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Avoiding Selective Pressure: Using Genomics to Design Anti-Virulence Drugs

Current antimicrobial drugs target genes that are essential for survival, creating

immense selection pressure for populations to develop resistance. One way around this

problem is for antibiotics to target virulence genes instead of essential genes. Because

many virulence genes do not affect survival, anti-virulence drugs would exert less

selection pressure. In the face of rising antimicrobial resistance, genomics and

bioinformatics offer new opportunities for understanding pathogenesis and identifying

new drug targets.

Antimicrobial resistance is widespread, rapidly rising, and deadly. A wide range

of bacterial strains that were previously susceptible to antibiotics are now resistant; these

include penicillin resistant *Streptococcus pneumoniae*, which causes pneumonia,

penicillin and tetracycline resistant *Neisseria gonorrhoeae*, which causes gonorrhea,

rifampicin and isoniazid resistant *Myobacterium tuberculosis*, which causes tuberculosis,

multiresistant *Shigella dysenteriae*, which causes dysentery, and multiresistant

Salmonella typhi, which causes typhoid.[1] Furthermore, resistance can spread rapidly: in

1987, less than 10% of *Shigella dysenteriae* isolates in Bangladesh were resistant to

nalidixic acid; in 1992, more than 90% of isolates were nalidixic acid-resistant.[2]

Resistance has even risen against vancomycin, the "antibiotic of last resort," increasing

from 0.3% in 1989 to 7.9% in 1993.[3] The threat posed by these drug-resistant bacteria

cannot be understated: infectious diseases are one of the leading causes of death in the

world, accounting for 13.3 million deaths (25% of all deaths) in 1998, and are related to

five of the ten leading causes of death in the U.S.[4] Even if other drugs can be used,

second- or third-line antimicrobials are often more expensive – the drugs needed to treat

multiresistant forms of tuberculosis are over 100 times more expensive than the first-line

drugs used to treat non-resistant forms.[5] Resistant bacteria are also more difficult to treat,

and patients may suffer longer while doctors try different antibiotics.

Resistance is a natural biological phenomenon. Although mutations are rare,

occurring in about 1 in 1,000,000 or 1 in 10,000,000 cells,[6] the rapid reproduction and

huge numbers of bacteria increase the frequency of mutations within a population. Once

a resistance gene arises, it can be spread between bacteria, even across the species barrier,

through transduction, the transfer of bacterial DNA by a virus; transformation, the

incorporation of bacterial DNA from the environment; and conjugation, the exchange of

genetic material between bacteria. Furthermore, genes coding for resistance are

frequently found on transposons, small units of DNA that are readily exchangeable.

Adding to the natural evolution of resistance, current antibiotics create immense

selection pressures that encourage the spread of resistance. Because antibiotics kill non-

resistant bacteria, the remaining resistant bacteria face greatly reduced competition for

nutrients and will rapidly proliferate, resulting in a resistant population. Furthermore,

antibiotics often kill "innocent bystanders," benign bacteria that would otherwise limit

the spread of pathogens by competing for resources; even worse, the surviving bacteria

often become reservoirs of resistance genes, passing on these traits to foreign pathogens.[7]

Selection pressure favoring resistant bacteria might be reduced if antimicrobials

targeted virulent genes instead of essential genes. Pathogens want to survive; the reason

there is immense selection pressure on a population to evolve resistance is resistance is

necessary for survival. If drugs inhibited virulent genes but did not kill the microbes, this pressure would be reduced. In fact, pathogens find it to their advantage to mitigate their virulence, provided they can do so without compromising their livelihood.[8] Furthermore, these "harmless" bacteria could actually help protect against other diseases because they would compete with, and thus limit the proliferation of, other pathogens.[9] Anti-virulence drugs would allow humans and microbes to coexist, similar to the relationship between humans and the millions of harmless bacteria that live in their guts.

