Title: Reservoirs of Antibiotic Resistance - Bioinformatics
Contract number: 5 U24 AI050139-03
Dates: 8/15/2002 through **5/31/2008**

A. SPECIFIC AIMS

1) To use bioinformatics methods and tools to build a Web-based, interactive resource for information and data on resistance phenotypes and genotypes of reservoir bacteria, consisting of the following integrated elements:
   a. A website.
   b. A searchable isolate database to which researchers can contribute data.
   c. A searchable literature database populated by annotated citations of publications characterizing the isolate data.
2) To maintain and build upon the ROAR network of scientists interested in and working on resistance in bacterial reservoirs:
   a. To solicit and fund research on the ecology and/or evolution of resistance in commensals.
   b. To disseminate information through the ROAR website, listserv, scientific papers, symposia, conferences, and other appropriate channels.
3) To apply quantitative and phylogenetic methods to ROAR isolate data in order to better understand the ecology and evolution of resistance in commensals by:
   a. Examining the ecological pattern of antibiotic resistance in commensals, and
   b. Determining the relative importance of gene transfer and clonal spread in the rise of resistance.

B. STUDIES AND RESULTS: Closeout Report

1) To use bioinformatics methods and tools to build a Web-based, interactive resource for information and data on resistance phenotypes and genotypes of reservoir bacteria

The ROAR project has completed building an interactive website (www.ROARproject.org.) with detailed information on resistance phenotypes and genotypes of reservoir bacteria. It contains two subsites: 1) A uniquely synthesized and annotated library of references on antibiotic resistance in commensal flora, and 2) An isolate database of solicited genomic data from multiple sources to facilitate cross data-set analyses. The website is open to the public and scientific community with the two related quality-controlled databases administered online in a password-protected area. The databases have over 300 registered users as of May 31, 2008. From an "administration" page, APUA staff can manage and edit content, enter and annotate literature database citations, upload isolate data, and view website usage statistics. (http://apua.weblogz.org/ROAR/admin/login.php. (username:NIH; password: 1234) Most recently, new database function was developed to include a finalized "data dictionary" and to provide greater facility for database searches.

Citations in the ROAR Literature database are currently grouped under four topics: Resistance in Commensals (1164), Bioinformatics (260), Mathematical modeling (40) and *Escherichia coli* (284). Literature annotation fields have been tested and finalized for a total of 53 possible variables (see admin site > literature database: "add"). The library currently contains over 1800 citations, with new additions and annotation continuing on a regular basis using PubMed literature search keywords and citation services, supplemented with publication cross-referencing. During the annotation process, citations describing datasets of interest have been flagged to enable the selection of potential gene-based isolate datasets that can be readily solicited for populating the ROAR isolate database. The literature data base and isolate data solicitations are ongoing.

The isolate database is currently populated with genetic data from 3352 isolates which include all data generated by the year 1-5 consortia (see list of consortium studies below). In addition, *E. coli* data were acquired and added from the National Antimicrobial Resistance Monitoring System (NARMS; http://www.fda.gov/NARMS), provided to us by Steering Committee member Dr. David White, director of the NARMS program.

An interactive web-based dynamic data entry template for direct downloading of isolate data was explored and tested, but full implementation has presently been deferred due to quality control concerns. The APUA

screened-entry system is currently operating effectively. Further refinement of the dynamic template is possible and still under consideration.

Although the project is officially closed, APUA seeks to maintain these functions through other funding sources. ROAR project publicity is scheduled through an ASM 2008 Boston symposium address entitled *APUA Global Projects on Antimicrobial Resistance*, presented by former ROAR principal investigator, Dr. Michael Feldgarden.

2) To maintain and build upon the ROAR network of scientists interested in and working on resistance in bacterial reservoirs

A scientific network of recognized experts in the field was assembled to form the ROAR Steering Committee. This committee was subsequently expanded to a total of 35 members in order to include selected ROAR consortium participants. APUA has convened members of this group annually since the project began, with NIAID representation at the majority of meetings. The group met most recently at the 107th General Meeting of the American Society for Microbiology, May 21-25, 2007. Meetings of this expert group have focused on identifying major knowledge gaps, setting and prioritizing a research agenda, developing RFPs to further ROAR objectives, reviewing RFP responses, and providing direction for the development of website content, as well as sharing relevant individual research activities.

The ROAR Network Listserv (https://elist.tufts.edu/wws/info/roar) currently has over 350 members, with APUA taking requests for new membership on a regular basis. Comprised of interested investigators from diverse scientific fields, this listserv acts as an active forum for timely discussion of resistance in commensals and has been a valuable resource for the dissemination of ROAR-related scientific news, data solicitations, and announcements and deliberations around controversial scientific policy questions such as antibiotic use in animal feed. Discussions are archived by date and topic. The APUA staff will continue to moderate the Listserv as funding allows.

