-laboratory studies and the collaborative study results. $APOD_{(0)}$ calculated from pooled valid collaborative data is termed $CPOD_{(0)}$.

(**d**) *Inclusivity.*—Strains, isolates, or variants of the target agent(s) that the method can detect. Each SMPR contains a list of strains or variants called the inclusivity panel that the assay must be able to detect.

(**e**) *Exclusivity.*—Nontarget agents, which are potentially cross-reactive, that must not be detected by the method. Exclusivity is determined by testing near-neighbor species (or other molecules) listed in the SMPR's exclusivity panel.

(**f**) *Environmental interference.*—The ability of the assay to detect the target agent in the presence of nontarget environmental organisms or substances. Environmental interference is evaluated using a variety of nontarget organisms and/or substances listed in an environmental factors panel developed for each class of assays. These may include air and soil background organisms, DNA viruses, microbial eukaryotes, higher eukaryotes (freshwater amoebae, fungi, arthropods, mammals, avian, plants), soil samples, biological insecticides, powders and chemicals, and swab materials. The environmental factors panel varies depending on the technology and intended use of the method.

SMPRs

Standard Method Performance Requirements for DNA-Based Methods of Detecting Bacillus anthracis in Field-Deployable, Department of Defense Aerosol Collection Devices, AOAC SMPR 2016.006 (7)

B. anthracis is a Gram-positive, spore-forming rod and the causative agent of anthrax. The CDC classifies *B. anthracis* as a Category A bioterrorism agent, and because of the history of intentional dissemination through the postal system in October 2001, this agent was given high priority for development of SMPRs.

Similar projects in 2004 and 2007 preceded this project and served as a starting point for the DoD initiative. The 2004 project developed acceptance criteria for immunological "hand-held assays" for detection of *B. anthracis* spores. A year-long discussion was required by the federal agencies and other SPADA members to develop consensus on the inclusivity and exclusivity panels for *B. anthracis*. The main issue was to develop an inclusivity panel that adequately represented the genetic diversity of *B. anthracis*. AOAC normally requires 50 representatives of a species for inclusivity testing. In the case of *B. anthracis*, SPADA recognized that this species is relatively homogeneous and that 15 inclusivity strains could adequately represent its genetic diversity based on variable number tandem repeat analysis. The number of inclusivity strains was further reduced to 14 in 2016.

The original exclusivity panel developed in 2004 consisted of 20 close relatives of *B. anthracis*, including representatives of five *Bacillus* species. *B. cereus* and *B. thuringiensis* are the closest relatives and represent the largest analytical challenge. The number of exclusivity panel members was also reduced from 20 to 14 species in the current 2016 SMPR.

The concept of AMDL for qualitative methods was first developed for *B. anthracis* PCR methods. The AMDL is not necessarily the lowest concentration that a method can detect, but it is instead a low level at which a method is required to reliably detect the analyte (in this case, *B. anthracis)*. The AMDL is composed of an analyte level (mass or concentration), a detection requirement, and a level of confidence.

For *B. anthracis* detection in aerosol collection samples, SPADA set the AMDL at 20,000 spores per filter or 2000 spores/mL buffer, balancing the practical capabilities of PCR methods and the needs of the testing community. SPADA also determined that the detection rate, known as the POD, at the AMDL should be at least 95%, but more specifically, the 5% lower confidence limit on the POD should be 0.95 or higher to represent the requirements of the testing community. This acceptance criterion can be met by testing 96 replicate test portions containing the analyte at the AMDL with no more than one failure. There are other testing schemes that can meet the POD ≥ 0.95, but a discussion of other testing schemes is beyond the scope of this document.

Each strain identified in the inclusivity panel is tested once at the AMDL. If the candidate method detects the strain, it passes the test. If the candidate method does not detect the inclusivity strain, then a further 96 replicates are tested, and the candidate method must detect all 96 replicates to meet the acceptance criterion, therefore demonstrating a POD ≥ 0.95 at the AMDL.

