Does the possession of virulence factor genes mean that those genes will be active?

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ABSTRACT

There are a number of relationships the host can establish with the microbes we ingest. For the vast majority of microbes, they have a short-lived liaison with the human host. Either they are destroyed by the stomach acid or bile, or can not establish even a temporary residency in the gastrointestinal tract. Early in life the mucosal surfaces of the body establishes a resident, and generally stable, normal flora. These normal flora microbes, the majority of which are bacteria, have specific receptors for specific areas of the alimentary tract. If the foreign microbe can establish residency, it then may transiently or permanently become part of the normal flora. However, in order to produce disease, it must possess an additional set of virulence factors. While some of these are known, many are not. Those that are known include enzymes, such as protease, lipase, and esterase. Accordingly, VFAR may not be associated with human disease and its presence or absence has no public health meaning.

Key words | gene functionality, virulence factor

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INTRODUCTION

The 1996 Safe Drinking Water Act amendments require the EPA (US Environmental Protection Agency) to publish every five years a list of currently unregulated contaminants in drinking water that may pose risks. In addition, it must determine whether or not to regulate at least five contaminants. In May of 1997 the EPA convened a workshop on microbiology and public health to develop a list of pathogens for possible inclusion. The basic criteria were (1) public health significance, (2) known waterborne transmission, (3) occurrence in source water, (4) effectiveness of current water treatment and (5) adequacy of analytical methods. The results of the deliberations were adopted by NDWAC (National Drinking Water Advisory Council) and subsequently utilized by the EPA to select microbiological contaminants placed in the 1998 CCL (Candidate Contaminant List) draft. Microbiological contaminant candidates included adenoviruses, *Aeromonas hydrophila*, caliviruses, coxsackieviruses, cyanobacteria, echoviruses, *Helicobacter pylori*, Microsporidia (*Enterocytozoon* and *Septata*) and *Mycobacterium avium intracellurae* (MAC). Of these,

Aeromonas monitoring was added to the list of unregulated contaminants to be monitored by the larger utilities (Havelaar *et al.* 1987; EPA 1999*a,b*). Subsequently, the EPA recognized that only some clones of *Aeromonas* possessed virulence factors that allowed them to be potential human pathogens. Accordingly, the EPA modified the monitoring scheme to include the sending of isolates of this genus to the EPA Research Laboratory in Cincinnati for virulence factor analysis (EPA 1996, 1997*a,b,c*, 1998, 1999*a,b,c,d*, 2000*a,b*).

In 2004, the EPA, in the Code of the Federal Register (*Federal Register*, **69**, April 2, 2004) published "Drinking Water Contaminant Candidate List 2", which included the same microbes as 1998, but also included a review of the National Research Council (NRC) report "for developing a more comprehensive and transparent CCL listing process". As part of that notice, the EPA requested comments on the NRC's recommendation to pursue not indicators of fecal pollution, or the pathogens themselves, but surrogates of virulence factors, which it called "Virulence Factor–Activity Relationship", or VFAR. Essentially, one first

establishes the important virulence factors of gastrointestinal pathogens. Once these virulence factors are known, a model is constructed in which chemical (e.g. a protein, adhesion) structures demonstrating those virulence factor activities are generated. One may then analyze a drinking water sample for chemical structures that have sufficient public health importance, rather than performing a biological assay. The successful implantation of the VFAR strategy depends on detailed knowledge of the broad range of virulence factors, which include, as shall be described, functions of 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. Armed with this large bank of genetic structures (e.g. Adam 2000; DOE 2000), a format such as microarray analysis (simultaneous amplification of many individual DNA sequences on a chip) or multiplex PCR is designed (DeRisi & Iyer 1999; Sulaiman et al. 1999; Anthony et al. 2000; Xiao et al. 2000; Nesic et al. 2004). Inherent in the success of the VFAR strategy is the assumption that the DNA sequences of consequence are stable in the microbe. Ideally, DNA sequences of importance will be shared amongst the pathogens of interest, hence the analysis for "Activity Relationships". This paper is limited to the question: does the possession of virulence factor genes mean that those genes will be active, and the sub-question, is there further information that is required to answer the question?

