

Approaches to Biohazard Analysis of Select Biodefense Vaccine Candidates

Venkat Rao

Computer Sciences Corporation, National and Defense Programs, Alexandria, Virginia

Abstract

Biohazard assessment of biodefense vaccine candidates forms the basis for a facility- and activity-specific risk assessment performed to determine the biosafety levels and general safety standards required for biological product development. As part of our support to the U.S. biodefense vaccine development program, the author performed a systematic biohazard assessment of potential vaccine candidates with the primary objectives to: (a) identify and characterize hazard elements associated with the wild-type and vaccine strains; (b) provide biohazard information on the vaccine candidate to assist Phase 1 clinical trial facility sites; (c) provide a baseline agent and facility-specific risk assessment at clinical trial facilities interested in performing Phase 1 clinical trials; (d) provide comparative hazard profiles of the vaccine candidates with Material Safety Data Sheet (MSDS) for wild-type to identify and establish appropriate protective biosafety levels; and (e) support a determination of the personal protective equipment as required under the OSHA guidelines. This paper describes the biohazard analysis of two vaccine candidates, Venezuelan Equine Encephalitis Virus Strain 3526 and Francisella tularensis LVS, a viral and bacterial agent, respectively. As part of the biohazard assessment, we performed a thorough review of published literature on medical pathology, epidemiology, pre-clinical investigational studies, and environmental data on the etiologic agent subtypes and the vaccine candidates.

Using standard analytical procedures, the data were then analyzed relative to two intrinsic hazard parameters—health hazard and environmental hazard. Using a Weight-of-Evidence (WoE) approach, the potential hazards of etiologic agent wild-subtypes and vaccine candidates were ranked under three main categories: Public Health Hazard; Environmental Hazard; and Overall Hazard. A WoE scoring system allows for both a determination of the intrinsic hazard of each vaccine and also allows for a comparison of values between vaccines. The information in this hazard assessment, and the WoE scores in particular, provided a systematic analytical framework to begin facility-specific risk assessments for follow on manufacturing and Phase 1 clinical trials.

1.0 Introduction

In general terms, biohazard is the intrinsic property of an infectious agent that determines the nature and severity of adverse effects on the affected population. Biotechnology-derived biodefense medical countermeasure development requires that candidate products under development undergo a systematic biohazard assessment with the goals to define and identify the intrinsic biohazard elements associated vaccine candidates as opposed to the wild-type virulent strains. Essentially, biohazard assessment is qualitative in nature and based on a combination of extensive review and analysis of published literature on the wild-type and their vaccine counterparts. As part of this effort, published data from epidemiological investigations, laboratory investigations on experimental animals, and environmental studies are reviewed to develop a systematic analytical framework based on the macromolecular modifications between wild-type and attenuated vaccine strains, biochemical characteristics for biohazard based on phylogenetic analysis of species and strains of wild-type and attenuated organisms.

Incorporating biohazard assessment as part of the product development process is essential when developing highly sophisticated biodefense-oriented medical countermeasure products for military and civilian uses. A well-conducted biohazard assessment could guide: (a) evaluation of the technical base for selection of candidates for biologics product development; (b) biosafety-related decision in the selection of facilities to conduct various production, testing, and evaluation activities; (c) process development and validation; (d) manufacture process and controls-related decisions; (e) pre-clinical animal testing; (f) data incorporation and preparation of the Investigational New Drug (IND) application for filing with the U.S. Food and Drug Administration (FDA); (f) biosafety-related decisions in clinical trials; and (g) successful filing of the new biologics licensure application with the FDA. Evidently, biohazard assessment could have a significant impact directly and indirectly across the entire product development spectrum.

This paper outlines a WoE-based biohazard assessment derived from two interrelated analytical approaches to biohazard evaluation for biodefense vaccines. The first approach is based on the macromolecu-

lar modification through bioengineering on wild-type strains to yield vaccine candidates. Our studies on the Venezuelan Equine Encephalitis (VEE) vaccine candidates are used as an illustrative example of this approach. The second approach is based on a phylogenetic analysis together with comparative biomarkers associated with toxicity assessment of wild-type and vaccine strains. Our studies on the *Francisella tularensis*, wild-type, and vaccine strain, Biovar LVS (NDBR, 101), are used as an illustrative example of this approach.

2.0 Methods and Results

2.1 Biohazard Analysis Principles

The biohazard analysis follows the chemical and biological risk assessment components that include: (a) hazard assessment, which involves an evaluation of the intrinsic hazard characteristics attributable to the phylogenetic, biochemical, or macromolecular properties linked to hazard attributes; (b) dose-response evaluation, which in the case of a biological agent involves parameters such as minimal dose for infectivity, pathogenicity, environmental transmission, and distribution in the ecosystem populations; (c) exposure assessment such as those involved in occupational, clinical, and general environment-related activities using a set of realistic exposure scenarios; and (d) risk characterization, a formalized approach to combine the characteristics of hazard, toxicity, and exposure to derive a measure of risk associated with the biological agent.

2.1.1 Biohazard Analysis Based on Macromolecular Modifications: Rao et al. (2006) describes the biohazard assessment approach based on the macromolecular modification through bioengineering of wild-type strains to yield vaccine candidates as in the case of V3526, the Venezuelan Equine Encephalitis (VEE) vaccine strain. Figure 1 illustrates macromolecular construction of V3526 that results in modifying pathogenicity but retaining the immunogenic potentials. Briefly, V3526 is a live-attenuated vaccine candidate constructed by site-directed mutagenesis of the full-length cDNA V3000 clone with the desired attenuating mutations (Davis et al., 1991; Grieder et al., 1995). The deletion mutation eliminates the sequence encoding a furin protease cleavage site, which is utilized during maturation of the viral glycoproteins. The deletion results in the inclusion of the E3 glycoprotein in the spikes of the V3526 virion.

