Virulence factor–activity relationships: workshop summary

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ABSTRACT

The concept or notion of virulence factor–activity relationships (VFAR) is an approach for identifying an analogous process to the use of qualitative structure–activity relationships (QSAR) for identifying new microbial contaminants. In QSAR, it is hypothesized that, for new chemical contaminants, their potential acute or chronic toxicity may be reasonably estimated on the basis of structural relationships to other known toxic contaminants. Thus the parallel that is being attempted for pathogenic microorganisms is that known virulence factors may be used as predictors for identifying undiscovered pathogens and microbial causes of emerging diseases. Advances in molecular biology, genomics and proteomics have led the Committee on Drinking Water Contaminants of the National Research Council, as requested by the EPA, to recommend the VFAR approach as a potentially more systematic and scientific process for the selection of microorganisms for inclusion in the Contaminant Candidate List (CCL).

Key words | immune response, pathogenesis, virulence factors, waterborne

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In response to the National Research Council (NRC 2001) recommendations, the US EPA organized a workshop (at Wyndham Baltimore Inn Harbor Hotel, Baltimore, MD, October 28–29, 2004) to explore the feasibility of incorporating a VFAR approach as a tool for the selection of potential microbial contaminants for inclusion in the CCL. Currently there are information gaps and technological challenges for the immediate application of VFAR. For example, it is not known yet how well virulence factors and their relationship to public health impacts will parallel chemical structural–activity relationships; it is not known how many virulence genes have yet to be discovered, sequenced and the relationship between the various components sufficiently understood to assess their significance; the effect of host susceptibility factors is not fully understood; it is unclear if the concept of VFAR is applicable to obligate parasites such as viruses. Thus the purpose of the VFAR workshop was to seek advice from experts in the area of microbial virulence to identify information gaps,

short- and long-term research needs that would help define the role of VFAR in the CCL process, and help evaluate the merit of virulence factors for both current CCL pathogens as well as unknown pathogens.

The invited experts addressed the following topics:

- the EPA's interest in VFAR (presented by Dr Jeffery K. Griffiths of Tufts University, Boston, MA and not included in this special issue),
- virulence factors and their mechanisms of action (Casadevall & Pirofski 2009),
- linking the detection and identification of virulence factors to pathogenesis (Edberg 2009),
- diversity of microbial virulence factors (Chopra *et al.* 2009),
- VFAR for the detection and identification of viruses and protozoa (Fayer *et al.* 2009),
- VFAR for the detection and identification of pathogens that have emerged in the past 30 years (Cangelosi 2009),
- approaches to find future emerging pathogens in addition to VFAR (Sobsey 2009),

- available technology for examining virulence factors now and in the future (presented by Dr Timothy Straub of the Pacific Northwest Natural Laboratory, Richland, WA and not included in this special issue).

This summary is an attempt to identify the key issues from each topic as addressed in its respective paper and workshop presentation.

US EPA'S INTERESTS IN VFAR

The VFAR approach reflects the EPA's interest in utilizing a rational and systematic tool for identifying potentially pathogenic organisms in water. The selection process for inclusion of microorganisms in the CCL has been primarily a retrospective process based on a compilation of information from expert panels. The EPA is interested in pursuing an approach for the selection of CCL organisms that is more systematic and more transparent.

In 2001, the National Research Council recommended that the EPA consider the use of virulence factor–activity relationships as part of the process for classifying microbials for regulatory consideration. The underlying basis for the recommendation is that full genome sequencing of microbials is occurring and is likely to continue in the future. Analysis of such sequences could be used to deduce genes and their transcription mechanisms based on the field of genomics. The translated proteins and likely structures could be deduced based on knowledge from the field of proteomics. The relationship that the proteins, and potentially more complex molecules, have with known virulence factors would be used as the rationale for considering the organism as a potential pathogen. Griffiths *et al.* (presented and not included in this special issue) provide a description of the VFAR concept and some of the EPA's interests in assessing the merit of VFAR as a tool for the selection of potential pathogens into the CCL.