VIRULENCE FACTORS, THE MICROBE AND THE HOST

$$\text{Disease} = \frac{(\text{number of microbes}) \times (\text{virulence factor of microbes})}{\text{specific immunological status of the host target organ system}}$$

The process to disease

There are a number of relationships the host can establish with the microbes we ingest. For the vast majority of microbes, they have a short-lived liaison with the human host. Either they are destroyed by the stomach acid or bile, or cannot establish even a temporary residency in the gastrointestinal tract. Early in life the mucosal surfaces of

the body establishes a resident, and generally stable, normal flora. These normal flora microbes, the majority of which are bacteria, have specific receptors for specific areas of the alimentary tract. Accordingly, once in place, in order for a "foreign" microbe to attach it must have that same attachment mechanism (e.g. glycocalyx or adhesion) and must find a vacant "parking spot". After it attaches, it must have the ability to survive the hostile environment of the gastrointestinal tract, which includes lowered oxygen tension, high salt concentration, bacteriocins, various host-cell-produced proteases, lipases and esterase, etc (Duncan & Edberg 1995) (Table 1). If the foreign microbe is resistant to particular antibiotics, and the host is receiving those antibiotics, there is a marked facilitation to establish residency. For example, *Pseudomonas aeruginosa*, when ingested, will not establish residency in the gastrointestinal tract of humans. However, if patients are administered antibiotics such as ampicillin, to which this species is innately resistant, residency (also known as colonization) can occur. When the antibiotics are discontinued, the

Table 1 | The host defenses of the gastrointestinal tract (from Duncan & Edberg 1995)

Site	Major defenses
Oropharynx	Lysozyme
	Production of liquids
	Normal flora (attachment and bacteriocins)
	IgA antibody
	Proteolytic enzymes in saliva
Esophagus	Peristalsis
Stomach	Acid pH
	Proteolytic enzymes
Small intestine	Peristalsis
	Mucin production
	Bile acids
	IgA antibody
	Primary lymphoid system (Peyer's patches)
	Epithelial shedding
	Normal flora
Large intestine	Peristalsis
	Normal flora
	Epithelial shedding
	Mucin production

colonization ceases (Hardalo & Edberg 1997). The ability to withstand stomach acid and bile, and the possession of adhesion glycocalyxs or other molecules for specific target cells, are microbial virulence factors (Sarker & Gyr 1991).

If the foreign microbe can establish residency, it then may transiently or permanently become part of the normal flora. However, in order to produce disease, it must possess an additional set of virulence factors. While some of these are known, many are not. Those that are known include enzymes, such as protease, lipase or esterase (Table 2). In addition, the end product of these factors can be assayed. Two such end products are cytotoxicity and cell invasion. Some microbes, such as viruses, can cause disease not by the production of extracellular virulence factors but by invading the host cell and causing its disruption (Payment *et al.* 1991).

Disease occurs functionally when there is a pathologic change to the host target cell or organ. For health risk assessment purposes, we generally are alerted to these changes by the induction of signs (e.g. fever, malaise) or symptoms (e.g. cramping, diarrhea). Medical epidemiologists have referred to the overall cluster of signs and symptoms as an acute gastrointestinal event (ACGE) or HCGI (highly credible gastrointestinal illness) (Payment *et al.* 1991). However, it is well known in medicine that a certain percentage of infections by a given microbe may not result in obvious disease, but be sub-acute (Barnes & Dourson 1988; Rusin *et al.* 1997; Haas *et al.* 1999; Schaub & Oshiro 2000; Selevan *et al.* 2000). In this situation, the microbe apparently possesses the needed virulence factors, but host immunology limits the interaction not to discernable disease but to infection. These patients may transmit the microbe to other patients and contaminate the environment. Also, they may suffer the same sequelae from infection as those patients with disease. Lastly, some patients may recover clinically from the disease but continue to shed the microbe for a period of time. This situation is known as the carrier state.

EFFICACY OF VFAR, AND VIRULENCE FACTOR, ANALYSIS: CONSIDERATIONS

The National Research Council (NRC) has recently published the results of its analysis of EPA research and goals and recommended that it concentrate its energies for microbiological protection of drinking water to Virulence Factor Associated Relationships, or VFAR. A complete description of VFAR is to be found in this issue. In summary, the NRC said that the EPA should proceed down a pathway similar to that used by the pharmaceutical industry to create new drugs. For several years, one process of new drug generation has centered on molecular modeling. For example, basic research has demonstrated that certain antigenic types of human papilloma virus are associated with the development of cervical carcinoma. Molecular modelers would then generate an accurate picture of the antigenic protein molecule on the surface of the virus that attaches to its specific receptor on its target epithelial cell. Employing this proteonomic information, molecules that have an inhibitory shape to this receptor would be generated and administered to the patients in the hope that they would prevent binding of the virus and hence limit or prevent the disease. This modeling process precludes the often haphazard means of classical drug discovery and can be considerably time- and money-saving. However, it depends on a detailed knowledge of the underlying pathogenesis of the disease and exact, or very close, information about the structure of the various ligands.