Figure 2 illustrates site-induced mutation at the furin cleavage site that locks the potential for reversion to a more pathogenic form during successive replications. The combination of these two mutations (deletion and substitution) results in a stable infectious virus with low pathogenicity (Davis et al., 1991). V3526 safety and reactogenicity have also been studied in a variety of animal models and have consistently shown lower neurovirulence and minimal potential for vector transmission (Hart et al., 2000; Ludwig et al., 2001).

2.1.2 Biohazard Analysis Based on Phylogeny, Genetic and Biochemical Markers: Our studies on the *Francisella tularensis*, wild-type and vaccine strain, Biovar LVS

Figure 1
Construction Strategy of V3526 Fundamentally Modifies Pathogenicity Without Concomitant Loss in Immunogenic Properties.

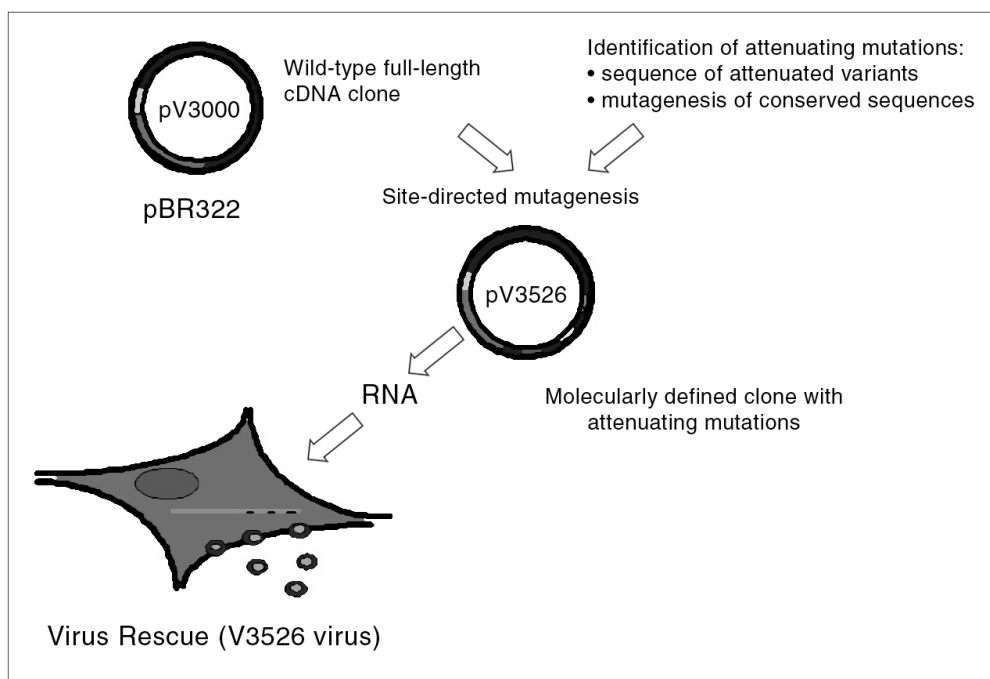
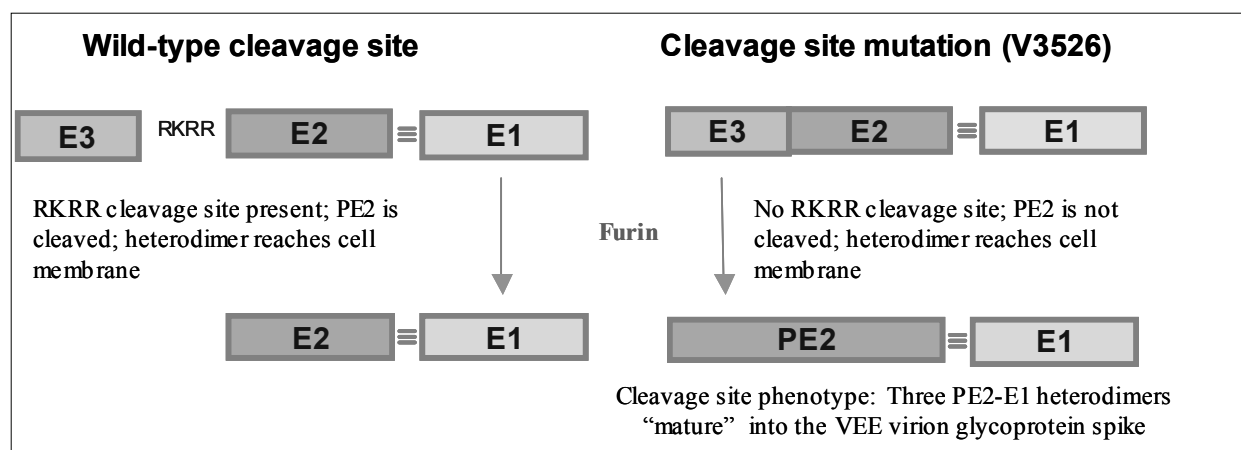


Figure 2

Lethal Mutation Due to Furin Cleavage Locks V3526 Potentials for Reversibility with Viability.



(NDBR, 101), are used as an illustrative example for this approach. Phylogenetic analysis of *F. tularensis* involved mapping of the biochemical, bacteriological, and molecular data at the subspecies and biovar levels to identify biomarkers for pathogenicity and infectivity. The biohazard analysis consists of a systematic comparative analysis of these biomarkers' presence and distribution in the subspecies and biovars for both wild strains and potential vaccine candidates.