Conversely, near neighbors must meet an acceptance criterion of a 95% upper confidence limit on the POD of 0.05 or lower. In other words, the detection rate or POD for exclusivity panel members should be less than 5% (detection is unwanted because these are not target species) with a 95% one-sided confidence limit. Exclusivity species are tested at a concentration of 10 times the AMDL. One replicate of each strain is tested initially. If the candidate method detects an exclusivity panel member, then 96 additional replicates are tested with no additional failures to meet the acceptance criterion.

In addition to the inclusivity and exclusivity panels, an environmental factors study was developed to test for assay interference, both positive (cross-re-activity) and negative (inhibition of analyte detection). This environmental factors study includes both an extensive list of environmental organisms and a list of various potential interferents. The environmental organism panel contains organisms that could be encountered in aerosol collection samples, such as other biological threat agents, bacteria found in air and soil, DNA viruses, microbial eukaryotes, DNA from higher eukaryotes, and biological insecticides.

Standard Method Performance Requirements for DNA-Based Methods of Detecting Brucella suis in Field-Deployable, Department of Defense Aerosol Collection Devices, AOAC SMPR 2016.009 (8)

Brucella is an intracellular bacteria pathogen and the causal agent of brucellosis. Four species of *Brucella* cause the majority or brucellosis infections in humans: *B. melitensis, B. suis*, *B. canis*, and *B. abortus*. Ten currently recognized species (often with strong host-specificity) are further divided into biovars.

The inclusivity panel includes available type strains of *B. suis* biovars from 1 to 4 as well as the genomic sequences for biovar 5 and a Chinese vaccine. The exclusivity panel includes 23 strains and species of *Brucella*, including biovars of *B. abortus*, *B. melitensis*, *B. canis*, *B. microti*, B. *neotomae*, *B. ovis*, *B. ceti*, *B. pinnipedialis*, *B. inopinata*, *B. papionis*, *B. vulpis*, and, in addition, *Agrobacteriumtumefaciens*, *Ochrobactrum anthropi*, and *Ochrobactrum intermedium*.

The Brucella Working Group is aware that *B. canis* can infect humans. There are approximately 100 cases of human brucellosis annually. There is also a close relationship between *B. suis* and *B. canis*. In fact, the taxonomic classification of all *Brucella* spp. has been debated for decades, with some scientists proposing that all *Brucella* spp. be reclassified as *B. melitensis* based on results of DNA-DNA hybridization and that the current species be reclassified as biovars. However, the classic taxonomic scheme for the *Brucella* spp. and existing biovars was reapproved in 2003 based on host specificity, phenotypic characteristics, varying virulence, and genotyping data (9). For these reasons, and based on direction from DoD to focus on *B. suis*, the working group developed this SMPR specific for the detection of *B. suis*.

The Brucella Working Group is aware that Russian vaccines use *B. abortus* SR82 and *B. abortus* 7579, and possibly other strains. These vaccine strains were not available at the time this SMPR was adopted. Consequently, the working group decided not to include these vaccine strains in the exclusivity panel.

Standard Method Performance Requirements for DNA-Based Methods of Detecting Burkholderia pseudomallei in Field-Deployable, Department of Defense Aerosol Collection Devices, AOAC SMPR 2016.009 (10)

Burkholderia pseudomallei is a Tier 1 select agent and an aerobic, Gram-negative bacillus that causes the disease melioidosis. It is common in many tropical regions but is particularly important in northern Australia and Southeast Asia, where virulent strains can be easily

isolated from water and soil. For biothreat concerns, there are two closely related species of *Burkholderia: pseudomallei* and *mallei. B. mallei* causes a distinct disease (glanders) and is actually a clonal derivative from *B. pseudomallei*. In contrast to *B. pseudomallei, B. mallei* has much lower diversity, and there is no evidence of horizontal gene transfer, although large chromosomal deletions have occurred and can affect assay design. DUSA directed the working group to focus on *B. pseudomallei* but to consider how the inclusivity and exclusivity panels might be designed to accommodate evaluations of the *B. mallei* species. Because of their close relationship, the *B. mallei* inclusivity panel serves as a partial exclusively panel for *B. pseudomallei* and conversely for the reverse comparison.