Until recently, scientists lacked the technology to develop anti-virulence drugs. The antibiotics of the 1950s and 60s were discovered by whole-cell screening approaches – scientists either used combinatorial chemistry or looked directly for natural compounds that killed microbes. Although these methods successfully discovered many antibiotics, they employed a trial-and-error methodology, the goal of which was to discover compounds that killed the microbe *in vitro* rather than stopped virulence *in vivo*.

Today, genomics and bioinformatics enable the rational drug design of antimicrobials targeted specifically at pathogenic genes. By using techniques such as *in vivo* expression technology (IVET), differential fluorescence induction (DFI), and signature-tagged mutagenesis (STM), scientists can discover which genes are activated by microbes during the process of infection; these genes are often inactive, and thus undetectable, *in vitro*. Genomics and bioinformatics can also be employed in the functional analysis of these gene sequences. These technologies also allow scientists to use comparative genomics to identify the best drug targets. Finally, DNA microarrays can be used to construct expression profiles, aiding target identification as well as allowing mechanism of action studies at all discovery phases.[10]

In IVET, a vector that contains a promoterless resistance gene is randomly inserted into bacterial genomic DNA fragments. The host is then infected, incubating the modified bacteria *in vivo*. The microbes are then recovered. Because the vector is constructed so that the indicator gene is expressed only when the modified bacteria gene is activated,[11] scientists can detect which genes are activated by screening for antibiotic resistance. Because the incubation time can be varied, IVET can be used to generate temporal information on gene expression.

DFI is used to identify infection-specific processes. First, green fluorescence protein (GFP) is fused to different bacterial promoters; GFP is used because it can usually be expressed in bacteria without disturbing its pathogenicity.[12] When these modified microbes infect hosts, transcription factors bind to promoters, activating both virulence gene and GFP at the same time. Fluorescence activated cell sorting can therefore be used to identify microbial genes that were specifically activated during infection.[13]

Genes that are required for the onset and maintenance of an infection are identified by STM. First, unique sequence tags are inserted into random bacterial genes so that the inserts render the genes inactive. Then, hosts are infected with these microbes, and any bacteria that grow and establish an infection are recovered. Hybridization techniques are then used to compare the inocula with the recovered pools to discover which tags are missing from the recovered fraction; the missing tags correspond to genes that are vital for infection and, because mutated, were not able to establish an infection.[14]

Once these pathogenic genes are identified, their sequences are compared with known libraries to establish their function and identify potential drug targets. Linear comparisons of DNA and protein sequences allow the assignment of functions to genes

identified through DNA sequencing via similarity to characterized proteins.[15] Other

sequence-based clues can also be used in functional analysis; for example, computers

could also be used to search for tandem repeats at the 5' ends of genes; such repeats are

often found in certain virulence genes.[16] *In silico* analysis could be used to identify genes

that are coregulated with known virulence genes, suggesting new virulence factors.[17]

Programs can also search for conserved units of proteins, called motifs, to discover

classes of drug targets; for example, surface-associated proteins are more accessible and

thus easier to inhibit because drugs do not need to penetrate into the cell, and therefore

would not be as susceptible to microbial defense mechanisms.[18]

Another advantage of rational drug design lies in the field of comparative

genomics. Over 64 complete microbial genomes have been published.[19] Scientists can

compare the genomes of pathogens that cause a specific disease to identify conserved

genes; these genes are potential targets of drugs that would effectively treat not just

specific microbes, but clinical conditions.[20] Furthermore, with knowledge of the human

genome, comparisons between human and microbial genomes can help screen for drug

targets that are present in pathogens but not present in humans or benign microbes.

Finally, DNA microarrays are a powerful tool that can be used to analyze gene

expression profiles. Microarrays are chips that are spotted with thousands of different

genes. To detect which genes are expressed by an organism, purified mRNA is labeled

with a fluorescent dye and base-pairs to the microarray. Because the location of the DNA

on the microarray is known, the mRNA, and thus the expressed genes, can be identified.[21]

From the perspective of drug discovery, the patterns generated from the analysis

of all genes that are expressed under defined conditions can give clues to the function of

previously uncharacterized genes, as well as provide information about cellular responses to treatments with small inhibitor molecules.[22] For example, scientists can use microarrays to identify genes that are expressed in virulent, *in vivo* bacteria by comparing expression profiles with *in vitro* bacteria.[23] Microarrays can also be used to discover why some antibiotics no longer work and identify new drug targets on the resistance genes. Toxicology tests could profile gene expression in host cells to understand whether an antibacterial compound is likely to have adverse effects on eukaryotic cells in the host.[24] Understanding how drugs affect gene expression would streamline drug development.