Figure 3 illustrates the phylogeny of *F. tularensis* used in the biohazard analysis. Briefly, the genus Francisella has two species—*F. tularensis* and *F. philomiragia*. Of the two, *F. philomiragia* is less virulent, mostly waterborne, and relatively rare in occurrence. There are four subspecies of *F. tularensis* with a unique ecosystem and global distribution pattern. *F. tularensis* subspecies nearctica (biovar type A) is the most virulent among the subspecies and found predominantly in mammalian species and arthropod vectors in North America. *F. tularensis* subsp. holarctica (a.k.a. palaeartica, biovar type B) is moderately pathogenic, mostly waterborne, with multiple biovars distributed in Asia, Europe, and North America. *F. tularensis* subsp. mediaasiatica was isolated only from central Asia and former Soviet Republics. Finally, the *F. tularensis*, subsp. novicida was isolated from water samples in Utah (North America). Differences in pathogenicity and virulence among the *F. tularensis* subspecies and biovars were determined using a combination of taxonomic information, growth characteristics, biochemical analysis, and phylogenomic analysis.

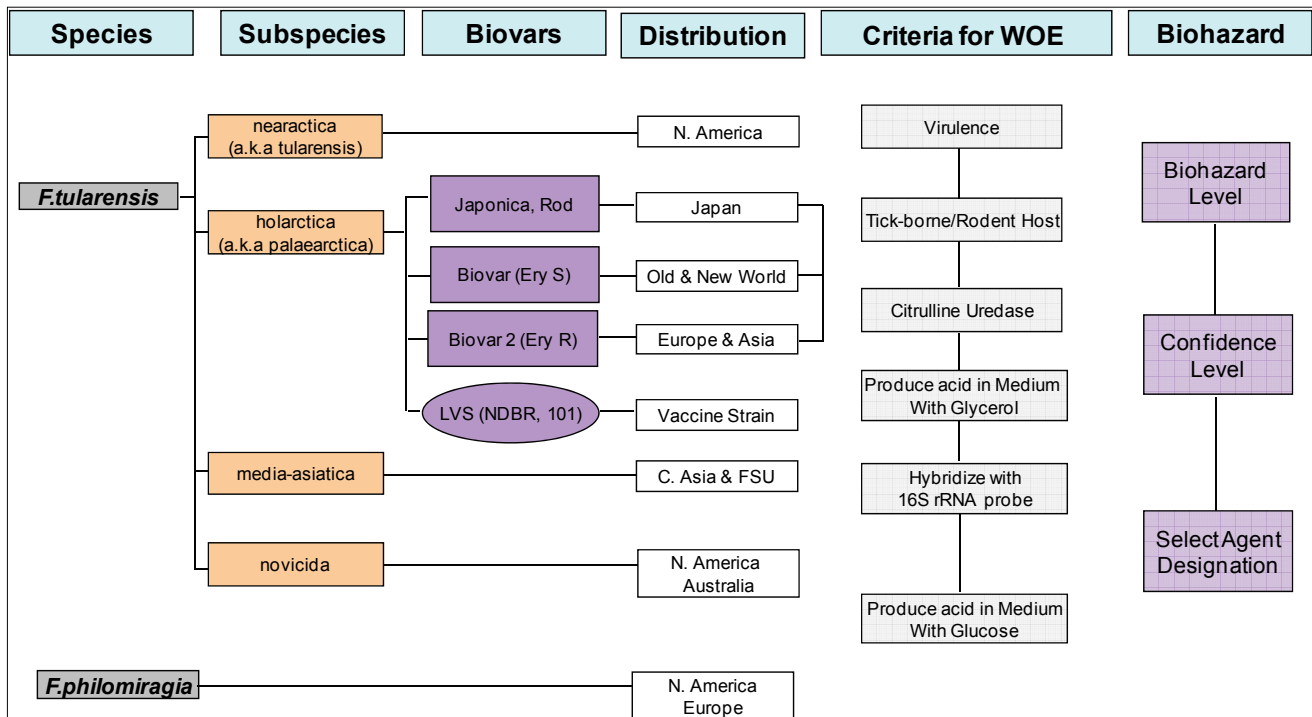
Scientists have adopted more extensive genetic analysis to establish the evolutionary relationships among various subspecies of *F. tularensis*. Phylogenetic analysis based on unidirectional genomic deletion events and single nucleotide variations in four subspecies of *F. tularensis* indicated that the highly virulent *F. tularensis* subsp. tularensis (type A) appeared before the less virulent *F. tularensis* subsp. holarctica (type B)

(Svensson et al., 2005). These phylogenetic investigations revealed specific, unidirectional gene loss in the attenuated vaccine strains of *F. tularensis* compared to the more virulent strains. The use of unidirectional deletions for phylogenetic analysis is based on the assumption that these events stabilize in bacterial populations providing a sort of phylogenetic lineage. For example, Svensson et al. (2005) used microarray studies to identify large size regions of difference among *F. tularensis* strains. Direct repeat sequences within these regions were compared for various strains of *F. tularensis* to establish evolutionary relationships among various virulent and vaccine strains. These analyses revealed an evolutionary scenario where the highly virulent *F. tularensis*, subsps. tularensis (type A) originated before the less virulent *F. tularensis*, subsps. holarctica (type B). Attenuated strains of *F. tularensis* exhibited unidirectional gene loss compared to their virulent progenitors (Svensson et al., 2005).

Analysis of the phylogenomic sequence for regions identified as unique and associated with pathogenicity is increasingly used in biohazard analysis. Using the genomic data for the most virulent *F. tularensis*, strain SHU S4 (biovar A), sequence repeats, generally known as variable-number tandem repeats (VNTR), were identified and compared with other known genomic sequence data for *F. tularensis* subspecies, biovars, and strains isolated from a variety of biological samples (Farlow et al., 2001). These investigations revealed VNTR sequences to be an effective discriminator to rapidly identify and characterize biovars and strains during the epidemiological investigation of an outbreak. These findings also indicated that genomic sequences data could be used to establish phylogenetic relationships and geographic distribution among various biovars and strains to locate a source of infection. These studies reveal clustering of VNTR loci, which were generally less diverse among biovar B isolates compared to those from infective biovar A isolates.