There is great genetic diversity within the *B. pseudomallei* species because of frequent recombination and horizontal transfer of genetic material. In pairwise comparisons, any two isolates of *B. pseudomallei* will be different at >10% of their gene content. Therefore, it is important for the inclusivity panel to capture as much of this diversity as possible to avoid assays with lower sensitivity. There are two major populations in *B. pseudomallei* species, Australian and Southeast Asian. Therefore, five isolates from each population are included in the inclusivity panel.

The exclusivity panel is composed of 19 *Burkholderia* spp. that have potential to cause diagnostic confusion (lower specificity) based on their close relation to *B. pseudomallei*. The *B. pseudomallei* exclusivity panel was designed so that it could also be used as an exclusivity list to evaluate methods designed to detect *B. mallei* species. Species identified as 3 to 19 in the SMPR could be used to evaluate the specificity of methods design to detect *B. mallei* species only.

Standard Method Performance Requirements (SMPRs) for Detection of Botulinum Neurotoxins Al andA2 in Field-Deployable, Department of Defense (DoD) Aerosol Collection Devices, AOAC SMPR 2016.011 (11)

Botulinum toxin is a neurotoxic protein produced by the bacterium *Clostridium botulinum* and related species. It is also produced commercially for medical, cosmetic, and research use. There are two main commercial types: botulinum toxin type A and botulinum toxin type B. Infection with the bacterium may result in a potentially fatal disease called wound botulism. However, most cases of botulism result from ingestion of the botulinum neurotoxin. The DoD's concern is the detection of aerosolized botulinum toxins.

> The working group determined that the AMDL can be done using just Al complex, but the AMDL would need to be higher, and 1.25 ng/mL was agreed. A2 to A8 were then removed from the inclusivity table.

Candidate methods must be able to detect botulinum neurotoxins Al and A2 in liquid samples.

Standard Method Performance Requirements (SMPRs) for Detection of Coxiella burnetii, AOAC SMPR 2015.011 (12)

Coxiella burnetii is a small Gram-negative bacterium, an obligate intracellular bacterial pathogen, and the causative agent of Q fever. The genus *Coxiella* is morphologically similar

to *Rickettsia* but with a variety of genetic and physiological differences. *C. burnetii* is highly resistant to environmental stresses, such as high temperature, osmotic pressure, and ultraviolet light. It can survive standard disinfectants and is resistant to many other environmental changes.

C. burnetii is a highly infectious agent and wide-spread among livestock around the world. Although the culture process for C. *burnetii* is laborious, large amounts of infectious material can be produced. If used as an aerosolized biological weapon, *C. burnetii* may not cause high mortality but could provoke acute disabling disease. In its late course, Q fever can be complicated by fatal (e.g., endocarditis) or debilitating (e.g., chronic fatigue syndrome) disorders. The diagnosis of Q fever might be delayed because of nonspecific presentations. Effective antibiotic treatment is available for the acute form of the disease but not for the chronic complications.

The Q Fever Working Group agreed on a scope for the SMPR as field-deployable assays (preferred) that are specific for the detection of C. *burnetii* in collection buffers from aerosol collection devices. The working group also set the AMDL at 2000 genomic equivalents/mL of *C. burnetii* target DNA in the candidate method sample collection buffer. This concentration is based on AMDL levels established during the development of AOAC SMPRs for other threat agents. The theoretical minimum detection level for state-of-the-art technology is 1 genome equivalent. For validation purposes, homogeneity at very low concentrations, such as 1 genome equivalent per microliter, is problematic because of sampling error. Therefore, a minimum of 20 genome equivalents per microliter considers the sample error because of random distribution of the target. Most collection buffers are kept in the milliliter range, from which microliter range test samples are taken, so a 100-times scale up is needed. Therefore, the AMDL is set at 2000 genome equivalents per milliliter.