Despite the promise of anti-virulence antimicrobials, it remains to be shown that such mechanisms can be developed into viable therapeutic intervention strategies.[25] In order for anti-virulence antimicrobials to be successful, they need to demonstrate their efficacy and their advantage over traditional antimicrobials. Although a recent study showed that an experimental molecule that inhibited a virulence factor permitted infected mice to resist and overcome infection, it is important to keep in mind that this approach has not yet yielded a licensed drug.[26]

New advances in genomics and bioinformatics give hope that increasing microbial resistance may be countered by new antimicrobial drugs. But even if anti-virulence drugs are successful, history has shown that there are no magic bullets. The future impact of infectious diseases will be influenced by three variables: the relationship between increasing microbial resistance and successes in developing new antibiotics and vaccines, the basic quality of life of the poorest groups of countries with developing and transitional economies, and the degree of success of global and national efforts to create effective systems of surveillance and response.[27]

Endnotes

[1] Dr. R. Williams, "Bacterial infections previously susceptible are now resistant to antibiotics," Antimicrobial Resistance, World Health Organization, 9/3/01, accessed May 29, 2002, http://www.who.int/emc/slideshows/AMRMagnitudetrends/sld008.htm, slide 8.

[2] Dr. R. Williams, "Trends in antimicrobial resistance in *Shigella dysenteriae* type 1 isolates from Bangladesh," Antimicrobial Resistance, World Health Organization, 9/3/01, accessed May 29, 2002, http://www.who.int/emc/slideshows/AMRMagnitudetrends/sld017.htm, slide 17.

[3] "Vancomycin Resistant Enterococci," Clinical Topics, NICL Laboratories, 1996, accessed May 29, 2002, http://www.nicl.com/clinicaltopics/vre.html.

[4] Gail H. Cassell and John Mekalanos, "Developments of Antimicrobial Agents in the Era of New and Reemerging Infectious Diseases and Increasing Antibiotic Resistance," JAMA, 2001 Feb 7; 285(5): 601-5, accessed May 29, 2002, http://jama.ama-assn.org/issues/v285n5/ffull/jsc00411.html.

[5] "Antimicrobial Resistance," WHO Information Fact Sheets, World Health Organization/OMS, 2002, accessed May 29, 2002, http://www.who.int/inf-fs/en/fact194.html.

[6] "About Antibiotic Resistance," Alliance for the Prudent Use of Antibiotics, accessed May 29, 2002, http://www.healthsci.tufts.edu/apua/Q&A/Q&A_AR.html#2.

[7] Stuart B. Levy, "The Challenge of Antibiotics," Scientific American, 1998, Issue 398, accessed May 29, 2002, http://www.sciam.com/1998/0398issue/0398levy.html#link3.

[8] Joshua Lederberg, "Emerging Infections: An Evolutionary Perspective," Emerging Infectious Diseases, Vol. 4, Number 3, July-September 1998, Center for Disease Control and Prevention, accessed May 29, 2002, http://www.cdc.gov/ncidod/eid/vol4no3/lederberg.htm.

[9] Levy, http://www.sciam.com/1998/0398issue/0398levy.html.

[10] John Rosamond and Aileen Allsop, "Harnessing the Power of the Genome in the Search for New Antibiotics," Science, 2000 Mar 17; 287 (5460): 1973-6, accessed May 29, 2002, http://www.sciencemag.org/cgi/content/full/287/5460/1973.

[11] Klaus P. Schaefer, "The in vivo Selection for Induced Genes," Determination of Essential Genes, Lecture 3, Winter 2001, accessed May 29, 2002, http://www.uni-konstanz.de/FuF/Bio/Bioinformatik/biv203.htm.