The ROAR subcontracting consortia program proved to be an invaluable means for publicizing ROAR and accelerating coordinated research in this area. The Steering Committee and APUA staff developed criteria for RFP development and release, as well as a detailed review process. Initial advice recommended unrestricted solicitations in order to gauge the level of interest in particular commensal species. In order to facilitate prospective analyses however, the final RFP solicitation was targeted to one organism (*E. coli*). As a result of three RFPs that were developed and distributed, the ROAR staff received and reviewed over 130 project proposals from national and international sources, most of which were meritorious by various measures. Many were meritorious, but due to limited available funds, APUA and NIH approved scientific merit to award sub-grants to just 8 consortium projects, the last six of which were completed in 2007.

The ROAR-subcontracted Studies worked in diverse areas, reflecting the enormity of the initiative's subject, but developed insights with implications for the whole field:

- Susan Hollingshead, University of Alabama, "*Streptococcus mitis* biovar 1 and its potential as a reservoir for *Streptococcus pneumoniae*."
- Lisa Nolan and Timothy Johnson, Iowa State University, "Possible emergence of a plasmid-mediated reservoir of resistance genes among the *Escherichia coli* of poultry."
- Anne Summers, University of Georgia, "High throughput molecular genotyping of environmental and human Staphylococci carrying class I integrons."
- Dr. James Tiedje, "Exploring transfer, diversity, and distribution of antibiotic resistance genes residing in soil"
- Marilyn Roberts "Profiling of *mef* and *erm* resistance genes in oral pediatric isolates
- Erick Denamur, Andre Andremont, and Sylvain Brisse, "Phylogenetic Analysis of Antibiotic Resistance in Commensal *Escherichia coli*."

- David Gordon, Australian National University: "Antibiotic Resistance and Genetic Relationship of *E. coli* from Australia."
- Betsy Foxman, Probe Hybridization Array Typing: A High Throughput *E. coli* Typing Method

These ROAR studies have led to a deeper understanding of the prevalence of antibiotic resistance between clinical and environmental bacterial isolates, and the role of antibiotic selective pressure associated with human activity. They also revealed the diversity of resistance genes and virulence determinants and enabled the facilitation of commensal antibiotic resistance-based research. Their outcomes have been/are being published in scientific journals or presented in conferences (see E. Publications).

Consortium Summaries:

Dr. Tiedje's group found four classes of Tetracycline resistance genes as well as aminoglycoside and quaternary ammonium resistance genes in DNA extracted from soil, but only in soil manured by tetracycline-fed pigs. Numerous integrons were found in all soils, however, indicating that while resistance genes may be concentrated at sites of selection, genetic elements to disseminate them are widespread.

Dr. Roberts' project identified a variety of each of the two major types of genes expressing macrolide resistance, many of them linked to mercury resistance genes, in many genera of commensal oral flora of children who had mercury dental amalgams. Traces of mercury could thus co-select for greater persistence of the macrolide resistance genes and the chance of their transfer to neighboring pathogens.

To begin to explore systematically the long-suspected contribution of commensal mouth flora to the resistance gene complement of *Streptococcus pneumoniae* **Dr. Hollingshead** and colleagues developed MLST systems to elaborate the population structure of collections of *Streptococcus mitis* and delineated their resistance genes.

Dr. Summers' group worked with a variety of collaborators to develop a battery of methods to discriminate strains of staphylococci, including plasmid profiling and fingerprinting, phenotyping and genotyping, planar microarray and leveraged compete plasmid sequencing, the latter now spinning off further work to explicate the mobility mechanisms of staphylococcal genetic elements.

The work of **Dr. Nolan**'s laboratory was to design multiple multiplex PCR panels to screen over two thousand isolates of *Escherichia coli* for plasmid virulence and resistance traits, and to observe presumptive flow of these between commensal and pathogenic isolates from the collections different sources, which included poultry and distant humans. While the avian pathogenic *E. coli* had more resistance genes than those from other sources, it also emerged that all of the populations shared some resistance genes.

Dr. Gordon's project was to develop the classification of *E. coli* to advance understanding of its population structure, which is needed to understand its major role in commensal-pathogen spread of resistance. The contribution was to relate a new rapid PCR-based method to identify *E. coli* phylo-groups to the older more laborious method that had been in use.

Dr. Amabile Cuevas found no difference in prevalence of resistance among oral streptococci between groups presumptively with high and low exposure to antibiotics, although the former has more multiply resistant strains. Further work suggests a selective pressure in the environment that favors emergence of low-level resistance to fluroquinolones.