The working group developed an inclusivity panel consisting of the *C. burnetii* phylogenic groups 1 to 6 and provided isolate examples for testing purposes. The exclusivity panel consists of four *Legionellae* species and/or strains and one *Rickettsiella* spp. These species or strains represent the only known closely related species.

Standard Method Performance Requirements (SMPRs) for Detection of Francisella tularensis in Aerosol Collection Devices, AOAC SMPR 2016.007(13)

F. tularensis is a Gram-negative bacterial pathogen and the causative agent of tularemia of rabbit fever. *F. tularensis* is classified as a Category A bioterrorism agent by the CDC because it can be easily spread by aerosol and is highly virulent. The *Francisella* genus is traditionally divided into three species, *F. tularensis*, *F philomiragia*, and *F novicida*. Of these species, *F. tularensis* is the only species that is clinically significant. *F. tularensis* has three subspecies, including *F. tularensis* subspecies *tularensis*, *F. tularensis* subspecies *holartica*, and *F. tularensis* subspecies *mediasiatica*. Of these subspecies, *F. tularensis* subspecies *tularensis* is highly virulent, whereas *F. tularensis* subspecies *holartica* is less virulent. Each of the three subspecies was originally represented on the inclusivity panel in a previous SMPR (unpublished), but *F. tularensis* subspecies *mediasiatica* was removed because of a lack of available strains in the United States. Strains of the remaining two subspecies *(F. tularensis* subspecies *tularensis* and *F. tularensis* subspecies *holartica*) were

chosen to cover the genotypes (types Al, A2, and B) and to provide some geographical diversity of isolates. There are nine *F tularensis* strains on the inclusivity panel, including genotypes Al, A2, and B. The exclusivity panel contains 10 near neighbors, including six strains of *F. philomiragia*, three strains of *F novicida*, and one strain of *Wolbachia persica*, which is a species closely related to *F tularensis*.

A major issue for development of an SMPR for *tularensis* was the proposed reclassification of *F. novicida*. In 2005, Sjöstedt proposed that *F. novicida* be reclassified as *F. tularensis* subspecies *novicida*, but this reclassification has not been officially recognized (14). For this reason, *F. novicida* was included in the exclusivity panel becasue the target for this SMPR is specifically *F. tularensis*.

Standard Method Performance Requirements (SMPRs) for Detection of Staphylococcal Enterotoxin B, AOAC SMPR 2015.013 (15)

SEB is an enterotoxin produced by the Gram-positive bacteria *Staphylococcus aureus*. It is a common cause of food poisoning, with severe diarrhea, nausea, and intestinal cramping often starting within a few hours of ingestion. The toxin is quite stable and may remain active even after the contaminating bacteria are killed. The toxin can withstand boiling at 100°C for a few minutes. Gastroenteritis occurs because SEB is a superantigen, causing the immune system to release a large amount of cytokines that lead to significant inflammation.

As a possible biological weapons agent, SEB is stable when transformed into an aerosol and can produce multiorgan and system-wide symptoms. In extremely high doses, SEB may induce shock and even death. Symptoms of an aerosolized attack differ from ingestion and include high fever, chills, headache, muscle ache, dry cough, and inflammation of the eye lids. Aerosolized SEB may also cause trouble with breathing, chest pains, and the development of fluid in the lungs. Typically, SEB is considered an incapacitating agent that causes debilitation for up to 2 weeks. Small amounts can debilitate large numbers of victims because only 1/4000 of a microgram is fatal to 50% of the exposed population, and 1/200 of a microgram can induce fatality in 50%) of the human population. The agent cannot be transmitted from person to person.