Figure 3

A Suggested Phylogeny-Based Schema for Biohazard Assessment of *F. tularensis*.



2.2 Weight-of-Evidence Methodology

The WoE procedure used in biohazard analysis is based on standard methodologies adopted by the U.S. Centers for Disease Control and Prevention (CDC) and the U.S. Environmental Protection Agency (EPA) in the development of a WoE scheme and designation of hazard codes for the chemical and biological toxicant effects on human health and the environment (CDC, 2000; Rao et al., 2004; U.S. EPA, 2000).

For instance, the WoE-based hazard assessment for biological agent virulent species/sub-types and vaccine candidates identified four categories of hazard criteria as the basis for a WoE-based ranking and prioritization for a potential public health and environmental hazard: (1) pre-clinical data on both the virulent species/subtypes and the vaccine strains; (2) clinical data derived mostly from epidemiological studies on the incidence and extent of morbidity and/or mortality from epizootic events and clinical reports; (3) environmental transport and fate data related to transmission and dissemination of the biological agents and vaccine candidates; and (4) other considerations relating to data on genetic homology of virulent strains and vaccine candidates, and data on the potential for reversion of vaccine strains.

2.2.1 WoE Criteria and Category Designations: Based on these WoE criteria biohazard designations, a data schema was developed on the basis of the preponderance of evidence and a prioritized ranking of the available data with a particular emphasis on the availability of

mechanistic data for virulence and pathogenicity (veterinary and human data). Attempts have been made to develop a WoE-based designation and ranking for biohazard following guidelines set up by the CDC and World Health Organization (WHO) biological agent risk categories for public health preparedness (WHO, 2001). The risk-based designations for the agents are as follows:

- (a) Risk Group 1: Agents *not associated* with disease in healthy adult humans.
- (b) Risk Group 2: Agents associated with human disease that is rarely serious, and for which preventive or therapeutic interventions are *often* available.
- (c) Risk Group 3: Agents associated with serious or lethal human disease for which preventive or therapeutic interventions *may be available*—high individual risk but low community risk.
- (d) Risk Group 4: Agents that are likely to cause serious or lethal human disease for which preventive or therapeutic interventions are *not usually* available—high individual and high community risk.

An example of one such WoE-based biohazard category would involve (Rao et al., 2004):

- (a) Category A designation is used when evidence indicating greatest potential for broad-based adverse impact on human and animal health and the general environment with limited or no availability of medical intervention.
- (b) Category B designation is used when evidence indicating potential, under the right conditions, for environmental dissemination resulting in illness, but of a lower order and

amenable to currently available medical intervention.

(c) Category C designation is used when there is limited or negligible threat of dissemination and transmission in the environment and potential for limited adverse effects.

(d) Category D designation is used for vaccine strains with demonstrated safety; few (one adverse event or fewer per million doses administered) adverse events.

Based on the WoE criteria and the category designation established from a systematic review of the available published literature, a comparative WoE-based biohazard ranking was developed for wild, virulent species, and vaccine strains.

Comparative biohazard-based ranking follows procedures established by the CDC, WHO, and EPA for WoE-based public health risk assessment and prioritization (CDC, 2000; U.S. EPA, 2000; WHO, 2001).

3.0 Results and Discussion

The intrinsic infectivity for VEE virus in experimental animals can be measured as an infectious dose (dose required to initiate infection) or a median lethal dose (LD₅₀). In humans, the infectious dose has been estimated following experimental subcutaneous inoculation and accidental aerosol exposure among laboratory workers. Infectious dose is one of the early indicators for hazard for microbial agents and contributes to the establishment of safe-handling and transportation procedures for infectious agents.

According to the published Material Safety Data Sheet for VEE virus, infectious dose is between 5 to 10 organisms by respiratory route and 10⁶ to 10⁸ organisms by ingestion (Health Canada, 2001). Infectious doses for the IA/B wild-type Trinidad strain range from 1 pfu up to 100 pfu depending on the animal species and route of exposure. In mice and hamsters the infectious dose can be as low as 1 pfu following parenteral inoculation. An infectious dose of Trinidad and TC-83 for humans by aerosol exposure is estimated to be between 10 to 100 pfu (Smith et al., 2000). In addition to having a relatively low infectious dose in several animal systems, VEE is also a highly infectious virus, with 90%-

100% of humans who are exposed to an infectious dose, regardless of the route of exposure, exhibiting clinical signs of illness.

Many animal safety, reactogenicity, and immunogenicity studies have compared the effects of V3526 with another vaccine strain, TC-83 (Hart et al., 2000; Ludwig et al., 2001; Turell et al., 1999). These studies have shown several differences in the safety profiles. In general, V3526 was found to elicit an immune response at least as robust as TC-83, without the attendant pathology observed with TC-83. Wild-type VEE virus is extremely lethal in several different strains of mice, including BALB/c and C3H/HeN. This susceptibility makes them an ideal animal system to test the pathogenicity, safety profile, and immunogenicity of VEE vaccine candidates.

In a recent comparative study evaluating the neurovirulence of vaccine strains, juvenile rhesus macaques were inoculated with V3526 and TC-83 intrathalamic/intraspinal (i.t./i.s.) or subcutaneous (s.c.), followed by extensive clinical and biochemical assessment over a 180-day period. The results indicated a transient clinical profile for V3526 demonstrating immunogenic properties but without a demonstrable neurovirulence in the clinically relevant subcutaneous route of administration (Fine et al., 2008).