Successful implementation of this chemical modeling process also depends on a situation that does not exist in microbiology—that the attachment of the drug to its receptor is static, and not highly mutable. Unlike chemistry, microbiology is highly dynamic. Virulence factors may not be naturally present when the microbe is in the environment but only induced after they enter the host, and under very specific microenvironmental circumstances (e.g. pH, salt, heat) (see Table 3). These factors may be unknown, or difficult to reproduce in a laboratory setting.

Lye & Dufour (1991) analyzed a number of commonly accepted extracellular enzyme virulence factors (e.g. protease, lipase, esterases) from naturally occurring HPC drinking water bacteria, and generally found them lacking. As part of the discussion of this paper, they suggested that a potential strategy for drinking water analysis could be to analyze samples for virulence factors rather than specific microbes. Because virulence factors may be shared amongst a broad range of pathogens, public health protection could

Table 2 | Major microbial virulence factors active in the gastrointestinal tract

Virulence factor	Specific examples	Activity
Specific attachment to intestinal epithelium	Poliovirus, *rotavirus, V. cholerae*	Epithelial association prevents washing out
Motility	*V. cholerae*, certain *E. coli* strains	Ability to penetrate mucus to reach target call
Production of mucinase (neuraminidase)	*V. cholerae*	Assists in target cell attachment
Acid resistance	*M. tuberculosis, Helicobacter pylori*, parasite cysts, enteroviruses (hepatitis A, poliovirus, coxsackieviruses, echoviruses)	Passage through stomach
Bile resistance	*Salmonella, shigella*, enteroviruses, *Enterococcus faecalis, E. coli*	Survival to reach large intestine
Resistance to proteolytic enzymes	Enteroviruses, parasites	Survival to reach large intestine
Anaerobic growth	*Bacteroides fragillis, C. difficile*	Growth advantage
Local toxin production	*V. cholerae*, campylobacter, ETEC	Outflow of water
		Hyperperistalsis
		Pseudomembrane
Systemic toxin production	*S. dysenteriae*	Distant target organ affected
Ingestion of preformed	*C. perfringens, S. aureus, B. cereus, C. botulinum*	Outflow of water
		Systemic malfunction
Perforation of the mucosal epithelium	*E. histolytica*	Abscess
Epithelial cell invasion	*Cryptosporidium, Salmonella*, viruses	Outflow of water
		Hyperperistalsis
Attachment	*Giardia*, microspora, cyclospora	Prevention of nutrient absorption

Table 3 │ Signals in the gastrointestinal tract that regulate virulence expression

Bacterium	Environmental signal
Escherichia coli	Iron, temperature, carbon source
Salmonella typhimurium	Osmolarity, starvation, stress, pH, growth phase
Shigella sp.	Temperature
Vibrio cholerae	Osmolarity, pH, temperature, amino acids, CO_2, iron
Yersinia sp.	Temperature, ionized calcium

theoretically be enhanced by examining a drinking water sample for virulence factors.

VFAR analysis is an attempt to broaden this strategy since it analyzes for a class of virulence factors rather than each individual in the class.

NRC recommended that the EPA proceed along the VFAR pathway. The EPA has two basic charges: first, the development of health risk assessment parameters by which regulations governing treatment and pathogen removal are generated; and the approval of tests (including the frequency and volume of testing) of drinking water to protect the public health. These are very different end-products, and VFAR analysis may not fit equally well into each.

SYNOPSIS OF VIRULENCE FACTOR KNOWLEDGE STATUS

Pathogenicity is a complex series of events. A microbe must first reach its target organ. Next, it must attach to its host cell. It can then enter the host cell (invade), or not. Subsequently, it must multiply. It may or may not produce toxins. It must evade host destruction. It may or may not become systemic within the host body. It may or may not establish infection at sites other than its entry site. It must leave the host. It must survive in the environment. At each stage, a series of virulence factors are activated and then deactivated. Lastly, the microbe may transmit some or all of its virulence factors to other microbes via plasmids or phages, in the case of bacteria, or gene rearrangement, in the case of viruses. To date, our knowledge base of this complex system is only beginning to be understood, and then only with the major pathogens.