SEB is one of 24 known enterotoxins produced by *S. aureus*. The other enterotoxins are designated as SEA, SEC, SED, SEE, and SEH-SEV. The working group originally suggested that the DoD consider including SEA and SEC in the scope of the SMPR as the operational response would be the same. After much discussion, the stakeholder panel decided to focus this SMPR on SEB only; however, there might be the possibility of developing an SMPR for SEA, SEB, and SEC 1, SEC 2, and SEC3 at a later time. Therefore, the exclusivity panel for this SMPR includes SEA, SEC, SED, SEE, SEH, SEI, SEJ, and SEK (if available). The working group set the AMDL at 0.25 ng/mL of recovered SEB toxin in liquid and set the POD at the AMDL at ≥0.95.

Standard Method Performance Requirements (SMPRs) for DNA-Based Methods of Detecting Yersinia pestis in Field-Deployable, Department of Defense Aerosol Collection Devices, AOAC SMPR 2016.008 (16)

Y. pestis is a Gram-negative, rod-shaped bacterium belonging to the *Enterobacteriaceae* family. Human infection by *Y. pestis* takes three main forms: bubonic, pneumonic, and septicemic. All three forms are widely believed to have been responsible for several high-mortality epidemics throughout human history. Because of its high mortality and aerosol communicability, the CDC has classified *Y. pestis* as a Category A bioterrorism agent.

The challenges in developing inclusivity and exclusivity panels for *Y. pestis* include the genomic similarity of *Y. pestis* to *Y. pseudotuberculosis*, the lack of genetic diversity among isolates of *Y. pestis*, and the availability of strains.

The inclusivity panel for SMPR 2016.008 contains 16 *Y. pestis* strains chosen to cover: the Achtmann genotypes, known biovars, and strains lacking the various plasmids. Because the genetic variability of *Y. pestis* is not fully understood, SPADA added two additional strains, recent United States isolates, to the proposed inclusivity panel. The exclusivity panel contains 17 *Yersinia* non-*pestis* isolates that represent eight *Yersinia* species.

The AMDL was initially set at 500 cells per aerosol filter because the infective dose is low for these organisms. However, because of the many variables in aerosol collection, such as the flow rate through the collector, length of time of collection, whether indoor or outdoor, and potential volume of air affected by the release, it is difficult to equate an infective dose to any specific number of cells collected on an air filter. The *Y. pestis* working groups decided to recommend the same AMDL as was determined for *B. anthracis* (20,000 cells per aerosol filter; 2000 cells/mL aerosol collection liquid) based on what PCR methods could reasonably be expected to detect.

Standard Method Performance Requirements (SMPRs) for Detection and Identification of Variola Virus DNA, AOAC SMPR 2016.012 (17)

Variola virus, the causative agent of smallpox, is a solely human pathogen classified as a Category A agent. Smallpox was a feared disease that caused death in up to 40% of those infected. In 1980, the World Health Organization (WHO) declared smallpox to be eradicated, the only human disease to date, and the known remaining virus stocks are now held in two WHO collaborating centers. The WHO has published several restrictions on who can obtain *Variola virus* DNA and what manipulations can be conducted (http://apps.who.int/iris/bitstream/10665/205564/1/WHO_OHE_PED_2016.1_eng.pdf).

Unique challenges exist for development of detection assays for the presence of *Variola virus*. Because smallpox is eradicated and *Variola virus* is no longer found in nature, the predictive value positive (the proportion of true positives among those testing positive) is incredibly low. Therefore, the probability of a false-positive result is quite high, which could have significant negative impact on societal infrastructure when related to a Category A agent. One method to increase the predictive value positive of an assay is to incorporate detection assays for more than one target. There is greater confidence in a positive result when multiple regions of the genome are detected. However, the only way to confirm

whether viable virus at present is by propagation within tissue culture, which should only be attempted within one of the two WHO Collaborating Centers for Smallpox.