VIRULENCE FACTORS AND THEIR MECHANISMS OF ACTION

A thorough understanding of virulence factors and their mechanisms of action will be necessary to fully evaluate the

role VFARs may play in the selection of microorganism for future CCL. Casadevall & Pirofski (2009) provide a historic perspective of microbial pathogenesis and its relationship to the host.

Evolving concepts of microbial virulence

The concepts of microbial virulence and what constitutes a pathogen have changed and evolved over time. Initially, pathogens were considered to have specific properties that made them different from non-pathogens. These early notions of pathogens are now regarded as micro-centric, that is, the capacity to inflict disease resides in the microbe. As knowledge of the immune response and the various coordinated responses of the host to the pathogen increased, notions of pathogens shifted to a host-centric perspective, that is, the immunological status of the host affects the outcome of the disease. More recently, Casadevall & Pirofski (1999) proposed an inclusive approach to microbial–host interactions and microbial pathogenesis named the damage–response framework (Figure 1).

The damage–response framework

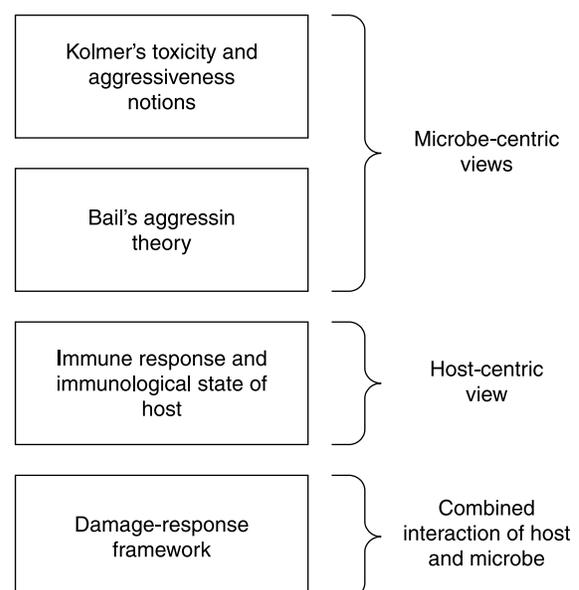In the damage–response framework, a virulence factor is defined as a microbial component that can damage a

Figure 1 │ The damage response framework.

susceptible host. Thus a pathogen is a microbe capable of causing host damage and virulence is the relative capacity of a pathogen to cause damage in a susceptible host. The damage–response framework takes into account that damage to the host could occur from conventional virulence factors such as toxins, microbial invasion or due to an excessive immunological response by the host. It has three basic principles:

- Microbial pathogenesis is the outcome of the inter-actions between two entities, a host and a microbe.
- The relevant outcome of the host–microbe interaction in a given host is damage in the host.
- Host damage can reflect the action of microbial factors, the host response or both.

Types of virulence factors

Virulence factors tend to fall into seven broad categories based on mode of action or mechanism of pathogenesis:

1. The ability to cause direct damage to a host.
2. The ability to enter a host.
3. The ability to evade host defenses.
4. The ability to grow in a host environment.
5. The ability to counteract host immune responses.
6. The ability to acquire iron and nutrients from the *host* environment (to differentiate it from the *water* environment).
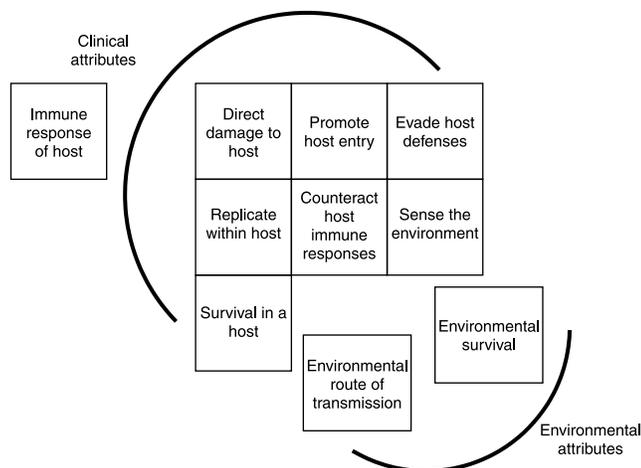7. The ability to sense environmental change within the host.