Virulence factor analysis in assessing drinking water bacteria dates to EPA's Lye & Dufour (1991) report in which it was suggested virulence factor analysis could be a useful adjunct to drinking water testing. Subsequently these authors with Stelma and others have continued this investigation into the difficult use of highly immunocompromised mice to study virulence factor expression (Stelma et al. 2004). My laboratory has published on virulence factor analysis in the last decade, the latest paper appearing in 2004 as part of a World Health Organization workshop held in Geneva (Edberg 1996, 1997; Edberg et al. 1996, 1997; Edberg & Allen 2004). In Canada, Payment has examined virulence factors in naturally occurring bacteria (Payment et al. 1994). These examples of research activity exclude many others working in the field and serve only to highlight the potential importance of this approach. However, virulence factor analysis is still in development. For a microbe to generate disease, a number of sequential virulence factors must be active. While clearly the genes that code for virulence factors must be present in the microbe, disease generation is a phenotypic phenomenon. The presence of a gene does not indicate if it will be active. The gene or genes may be suppressed; may be on a transposon or plasmid, and could be lost on subculture in vitro, etc. The microbial literature dating to the 1920s clearly demonstrates this dissociation between genotype and phenotype in regards to virulence expression. In fact, the loss of virulence factors in bacteria subsequent to their cultivation in vitro was called "dissociation".

DOES THE POSSESSION OF GENES MEAN THAT THOSE GENES WILL BE ACTIVE?—ITEMS TO RESOLVE

Biology and the plenipotentiary cell

Every cell in the human body has the genes to become any one of the four main–nerve, muscle, connective tissue, epithelial cells–and subtypes of cell therein (e.g. smooth muscle, striated muscle, cardiac muscle). The differentiation of cells from the zygote into these specialized cells does not occur by a selective, permanent loss of various genes from the genome of the embryo. Each cell of the human body

contains all the genes of that organism, but in any given cell type most of them are turned off or repressed. In plants, the totipotential of somatic cells can be demonstrated. For example, an entire tomato plant may be grown from a single differentiated cell. The concept that all cells of the human body are genetically identical is supported by the fact that they contain precisely the same amount of DNA. Therefore, the study of genetic evolution teaches us that the presence of a gene, even many thousands of genes, in a cell does not mean that the genes are active.

Enzyme induction and suppression

Jacob and Monod, who studied cellular differentiation in higher animals and developed the field of study called epigenetics, were able to model many of their hypotheses in bacteria. They found that bacteria possess accurately programmed mechanisms for regulating the relative amounts of different types of proteins that are produced. For example, in their breakthrough studies on lactose metabolism they found that enzymes (in this case lactose permease and the beta-galactosidase system) in bacterial cells change in concentration in response to changes in the availability of nutrients from the environment. Regulation of the rate of synthesis of these enzymes conserves the amount of RNA and protein that the bacterium must make. In essence, bacteria are following the governing laws of thermodynamics, in particular the second law, the law of entropy. As a single-celled entity with a relatively simple (compared to mammalian) genetic regulatory system, bacteria have the ability to turn on and turn off genes by efficient suppressor mechanisms.

Bacteria respond to molecules and conditions in their environment by two types of regulatory responses: enzyme induction and enzyme suppression. Accordingly, the bacterial cells conserved energy by only producing a baseline of enzymes (constitutive enzymes) but having the genes in the inactive form to respond to changes in the environment. Pseudomonads, for example, can induce enzymes in response to hundreds of different organic compounds. While it is not within the scope of this paper to review the process of bacterial genetics in detail, it is essential to note that there are genes that code for proteins that are gene repressors. These repressor molecules bind to a segment of the bacterial DNA, preventing the transcription of the structural gene to form mRNA. In the absence of an inducer the repressor molecule is bound to the structural DNA. When an inducer is present, it combines with the suppressor protein at a specific binding site to form an inactive repressor–inducer complex. This complex is not able to bind to the structural bacterial DNA, thus the structural DNA is unimpeded to transcribe. In bacteria, genes on the structural DNA are often in close proximity to each other, and are known as an operon. Therefore, a single repressor molecule may, with some enzyme systems, turn off a gene system by binding in the operon, in the operator. The operator, in effect, is the gene cluster switch. VFAR must account for microbial regulation.