Another challenge exists when attempting to design detection assays specific for *Variola virus* DNA. *Variola virus* is a member of the *Orthopoxvirus* family, whose members share a high degree of nucleotide similarity. Because of this high level of similarity, DNA detection assays must be thoroughly interrogated for specificity compared with these “near-neighbor” viruses. To further complicate matters, new members of the *Orthopoxvirus* family have been identified in recent years. These sequences must also be evaluated to determine if they have compromised the integrity of the detection assay. This requires vigilance in continued monitoring of the target of these assays.

An SMPR for *Variola virus* DNA was developed under SPADA in 2014 for Bio Watch and served as the foundation for the current SMPR specific to DoD application. Inclusivity and exclusivity panels are based on bioinformatic analysis. For exclusivity, there is a core group of poxvirus strains outlined in the SMPR (one from each major clade), which the detection assay should be tested against. Based on bioinformatics analysis of the target region(s), other virus strains with a higher degree of similarity compared with the strain listed in the core group should also be “wet-lab” tested in the detection assay. For inclusivity, at least one representative from each major clade of *Variola virus*, as well as any other strains that show differences within the target region, should be tested in the detection assay. Because only the two WHO Collaborating Centers for Smallpox can possess the whole genomic DNA of *Variola virus*, plasmid DNA containing <500 bp may be used to verify the sensitivity of the detection assay.

The *Variola* SMPR is notable as the first SMPR to incorporate the use of bioinformatics analysis, also known as in silico analysis. The SMPR includes sources, instructions on the use, and the application of bioinformatics analysis.

Standard Method Performance Requirements for Identification of Venezuelan Equine Encephalitis Virus (VEEV), SMPR 2015.012 (18)

Note: For the purposes of this manuscript, VEE, EEE, and WEE refer to the diseases Venezuelan equine encephalitis, Eastern equine encephalitis, and Western equine encephalitis. VEEV, WEEV, and EEEV refer to the causative agents of the diseases: Venezuelan equine encephalitis virus, Eastern equine encephalitis virus, and Western equine encephalitis virus.

VEEV is an *alphavirus* and is the causative agent of Venezuelan equine encephalitis (VEE). Other related diseases caused by a*lphaviruses* include Eastern equine encephalitis (EEE), Western equine encephalitis (WEE), Chikungunya fever, Semliki Forest disease, Barmah Forest fever, and Ross River fever. VEEV is a mosquito-borne viral pathogen. VEEV can affect all equine species, such as horses, donkeys, and zebras. After infection, equines may suddenly die or show progressive central nervous system disorders. Humans also can contract this disease.

VEE infection generally occurs when a person is bitten by an infected mosquito. VEE is highly infectious when aerosolized. Healthy adults who become infected by the virus may experience flu-like symptoms that may progress to neurologic disease. The disease is usually acute, prostrating, and of short duration. Illness begins suddenly with generalized malaise, spiking fevers, rigors, severe headache, photophobia, and myalgia. Nausea, vomiting, cough, sore throat, and diarrhea may follow. Full recovery takes 1 to 2 weeks. People with weakened immune systems and the young and the elderly can become severely ill or die from this disease.

VEE neurologic disease in humans is characterized by inflammation of the meninges of the brain and of the brain itself, thus accounting for the predominance of central nervous system symptoms. The overall mortality rate for VEE is less than 1% but is somewhat higher among children and older adults.

VEEV can be easily produced in large amounts and aerosolized for biological weapons purposes. As a biological agent, VEEV is relatively stable and can potentially injure thousands. Additionally, VEEV can be genetically manipulated, which may enhance its infectiousness and virulence for biological weapons purposes.