Figure 2 | Categories of virulence factors.

Although these broad categories are useful for understand the role different virulence factors play in infection, it should be kept in mind that virulence factors can overlap more than one category. The immune response of the host needs to be considered and, for waterborne or environmentally transmitted pathogens, an environmental route of transmission and survival are important attributes as well (Figure 2).

It is unclear at this point whether there are virulence factors that confer environmental resistance or whether environmental survival should be considered instead as a microbial attribute since it does not directly result in damage to the host. Nonetheless, from an environmental perspective, an effective pathogen needs to be capable of demonstrating toxicity, aggressiveness and survivability.

LINKING THE DETECTION AND IDENTIFICATION OF VIRULENCE FACTORS TO PATHOGENESIS

The successful implementation of VFAR in the regulatory framework will depend on detailed knowledge of a broad range of virulence factors including environmental persistence, entry and survival in the host, means of reproduction, means of producing disease, and the means by which the microbe leaves the host.

Once the virulence factors are identified, molecular analytical methods would presumably be used to detect the activity relationships of those factors to other potential pathogens in a water sample. Edberg (2009) cautions that it still remains to be ascertained whether possession of virulence factors means that those genes will be active and whether additional information would be required to determine if the microorganism possessing the virulence factors is indeed a pathogen.

In order for a microbe to produce disease, a number of sequential virulence factors must be active. Containing genes or DNA sequences associated with virulence factors is not sufficient since disease generation is a phenotypic phenomenon. In mammals and humans, each living cell, differentiated or not, contains all the genetic information needed for a whole new being. Similarly in plants, somatic cells contain all the genes necessary for the formation of the whole plant. The mere presence of those genes does not

imply that they are active. Nonetheless the argument could be made that, if the environment surrounding the cell changes, previously repressed genes could be activated.

Gene regulation in bacterial and eukaryotic cells (protozoa, yeast and fungi) is a complex process of induction, repression and modulation of gene expression. The VFAR concept needs to incorporate the key genetic elements associated with the expression of the virulence factors of concern. Virulence factors could potentially be divided into constitutive virulence factors, those expressed at all times, and inducible virulence factors, expressed in response to a host environment.

Some of the more traditional virulence factors, e.g. toxins or antibiotic resistance, may be coded in movable genetic elements such as transposons, plasmids or bacteriophages. The role these movable genetic elements play in virulence needs to be clearly understood in order to integrate them into a VFAR concept. As potentially movable virulence factors, there may be some inclination to develop analytical methods specifically targeting them.

Environmentally transmitted pathogens or those organisms that utilize a fecal–oral route of transmission must first survive host gastrointestinal tract defenses, successfully compete with normal gastrointestinal flora and express virulence factors conducive to gastrointestinal infections (Figure 3).

DIVERSITY OF MICROBIAL VIRULENCE FACTORS

The diversity of virulence factors that can be found in a single pathogenic bacteria is illustrated in *Aeromonas hydrophila* by Chopra *et al.* (2009). *Aeromonas* is listed in
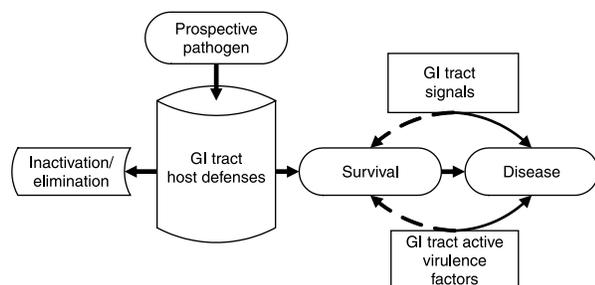
the CCL because of its potential to grow in distribution systems and its potential for both waterborne and water-associated illness. This organism causes an array of illnesses including diarrhea, septicemia, meningitis, skin wounds, respiratory tract and ocular infections. A variety of virulence factors have been found in *Aeromonas* including a capsule, endotoxin, flagella and fimbrae, lipases, hemolysins, cytotoxin, enterotoxins, proteases, type III secretion system and others. A considerable amount of work had to be performed in order to isolate and understand the mechanism of action of each of these virulence factors of *Aeromonas*.