Transposons, bacteriophages and plasmids: variability of gene expression and function

The DNA molecule of bacteria and viruses contains pieces of DNA, called transposons, that can move around the "genome". They are similar to lysogenic bacteriophages in that they can integrate into the bacterial DNA genome; however, transposons originate in the genome whereas lysogenic phages are extra-chromosomal. Moreover, lysogenic phages may revert from quiescent to active, both coding for and causing the production of virulence factors (such as diphtheria toxin) and can become a complete bacteriophage, multiply within the cell, and cause the cell to burst and infect other cells. Plasmids are circular, double-stranded DNA molecules that are found in the bacterial cell independent of the genome and can replicate independently of it. A plasmid is stably inherited, but is not required for the host cell's growth or reproduction.

Transposons can exert a number of important effects within the bacterial cell that can affect virulence: it can insert within a gene to cause a mutation or it can stimulate DNA rearrangement, leading to deletions of genetic material. In its simplest form, transposons are insertion sequences; others are more complex, and can carry multiple genes. Some transposons carry stop codons or termination sequences that can stop translation or transcription. Some transposons carry promoters and can activate genes. Transposons can be located within plasmids and participate in plasmid fusion and insertion. A number of different

transposons can insert into a single plasmid. For example, the insertion of several different plasmids, each coding for resistance to one antibiotic, can insert into a single plasmid, thus resulting in a multi-antibiotic-resistant bacterium, which can transmit this multi-resistance to other bacteria.

Plasmids, which can code for a broad variety of virulence factors, including toxins, antibiotic resistance and attachment adhesions, may not be stable within a bacterial cell. A bacterium can spontaneously lose a plasmid. Or the plasmid can be lost through an induction process such as ultraviolet light, molecules that intercalcate within DNA and high temperatures.

Accordingly, transposons, lysogenic bacteriophages and plasmids engender considerable variability into any process that analyses the presence, absence or activity of virulence factors and must be understood for VFAR.

Relationship between structure, function and avidity

In order for VFAR to be successful, there must be a detailed understanding of the interaction (i.e. structures, bindings, avidities) between the virulence site on, or factors from, the microbe and the corresponding binding site on the host. VFAR, being a relationship which would be representative of a group of closely related ligand–host interactions rather than a specific ligand–receptor interaction, is dependent on knowledge of the structure of both the microbe product, and the host receptor. One must understand not only how the ligand fits into the receptor but what is its avidity constant of binding. Inherent in VFAR is the choice of an avidity constant for the binding of the closely related structures that is stringent enough to include ligand–receptor combinations of importance, but not broad enough to include ligand–receptor combinations that have no clinical utility. By analogy to the drug discovery process in chemical pharmacy, from which VFAR is drawn, in order to develop a set of candidate therapeutic molecules, detailed knowledge of the three-dimensional structure of the ligand and the receptor is modeled so that one can construct analogous molecules of proper avidity to bind to their target. Information is just beginning to be accumulated regarding the actual structure of virulence factors and the interaction with their ligands. For example, many gram-negative pathogens including Escherichia coli, Shigella dysenteriae, Campylobacter species, and Helicobacter have a closely related toxic activity that results in the host cell of an arrested cell cycle and its subsequent death. This virulence factor (toxic activity in this case) is associated with the products of a bacterial operon that can generate three proteins, called CdtA, CdtB and CdtC. These three proteins combine to form a complex called cytolethal distending toxin (CDT). Only recently has the crystal structure of these proteins and the subsequent CDT been ascertained (Lilic et al. 2003). A typical question would be: should CDT, because of its commonality in a number of gram-negative bacteria, be chosen for VFAR analysis? Further information regarding possible mutation frequencies of the individual CDT constituents when changes in the avidity of the individual constituents result in a non-functional end product, the extent of CDT in pathogens and the extent of CDT in non-pathogens must be developed (Lilic et al. 2003). In addition, one must develop information regarding the control, feedback inhibition and induction of CDT and its constituents. Similarly, gram-negative bacterial pathogens inject virulence factors into host cells through a type III secretion system which require specialized bacterial chaperones. These chaperones can inject bacterial virulence proteins directly into host cells. Small changes in the shape of these molecules can alter their function (Stebbins & Galan 2003). The exact shape and avidity of interactions like these must be understood as a basis for VFAR analysis, and that knowledge is just being developed, and only at highly specialized laboratories.

Tadpoles, frogs and the microenvironment

As discussed above, the microenvironment is responsible for the activation of many virulence factor genes (see Table 3). Each cell in the tadpole has exactly the same genetic constitution as the frog. Yet, tadpoles remain tadpoles until the water temperature is right. Then, a broad range of genes are activated, suppressed, etc. Because a microbe has the gene to code for a specific virulence factor does not necessarily mean that it will become active. For example, a drinking water sample may contain spores of Clostridium botulinum. A genetic amplification would demonstrate the DNA sequences coding for this toxin. However, the spore will not activate in the host via ingestion.