[12] Zivile Katiliene, accessed May 29, 2002, http://www.public.asu.edu/~laserweb/woodbury/classes/bch561/student/Katiliene1.htm.

[13] Daniel W. Martin, "Regulation of Virulence in *Legionella pneumophilia*," Department of Microbiology and Immunology, East Carolina University School of Medicine, accessed May 29, 2002, http://www.ecu.edu/microbiology/fac_martin.htm.

[14] "Signature Tagged Mutagenesis – an explanation," Microscience, accessed May 29, 2002, http://www.microscience.com/stm.pdf.

[15] A. Allsop and R. Illingworth, "The Impact of Genomics and Related Technologies on the Search for New Antibiotics," Journal of Applied Microbiology, 2002; 92(1): 7-12.

[16] Guido Grandi, "Antibacterial vaccine design using genomics and proteomics," Trends in Biotechnology, 1 May 2001; 19(5): 181-188, accessed May 29, 2002, http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6TCW-42RMMT5-W&_user=145269&_coverDate=05%2F01%2F2001&_alid=45684521&_rdoc=1&_fmt=full&_orig=search&_qd=1&_cdi=5181&_sort=d&_acct=C000012078&_version=1&_urlVersion=0&_userid=145269&md5=2574c19201059892548739d2e6456e17.

[17] George M. Weinstock, "Genomics and Bacterial Pathogenesis," Emerging Infectious Diseases, Sept-Oct 2000; 6(5), accessed May 29, 2002, http://www.cdc.gov/ncidod/eid/vol6no5/weinstock.htm#Figure%202.

[18] Cassell and Mekalanos, accessed May 29, 2002, http://jama.ama-assn.org/issues/v285n5/ffull/jsc00411.html.

[19] "Microbial genomes and chromosomes in progress," TIGR Microbial Database: a listing of microbial genomes and chromosomes in progress, May 24, 2002, accessed May 29, 2002, http://www.tigr.org/tdb/mdb/mdbinprogress.html.

[20] Christopher M. Tang and E. Richard Moxon, "The Impact of Microbial Genomics on Antimicrobial Drug Development," Annual Reviews Genomics and Human Genetics, 2001; 2: 259-69, accessed May 29, 2002, http://genom.annualreviews.org/cgi/content/full/2/1/259?maxtoshow=&HITS=10&hits=10&RESULTFORMAT=&searchid=1022554736298_110&stored_search=&FIRSTINDEX=0&volume=2&firstpage=259&journalcode=genom#S4.

[21] Leming Shi, "DNA Microarray (Genome Chip) – Monitoring the Genome on a Chip," 2002, accessed May 29, 2002, http://www.gene-chips.com/.

[22] Rosamond and Allsop, accessed May 29, 2002, http://www.sciencemag.org/cgi/content/full/287/5460/1973.

[23] Grandi, accessed May 29, 2002,
http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6TCW-42RMMT5-W&_user=145269&_coverDate=05%2F01%2F2001&_alid=45684521&_rdoc=1&_fmt=full&_orig=search&_qd=1&_cdi=5181&_sort=d&_acct=C000012078&_version=1&_urlVersion=0&_userid=145269&md5=2574c19201059892548739d2e6456e17.

[24] Cassel and Mekalanos, accessed May 29, 2002, http://jama.ama-assn.org/issues/v285n5/ffull/jsc00411.html#r4.

[25] Rosamond and Allsop, accessed May 29, 2002,
http://www.sciencemag.org/cgi/content/full/287/5460/1973.

[26] Tang and Moxon, accessed May 29, 2002,
http://genom.annualreviews.org/cgi/content/full/2/1/259?maxtoshow=&HITS=10&hits=10&RESULTFORMAT=&searchid=1022554736298_110&stored_search=&FIRSTINDEX=0&volume=2&firstpage=259&journalcode=genom.

[27] Cassel and Mekalanos, accessed May 29, 2002, http://jama.ama-assn.org/issues/v285n5/ffull/jsc00411.html.