Ludwig et al. (2001) investigated the pathogenic potentials of V3526 and TC-83 on BALB/c and C3H/HeN mice following intracranial (i.c.) inoculation. Interestingly, BALB/c mice receiving V3526 via the i.c. route did not manifest signs of infection, but mice that received TC-83 i.c. consistently exhibited signs of disease, including inactivity and ruffled hair coats (Table 1). Even more pronounced differences in pathogenicity between TC-83 and V3526 viruses were observed following i.c. inoculation of C3H/HeN mice, a strain of mouse that is more susceptible to wild-type VEE infection than are BALB/c mice. The i.c. dose needed to kill 50% of C3H/HeN mice (LD₅₀) for TC-83 was calculated to be ~20 pfu (Table 1). In contrast, all C3H/HeN mice receiving an i.c. dose of V3526 survived. In addition, no clinical symptoms were observed following i.c. administration of 10⁶ pfu of V3526 into C3H/HeN.

Table 1
Pathogenicity of V3526 in Mice

VEE	BALB/c			C3H/HeN		
	LD ₅₀ (i.c.) ^a	MDD ^b	Sick Animals	LD ₅₀ (i.c.) ^a	MDD	Sick Animals
TrD (wild-type)	-0.7	10.3	NA ^c	N/D	N/D ^e	N/D
V3000	1.4	9.3	NA	N/D	N/D	N/D
TC-83	>7.2	NA	Yes ^d	1.3	12.1	NA
V3526	>5.7	NA	No	>6.0	NA	No

Based on Hart et al. (2000). Adapted from Rao et al. (2006). ^alog pfu; ^bMean days to death; ^cNot Applicable (N/A); ^dIn addition to transient signs of illness, mice exhibited long-term aggressive behavior; ^eNot Done (ND)

A key biohazard determination would involve an assessment of environmental transmission and dissemination of a biological agent. Table 2 summarizes a comparative assessment of mosquito infection and environmental dissemination of V3526 and TC-83. Whereas no significant differences in infection rate or dissemination rate were observed when the concentration of virus in the blood meal was equal to 10^6 pfu/ml, a significantly higher rate of infection was observed in the V3526 group fed on blood meal containing 10^7 pfu/ml or 10^8 pfu/ml of the virus. For example, as a vector of natural transmission life cycle of VEE, mosquitoes play a crucial role in the environmental distribution between animals and humans. A key criterion for biohazard is the determination of the ability of VEE-infected mosquitoes to transmit the virus by bite and mosquitoes to become infected by feeding on infected animals.

Turell et al. (1999) investigated VEE's potential for environmental transmission by inoculating three groups of mosquitoes with TC-83, V3000, or V3526. Maximum titer achieved by the virus in each group was determined (Table 3). The group inoculated with V3526 achieved the lowest titers suggesting that the probability of transmission of the virus by the mosquito to a susceptible host during feeding is less than that associated with V3000 or TC83. This was confirmed by additional repeated testing and analyses.

In a related study, V3526 and TC-83 passed five times sequentially in mice (i.e. inoculation) indicated that none of the V3526-inoculated animals exhibited clinical symptoms at any passage (Table 4). However, on the fifth passage, one of the mice inoculated with TC-83 exhibited VEE-like symptoms and died, suggesting that TC-83 had possibly reverted to a more virulent phenotype. No evidence of reversion was observed in hamsters that were infected with V3526 by intra-thoracic inoculated mosquitoes (data not included). Collectively, these studies demonstrated adequate evidence that the likelihood of V3526 reverting to a virulent phenotype is considerably low.

The WoE procedures used here are based on standard methodologies adopted by the CDC (CDC, 2000) and U.S. EPA (U.S. EPA, 2000) in the development of WoE schemes and designation of hazard codes for chemical and biological toxicant effect on human health and the general environment (Tables 5 and 6).

Table 5 is a summary of the WoE based on clinical and pre-clinical morbidity and mortality assessment criteria for VEE wild-type, TC-83, and V3526. Similarly, Table 6 is a summary of the WoE based on two key environmental criteria: dissemination and transmissibility.

Table 7 is a WoE summary for generally categorized as "other considerations" that takes into account data on genetic homology of the VEE virus and vaccine candi-

Table 2Mosquito Infection and Dissemination Rates: V3526 vs. TC83^a

VEE Strain	PFU/ml ingested	Number Tested	Infection Rate ^b (%)	Dissemination Rate ^c (%)
V3000	10^8	46	22	2
	10^7	47	19	0
	10^6	11	0	0
TC-83	10^8	47	2	0
	10^7	51	4	0
	10^6	29	3	0
V3526	10^8	54	80	19
	10^7	44	32	0
	10^6	21	5	0

^aBased on Hart et al (2000); Adapted from Rao et al. (2004); ^bPercentage of mosquitoes containing virus; ^cPercentage of mosquitoes containing virus in their legs

Table 3VEE Transmission (Virus and Vaccine Strains)^a

Virus Strain	Maximum Log Titer Following Inoculation (n)	Transmission Rate to Hamsters (n)	Hamster Mortality Rate (n)
V3000	7.1 (21)	81% (43)	94% (35)
TC-83	7.3 (6)	64% (36)	17% (23)
V3526	5.5 ^b (21)	18% (33)	0% (6)

^aBased on Hart et al (2000); Adapted from Rao et al. (2004); ^bInoculation: $10^{1.5}$ pfu/mosquito

Table 4Reversion in Mice: V3526 vs. TC-83^a

Brain Passage	V3526 ^b		TC-83 ^b	
	Illness/Total	Survivors	Illness/Total	Survivors
1	0/10	10	10/10	10
2	0/10	10	10/10	10
3	0/10	10	10/10	10
4	0/10	10	10/10	10
5	0/10	10	10/10	9/10