As part of this project, the DoD asked the VEE Working Group to consider whether EEEV and WEEV should be included as part of the scope of the SMPR. EEEV and WEEV are in the same antigenic complex as VEEV, and they are distantly related to VEEV. However, they are not genetically close to VEEV, so designing a molecular assay that would detect VEEV, WEEV, and EEEV might not be possible. Therefore, this SMPR was developed specifically for the identification of VEEV.

The working group established the AMDL at 5000 genome copies/mL for VEEV in aerosol collection liquid. This level is higher than the other bacterial biological threat agents because VEEV is an RNA virus that requires additional manipulations for PCR detection assays. The working group identified four serotype variants and representative strains for the inclusivity panel and identified nine viruses, including EEEV and WEEV, for the exclusivity panel.

Environmental Factors Study

Appendix O: Environmental Factors for Validating Biological Threat Agent Detection Assays (19)

The Environmental Factors study is common to all biological threat agent SMPRs, and it is designed to consider the types of evaluation needed to demonstrate that assays for the detection of biological threat agents are sufficiently rugged to maintain reliability when deployed in the field. Field applications include subway, bus, and airport sites, public and/or private buildings, and public spaces such as sports and entertainment facilities. PCR assays are typically developed for laboratory use in controlled environments. The deployment of PCR assays into an uncontrolled field increases the possibility of potential inhibition of the PCR amplification process and potential cross-reactivity with DNA fragments that are naturally occurring in the outside environment.

The Environmental Factors study is organized into three parts: environmental matrix study, environment organisms, and potential interferences. The environmental matrix study consists of evaluating stored, previously used environmental filters that previously produced negative results. In this study, half of the filters are used to evaluate for inhibition. There is concern that some naturally occurring potential inhibits can be anticipated and evaluated directly, but there might be naturally occurring potential inhibits that are not anticipated. Testing previously used environmental filters from a variety of regions and locales may expose an unanticipated inhibition and provide a baseline for estimating inhibition in field deployed PCR assays.

Part 2 of the Environmental Factors study, known as the Environmental Organisms section, is an evaluation of potential cross-reactivity with the DNA from a wide variety of organisms in the environment. There is concern that some naturally occurring potential cross-reactive strains can be anticipated and evaluated directly in the near-neighbor exclusivity panel, but there might be naturally occurring cross-reacting DNA fragments that are not anticipated. Environmental organisms were selected based on the expected prevalence of certain organisms in the environment, representation of the major genera expected in the environment, and availability of DNA from specific species representing the major genera. This section is noteworthy as the newly revised 2017 study now incorporates an option to use bioinformatics analysis, which greatly reduces the need for “wet” testing.

Part 3 of the Environmental Factors study, known as the Potential Interferents, was added to the Environmental Factors study in 2015. Part 3 considers the potential interferents that may be encountered in the DoD applications. The DoD applications include areas where active combat may be occurring and in other parts of the world. The DoD specific application required a reconsideration of the Environmental Factors section of the SMPR. The biggest concern was that combat situations are potential interferents, such as jet fuel, exhaust, obscurants (fog agents), burning vegetation, rubber, etc. Therefore, a new section on “Potential Interferents” was added to the Environmental Factors panel for the DoD-specific SMPRs.

Summary

SPADA has produced a set of performance standards for the top 10 priority biological threat agents to help method developers design assays and that method users can utilize to evaluate potential assays. These SMPRs describe the minimum performance requirements as determined by the most respected experts in the world. Four of these SMPRs *(Francisella tularensis*, *Burkholderia pseudomallei*, Variola Major, and *Brucella suis)* allow for the use of in silico analysis in lieu of direct testing under some circumstances in which inclusivity and/or exclusivity species or stains are not available. In another significant first, in silico analysis is offered as an option to reduce the required number of environmental organism DNA or RNA “wet” tests in the Environmental Study. It is expected that in silico analysis will reduce the amount of testing time and cost required and provide researchers with a much greater ability to determine potential cross-reactive DNA from environmental species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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