Of particular interest for the concept of VFAR are three enterotoxins from a diarrheal isolate of *A. hydrophila*: (1) the cytotoxic enterotoxin (*Act*) with a molecular weight of 49–52 kDa; (2) a heat-labile enterotoxin (*alt*) with a molecular weight of 44 kDa; and (3) a heat-stable enterotoxin (*Ast*) with a molecular weight of 71 kDa. Toxins are, perhaps, the virulence factors best suited for a VFAR approach. For example, work by Chopra *et al.* (2009) indicates that the *Act* molecule has several domains or biologically active regions. Two such regions are amino acid residues 245–274 and 361–405 and amino-acid substitutions in these regions can affect one or more of the biological activities of the toxin. Transposon mutants of *Act* also displayed altered virulence of one or more of its biological activities.

The structural knowledge gained by the various studies conducted by Chopra and his collaborators in combination with genomics and proteomics could be a starting point for finding similar toxin genes in other related microorganisms.

VFAR FOR THE DETECTION AND IDENTIFICATION OF VIRUSES AND PROTOZOA

The key questions are whether viruses have virulence factors and whether knowledge at the genomic level or proteonomic level could lead to predictions as to which viruses are likely to emerge as new human pathogens.

If a gene or gene by-product is deleted or removed from the virus and it renders it no longer infectious or capable of causing damage to a host cell, then it should be regarded as a virulence factor. Damage to a host cell can be interpreted

Figure 3 | Gastrointestinal tract infections.

in its most dramatic form, lysis by the action of late proteins, or in its mildest form, competing with the host cell for amino acids and nucleic acids for incorporation into viral-coded macromolecules in persistent infections.

Since viruses are obligate intracellular parasites and do not have free living forms, it has to be assumed that the VFAR concept would be best applied to (1) ascertain which animal viruses may be able to infect humans in the near future; (2) determine if mildly virulent strains may mutate to strongly virulent new strains; (3) determine if mutations or genomic changes would confer a non-environmentally transmitted virus sufficient resistance to be transmitted via the water route or (4) determine the likelihood that traditionally waterborne or environmentally transmitted viruses develop enhanced resistance to conventional treatment processes.

Severe acute respiratory syndrome (SARS) and avian influenza viruses have raised concerns over zoonotic transmission of viruses to humans. Fayer et al. (2009) point out recent literature suggesting that hepatitis E may occur as a zoonosis. Viruses in swine, deer and cats that are antigenically related to hepatitis E virus have been associated with human illness. In addition, other animals have been found to have antibodies to viruses possibly related to the hepatitis E virus. Although from an epidemiological perspective the evidence for hepatitis E zoonosis is currently relatively weak and limited to a few cases, there is some likelihood that this may occur in the future at a wider, perhaps epidemic, scale.

In protozoa the identification of virulence factors is more direct and follows more closely the traditional definitions for virulence that originated primarily from bacterial pathogens. The role of the host in protozoa and parasitic infections is very important, in particular, age and immune status of the host. With Cryptosporidium, age and immune status are strong determinants for species or strains that can cause illness (Fayer et al. 2009).

VFAR FOR THE DETECTION AND IDENTIFICATION OF PATHOGENS THAT HAVE EMERGED IN THE PAST 30 YEARS

Cangelosi (2009) points out that the successful application of VFAR to emerging waterborne pathogens will depend on the resolution of several issues: (1) the ability to distinguish between human primary pathogens and opportunistic pathogens; (2) virulence factors or traits are likely to exhibit a greater degree of variation in organisms which are not strict or obligate pathogens than those which can both live in the environment and infect human hosts; (3) the ability to distinguish between VFARs that affect humans from those affecting other mammals, identification of new virulence markers and demonstrating their functionality in the isolated organisms and (4) the demonstration of quantitative levels of expression.