^aBased on Ludwig et al (2001); Adapted from Rao et al. (2004); ^b10⁵ pfu was injected i.c. into each mouse

Table 5VEE Comparative WoE Biohazard Assessment Criteria—Public Health Hazard^a

Criteria	VEE-WT	TC-83	V3526
Morbidity			
Pre-Clinical	Causes fever and viremia in experimental animals.	Can cause temporary aggressiveness, viremia and fever in animals.	No indication of illness in experimental animals.
Clinical	Illness in humans likely and may require hospitalization	May cause temporary illness in 10-20% of vaccinees.	No clinical data
Mortality			
Pre-Clinical	Can causes high mortality in infected animals, especially equines.	May cause death in experimental animals at high doses	No deaths from vaccinations in experimental animals.
Clinical	Mortality in humans: 15% or less	No known human mortality	No clinical data

^aBased on Rao et al. (2004)

dates, and data on the potential for reversion of the vaccine strains.

The methodology used in the VEE hazard assessment represents one of the generic approaches that takes into account a comparative WoE scheme based on clinical, pre-clinical, environmental, and other key genetic data relating to the potential for reversion and genetic homology of the VEE wild-type and vaccine candidate strains. This approach extends existing standardized frameworks for health and environmental risk assessment to biohazard assessment of wild-type and vaccine candidate strains for biodefense medical countermeasures product development. Biohazard assessment performed using this approach constitutes the first step of a much more detailed analysis to characterize health and environmental risks.

The WoE methodology used in the biohazard assessment of *Francisella tularensis*, wild-type and vaccine strain, Biovar LVS (NDBR, 101), illustrates an alternative approach that involves mapping of the biochemical, bacteriological, and molecular data at the subspecies and biovar levels to identify biomarkers for pathogenicity and infectivity.

This phylogenetic-driven approach to biohazard analysis would consist of a systematic comparative analysis of these biomarkers' presence and distribution in the subspecies and biovars for both wild strains and potential vaccine candidates.

Identification and differentiation of *F. tularensis* subspecies are generally based on performing an analysis of biomarkers associated with the growth characteristics under culture conditions and biochemical analysis. Figure 4 illustrates the WoE criteria for the subspecies based on these growth characteristics: (a) virulence demonstrated from published literature and experimental evidence; (b) subspecies' ability to ferment glycerol and glucose in the growth medium and produce citrul-line ureidase enzyme; (c) differential requirement of subspecies for tick-borne/rodent hosts in the natural life-cycle propagation; (d) similarity and differences in the gene sequence for the 16S rRNA as revealed from RNA hybridization studies; and (e) similarities and differences in subspecies' ability to produce acid in the growth medium with glucose. Clearly, most of the WoE, except for the 16S rRNA, is based on the differences in growth characteristics under culture conditions, consistently

Table 6
VEE WoE Comparative Hazards Criteria—Environmental Hazard^a

Criteria	VEE-WT	TC-83	V3526
Dissemination	Under the right conditions, virus can cause an epidemic in the environment (refer to mode of dissemination)	Low probability of dissemination	Low probability of dissemination
Transmissibility	Mosquitoes to human; aerosols (laboratory)	Vaccine strain, no evidence of human-to-human transmission in contacts with volunteers immunized with TC-83 during clinical trials.	No data regarding human-to-human transmission. Pre-clinical data indicates that transmission by mosquitoes is unlikely

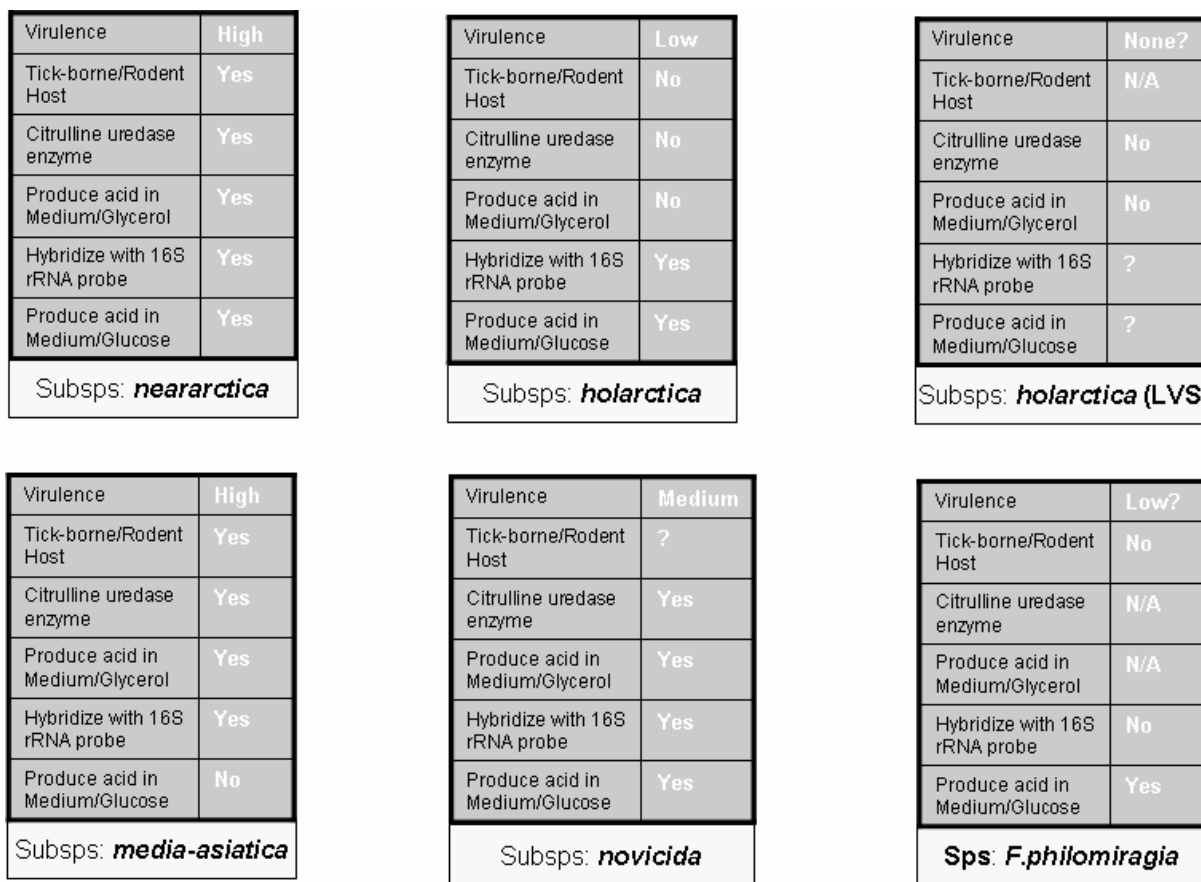
^aBased on Rao et al. (2004)