Many toxins and other virulence factors are coded for by bacteriophages. These may be found in a drinking water sample not associated with bacteria. Accordingly, the detection of the virulence gene does not mean a health risk.

Many relatively innocuous bacteria share a virulence factor with an actual pathogen but do not have the complete set of virulence factors necessary to cause infection. For example, *Citrobacter diversus*, which is not a gastrointestinal pathogen, can carry Vi antigen, which is a major virulence factor of *Salmonella typhi*. However, *C. diversus* does not have the virulence factors necessary to survive inside host cells and does not cause infection. Some bacteria have sets of virulence factors found in pathogenicity islands. If one gene is missing, either through deletion, suppression or mutation, the microbe will not cause infection. However, a number of virulence genes could be detected (Marshall & Hodgson 1998).

Cryptosporidium and ultraviolet light: an environmental model

Cryptosporidium has all of the genes necessary for a fully functioning nucleotide excision repair process. If one performed a probe for this set of genes, it might be deduced that *Cryptosporidium* can repair UV damage. Moreover, UV may not be a reliable disinfectant for *Cryptosporidium*. However, numerous studies demonstrate that UV is effective and that, once inactivated by UV, *Cryptosporidium* does not regain pathogenicity.

SUMMARY AND CONCLUSIONS

Each cell in the human body contains all the genetic code to make it any other cell. If one probes a muscle cell, one would find all the "nerve cell" genes. The production of a muscle cell is the result of phenotypic expressions, repressed genes, etc. If one probes the cell of a tadpole, one finds the genetic machinery of an animal that can breath air; if one probes the genes of a frog, one finds the genetic machinery of an animal that can remove oxygen from water. If one examines the genetic machinery of *Cryptosporidium parvum*, one finds that this parasite can

repair itself from ultraviolet light damage. Clearly, the presence of a gene does not mean that it is, or will, be active.

Moreover, even if the virulence factor coding gene was active, it would not mean that it was sufficient to cause disease. Disease is a process that has a number of steps, from entry into the body, survival of the microbe to passage to its target tissue, attachment to that tissue, interaction with the target tissue to cause disease (such as toxin production or disruption of the cellular machinery), survival on, or in, the target tissue, escape from the host, and survival in the environment (Regli *et al.* 1991; Steiner *et al.* 1997; Wadhwa *et al.* 2002). Additionally, in drinking water microbiology the ability to survive at low temperature, exist within inorganic and organic tubercles, and be resistant to oxidant disinfectants are also virulence factors (LeChevallier *et al.* 1991, 1997; Barker *et al.* 1992; Abbaszadegan *et al.* 1993, 1999; Canale *et al.* 1993). Accordingly, VFAR analysis must account for the fact that disease is multifactorial, with each segment of the process under its own regulatory control. An innocuous bacterium may possess some components of the virulence cascade. For example, *Citrobacter* can contain the Vi antigen that is a significant virulence factor for *S. tyhphi*; group C Streptococcus produces streptolysin 0, the major virulence factor that results in rheumatic heart disease, which is only caused by Group A Streptoccus; most gram-negative bacterium produce endotoxin, including many of the innocuous HPC; and *Pseudomonas aeruginosa* produces exotoxin A, which, as a protein can cause gastroenteritis, but because the species cannot easily establish residence in the gastrointestinal tract, it is not a cause of health risk from ingestion. Research is only in the early stages in putting together the pieces of the puzzle, and even knowing which are the essential pieces. In this regard, structure, function and avidity of virulence factor proteins must be known, as well as their genetic stability. How virulence factors remain stable within cells, or can be transferred amongst cells, also needs to be established.

Too many unknown variables exist to go directly to VFAR and bypass a diligent acquisition of knowledge of virulence factors and their expression. While the concept of predicting activity based on structure is quite powerful in the drug discovery arena, it remains only a notion in the world of medical microbiology. It is unlikely that how

the liver produces cholesterol will change; hence drugs designed to lower it based on molecular modeling can be efficient. However, microbes change, sometimes even as they are being measured and analyzed. Prior to VFAR, much must be learned about the virulence factors and their expression(s) and stability. Unlike eukaryotic cells, prokaryotic cells achieve diversity via high rates of mutation and exchange of genetic elements. VFAR can evolve over time as knowledge of virulence factors develops.

ACKNOWLEDGEMENTS

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