The predictive value of VFAR for emerging waterborne pathogens is challenged by the use of similar factors by non-pathogenic microorganisms for persistence in non-human habitats. These factors are likely to give a background signal and include adhesion factors, fimbria, scavenging mechanisms, quorum-sensing mechanisms and other similar factors involved in biofilm formation, adaptation to living within protozoa or animal host cells, and factors involved in the infection of non-human species.

Analytical sensitivity may limit the applicability of VFAR in environmental samples, where targeted genomic sequences are much lower in overall numbers relative to an abundance of non-target sequences.

OTHER APPROACHES FOR FINDING EMERGING PATHOGENS

Diverse viewpoints occur amongst scientist as to the feasibility of the VFAR concept to make useful predictions of new and emerging pathogens. Sobsey (2009) contends that the diversity of microbes, the variety of mechanisms and properties of virulence and pathways to pathogenicity are too varied to predict their emergence as human pathogens. In his viewpoint, the emergence of new waterborne pathogens will be stochastic or random and thus not amenable to the deterministic tools, i.e. genomics and proteomics, proposed for VFAR.

Sobsey (2009) proposes that alternative approaches to VFAR that rely on past experience and current knowledge of factors contributing to pathogen emergence be the basis for selection of emerging pathogens for the

CCL and regulatory consideration. Some of these factors may be:

- animal reservoirs of microbes which could lead to zoonotic infections and subsequent adaptation to human hosts,
- changes in environmental conditions that lead to new or broader geographic prevalence which would result in turn to new human pathogens,
- immunologically naive humans or human subpopulations migrating into areas where pathogens they have not previously been exposed to are prevalent,
- movement of genetic elements that confer human pathogenicity traits to new microbes,
- human activities that result in genetically altered microbes, e.g. antibiotic resistance, etc.

Active surveillance systems that incorporate these and other possible factors may be more effective at detecting emerging pathogens, although it could be argued that some of these factors would not necessarily lead to new or emerging pathogens but rather broader geographic distribution of previously known pathogens, and some of the factors discussed by Sobsey would not necessarily lead to environmentally transmitted pathogens.

AVAILABLE TECHNOLOGY FOR EXAMINING VIRULENCE FACTORS NOW AND IN THE FUTURE

One of the challenges with the VFAR approach is one of definition of its purpose. The proposals developed by the National Academy of Sciences and the National Drinking Water Advisory Council often equate a molecular-based approach for detection of pathogens in water with VFAR. In other words, the analytical tool used to query the environment, i.e. microarrays, is often confused with the purpose for querying the environment for new or potential pathogens.

As indicated by Straub et al. (presented but not included in this special issue), microarrays or other techniques used for multiplexed querying of environmental samples is dependent on the ability to concentrate the organisms from a relatively large volume of water and on the removal of assay inhibitors. Assuming these technological obstacles are overcome, microarrays can essentially be used as

applied genomics. Their potential application can be summarized as follows:

- Microarrays can be used as a detection platform for known sequences.
- Multiple sequences can be queried by an array.
- Microarrays rely on hybridization as a detection method.
- Pathogen or virulence factor sequences from environmental samples may need to be amplified to achieve sufficient sensitivity.
- Capture-and-detect or sandwich arrays may increase the sensitivity of the assays.
- Expression arrays may be used to determine if virulence factors are being actively expressed.
- Post-transcriptional and post-translational regulation will be more difficult to address.
- Specificity of detection needs to be designed into the hybridization sequences selected.

RECOMMENDATIONS

In addition to the information gaps and research needs identified in each paper, five general recommendations came out of the workshop:

1. Overall, the VFAR approach may be too simplistic to apply to a wide range of virulence factors.
2. It may be a useful tool in a limited context where it could be potentially applicable, e.g toxins.
3. It may be better suited if used as part of a selection criteria and not as the sole criterion.
4. The analytical technology needed to support the approach needs further development.
5. It is important to avoid making the analytical tools of molecular biology, probes and microarrays synonymous with the VFAR approach.

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