Table 7
VEE WoE Hazard Assessment Criteria—Other Considerations^a

Criteria	VEE-WT	TC-83	V3526
Homology	Genetically diverse	Heterogeneous virus population Attenuated via passage in cell culture.	Homogeneous virus population. Fully sequenced genome. Attenuated by deletion mutations.
Reversion	N/A	Demonstrated reversion in animals and cell culture	No demonstrated reversion after five serial passages in animals or cell culture.

^aBased on Rao et al. (2004)

Figure 4
A Suggested Phylogeny-Based WoE Scheme for Biohazard Assessment of *F. tularensis*.



reported from published literature for various subspecies and biochemical analyses. Use of molecular evidence, such as 16S mRNA to classify *F. tularensis*, was considered more reliable to a classification schema on the basis of biochemical analysis (Sandstrom et al., 1992). More recent investigations have indicated genetic markers such as variable-number tandem repeats (VNTR) and single nucleotide variations (SNV) as better discriminators of the subspecies and strains identification (Farlow et al., 2001; Svensson et al., 2005). The WoE criteria listed in Figure 4 represents a combination of both traditional biochemical and molecular markers consistently reported in the literature as key to discriminate the *F. tularensis* species, subspecies, and strains. This forms the basis for the WoE-based biohazard assessment shown for various species and subspecies including the vaccine strains (Figure 4).

This paper described a WoE-based framework for the biohazard analysis and demonstrated an example of such an approach to two vaccine candidates, VEE virus strain 3526 and *F. tularensis* LVS, a viral and bacterial agent, respectively. As part of the biohazard assessment, the author performed a thorough review of published literature on medical pathology, epidemiology, pre-clinical investigational studies, and environmental data on the etiologic agent subtypes and the vaccine candidates. Using standard analytical procedures, the data were then analyzed relative to two intrinsic hazard parameters—health hazard and environmental hazard. Using a WoE approach, the potential hazards of etiologic agent wild-subtypes and vaccine candidates were ranked under three main categories: Public Health Hazard, Environmental Hazard, and Overall Hazard. A WoE scoring system allows for both a determination of the intrinsic hazard of each vaccine and also allows for a comparison of values between vaccines. The information in this hazard assessment, and the WoE scores in particular, provided a systematic analytical framework to begin facility-specific risk assessments for follow-up on manufacturing and Phase 1 clinical trials.

4.0 Conclusions

In conclusion, we attempted to define and develop systematic approaches to performing biohazard assessments of hazardous biologics product candidates with potentials for adverse health and environmental impacts. Our attempts to adopt an existing framework for health and environmental risk assessment indicate that a WoE scheme offers a consistent framework to compare wild-type strains with vaccine candidates. Using two distinct but interrelated analytical approaches involving a recombinant candidate vaccine (Venezuelan Equine Encephalitis virus) and a phylogenetics-driven biomarker analysis (*Francisella tularensis*), the author demonstrated the value of the WoE scheme for comparative

biohazard assessment of biodefense medical countermeasure products.

Biohazard analysis is the key step in the determination of: (a) facility selection for process development and validation; (b) the biosafety and biosecurity containment levels; (c) compliance requirements under existing environmental and occupational safety regulations; and (d) submission requirements to regulatory agencies to obtain a license for commercial development.

Author's Note

This submission is based on the paper presented at the CBMTS—Industry V: Fourth World Congress on Chemical, Biological, and Radiological Terrorism, Dubrovnik, Croatia. April 15-20, 2007.

5.0 References

- Centers for Diseases Control and Prevention (CDC). (2000). Biological and chemical terrorism: Strategic plan for preparedness and response, recommendations of the CDC strategic planning workgroup. *Morbidity and Mortality Weekly Reports*, 49, 1-14.
- Davis, N. L., Powell, N., Greenwald, G. F., Willis, L. V., Johnson, B. J., & Smith, J. F. (1991). Attenuating mutations in the E2 glycoprotein gene of Venezuelan Equine Encephalitis virus: construction of single and multiple mutants in a full-length cDNA clone. *Virology*, 183, 20-31.
- Farlow, J., Smith, K. L., Wong, J., Abrams, M., Lytle, M., & Keim, P. (2001). *Francisella tularensis* strain typing using multiple-locus, variable-number tandem repeat analysis. *Journal of Clinical Microbiology*, 39(9), 3186-3192.
- Fine, D. L., Roberts, B. A., Terpening, S. J., Mott, J., Vasconcelos, D., & House, R. V. (2008). Neurovirulence evaluation of Venezuelan Equine Encephalitis (VEE) vaccine candidate V3526 in nonhuman primates. *Vaccine*, 26(27-28), 3497-3506.
- Grieder, F. B., Davis, N. L., Aronson, J. F., Charles, P. C., Sellon, D. C., & Suzuki, K. (1995). Specific restrictions in the progression of Venezuelan Equine Encephalitis virus-induced disease resulting from single amino acid changes in the glycoprotein. *Virology*, 206, 994-1006.
- Hart, M., Caswell-Stephen, K. K., Bakken, R., Tammariello, R., Pratt, W., & Davis, N. (2000). Improved mucosal protection against Venezuelan Equine Encephalitis virus induced by the molecularly defined, live attenuated V3526 vaccine candidate. *Vaccine*, 18, 3067-3075.
- Health Canada (2001). Material Safety Data Sheet—Venezuelan Equine Encephalitis Virus. Office of Laboratory Security. Last updated 2001-05-25.
- Ludwig, G. V., Turell, M. J., Vogel, P., Kondig, J. P., Kell, W. K., & Smith, J. F. (2001). Comparative neurovirulence of attenuated and non-attenuated strains of Venezuelan Equine Encephalitis virus in mice. *The American Journal of Tropical Medicine and Hygiene*, 64(1-2), 49-53.
- Rao, V., Hinz, M. E., Roberts, B. A., & Fine, D. (2004). Environmental hazard assessment of Venezuelan Equine Encephalitis virus vaccine candidate strain V3526. *Vaccine*, 22(20), 2667-2673.
- Rao, V., Hinz, M. E., Roberts, B. A., & Fine, D. (2006). Toxicity assessment of Venezuelan Equine Encephalitis virus vaccine candidate strain V3526. *Vaccine*, 24(10), 1710-1715.

- Sandstrom, G., Sjostedt, A., Forsman, M., Pavlovich, N. V., & Mishankin, B. N. (1992). Characterization and classification of strains of *Francisella tularensis* isolated in the central Asian focus of the Soviet Union and in Japan. *Journal of Clinical Microbiology*, 30(1), 172-175.
- Smith, C. G., Veenhuis, P. E., & MacCormack, J. N. (2000). Bioterrorism. A new threat with psychological and social sequelae. *North Carolina Medical Journal*, 61(33), 150-163.
- Svensson, K., Larsson, P., Johansson, D., Byström, M., Forsman, M., & Johansson, A. (2005). Evolution of subspecies of *Francisella tularensis*. *Journal of Bacteriology*, 187(11), 3903-3908.
- Turell, M. J., Ludwig, G. V., Kondig, J., & Smith, J. F. (1999). Limited potential for mosquito transmission of genetically engineered, live-attenuated Venezuelan Equine Encephalitis virus vaccine candidates. *The American Journal of Tropical Medicine and Hygiene*, 60(6), 1041-1044.
- U.S. Environmental Protection Agency (EPA). (2000). *Supplementary guidance for conducting risk assessment of chemical mixtures*. EPA/630/R-00/002. Washington, DC: Risk Assessment Forum.
- World Health Organization (WHO). (2001). *Ad Hoc expert consultations on risk assessment of microbiological hazards in foods*. Report on the joint FAO/WHO expert consultation on risk assessment of microbiological hazards in food. Geneva, Switzerland: WHO Headquarters.

Preliminary Risk Assessment of a Novel Antifungal Defensin Peptide from Chickpea (*Cicer arietinum* L.)

Aparna Islam

International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi, India

Abstract

Risk assessment of an antifungal peptide is a prerequisite before using the corresponding gene to produce broad-spectrum, fungal-resistant GM crops. In this study, a new, antifungal defensin peptide (Ca-AFP) isolated from chickpea (Cicer arietinum L.) was evaluated for biological activity, stability, and range of toxicity to determine risks in using the gene for transgenic crop production. The biological function of Ca-AFP was found to be highly stable in extreme pH (range from 2 to 10) and temperature (up to 100°C) conditions. The peptide also showed unaltered efficiency in a wide range of temperatures (from 15° to 42°C) indicating a wide functional temperature. Despite extreme stability, it was non-toxic to non-target organisms (e.g., bacteria and insect cell lines). When the viability of human cell lines together with erythrocytes were tested, the peptide was also found to be non-toxic. Only one B-cell epitope was determined with this amino acid sequence. Immunological tests with mice failed to develop any antibody, indicating the non-immunogenic nature of the peptide, and thus, the negligible possibility of it being an allergen. All these preliminary assessments suggest that Ca-AFP is a noble peptide worthy of exploring its efficiency in transgenic crops to combat fungal pathogens.

Keywords

Antimicrobial compound, toxicity, biological activity stability, human cell, allergicity

Introduction

Risk assessment is a prerequisite for the commercialization of any genetically modified (GM) crop, as regulatory bodies require information regarding the potential risk of releasing them into the environment (Dutton et al., 2003). With the increasing trend to produce GM crops commercially, concerns over the safety of these crops and crop products have increased very relevantly as demonstrated by several Multilateral Environmental Agreements, such as the Convention on Biological Diversity and the Cartagena Protocol of Biosafety (Young, 2004). The main biosafety concerns at the present time include the safety of human health, non-target organisms, and the environment, as well as the risk of horizontal gene flow (Snow et al., 2005). Therefore, any gene or gene product with potential beneficiary impact must be assessed for its risks before using it to produce any GM crop.

A large portion of research is going on to develop GM crops with resistance to biotic stresses like insects, weeds, viruses, and fungi. Among the pathogens, fungi have been identified as the most notorious group since they cause 70% of the major crop diseases (Agrios, 1997). At present, applying fungicides is the most widely practiced method to reduce crop losses from fungal attack. However, due to an absence of natural resistance, an intensive search is going on to develop a safer and more effective fungus-control method. Consumers' concern over a possible environmental impact associated with exposure to fungicides also acts as a driving factor.