Dr. Foxman and co-workers have developed Probe Hybridization Array Typing (PHAT) to be a more rapid and readily digitized method that will correlate with MLST typing while also detecting faster evolutionary changes to better monitor events in the real-world spread and transfer of resistance genes.

These studies illustrate the diverse kinds of work that will be needed to develop a coherent overview of the contribution of the world's vast populations of commensal microbes to the emergence and spread of antimicrobial resistance in pathogens.

3) To apply quantitative and phylogenetic methods to ROAR isolate data in order to better understand the ecology and evolution of resistance in commensals

Based on the recommendation of our bioinformatics consultants and Steering Committee, we determined that the original mathematical modeling approach is inappropriate for the kinds of data that are currently available. Quantitative methods were evaluated in order to determine the most appropriate methods for analysis of the ROAR antibiotic susceptibility testing data. An in-depth review of the available literature-based genetic data revealed that the expanding breadth of technologies and approaches available to investigators in the field of genetic typing makes direct comparisons through a database extremely challenging, and a core methodology was needed.

The consensus of the ROAR steering Committee was that the project could be advanced through the development of a core methodology, in particular, one that could be applied to *E. coli.* Thus the final set of the consortium studies was designed and funded in part to advance the technologies needed for consistent data output for this organism. The studies of Dr. Foxman and Dr. Gordon in particular (see above), were designed for this purpose.

As a result of funded consortium study output as well as other independently solicited data, there are a total of 14 data sets in the ROAR isolate database representing 3352 isolates: 2892 *E.coli*, 169 *Streptococcus*, 182 *Staphylococcus*, 33 *Neisseria*, 32 *Leuconostoc*, 2 *Gemmella*, 9 *Aerococcus*, and 35 *Enterococcus* spp.

More recently, the concept of developing a standardized method (GETEC, Genome-enabled Typing of *E. coli*) was conceived and developed at APUA. Multilocus sequence typing data generated by the ROAR project (Dr. D.M. Gordon) are serving as the basis of a collaborative effort between APUA and the Broad Institute's Microbial Sequencing Center. A white paper has been submitted for review to NIAID to sequence 103 commensal *E. coli* genomes in order to create a community resource that will provide the foundation for a broad range of research interests including, but not limited to, the genetic mechanisms of pathogenesis, antibiotic resistance, and human adaptation. This project will also enable the determination of the signatures of emerging infectious disease. The resulting standardized method, attributable to ROAR for its initiation and conceptualization, will be a major contribution to the field.

C. SIGNIFICANCE

The ROAR isolate and literature databases are unprecedented online resources on resistance in commensal bacteria for researchers, clinicians, and public health professionals. The database framework encourages data sharing and the development of statistical methods. The literature database compiles annotated, comprehensive information that is not readily available from other sources.

The expansion of the ROAR Network of scientists through the RFP, website, and listserv has fostered scientific interest, advancement and communication among diverse stakeholders regarding resistance in commensals. The project established relationships and increased understanding across related, but traditionally distinct disciplines such as veterinary medicine, ecology, and clinical medicine. ROAR consortium funding has supported the first coordinated research network and application of a novel population biology approach to antimicrobial resistance. As the ROAR isolate database becomes populated with more standardized and focused data, cross-dataset analyses will be facilitated to enable researchers to examine the ecology and spread of antibiotic resistance.

In an ongoing collaborative effort with CORE, the literature database is being updated and 'mined' to evaluate the current state of knowledge on antibiotic resistance in commensal flora. Efforts are underway to evaluate its ability to answer key research questions in this field. Participants of the ROAR Steering Committee will again

convene in June 2008 to share the newest information and to formulate and prioritize research agenda for advancing this area of research.

Additionally, plans have been initiated to further augment the isolate database with a minimum of 500 strains from international sources. Genetic data will be shared with APUA from The United States Army Medical Research Institute for Infectious Diseases (USAMRIID) as a result of a collaboration in which commensal bacterial strains will be imported from APUA–affiliated Chapter countries.

Another applied goal is to provide epidemiologists and researchers with a comprehensive library of phylogenetically informative SNP's to develop tracking methods for detection of epidemic spread of drug-resistant *E. coli* clones. This information could lead to better understanding and control of the spread of resistance, and perhaps even prevent the continuous emergence of novel resistant stains.

The ROAR project represents an unprecedented effort to assemble scientific insights, resources, and methods that can lay the groundwork for prediction of emerging resistance. Such steps help provide a scientific basis to inform public policy that can be implemented to decrease the incidence of resistant infections, morbidity, mortality, and associated costs.

D. PLANS

APUA plans to continue all aspects of the ROAR project and resources as funding allows. We will work with the Broad Institute to advance the GETEC project and continue to work with USAMRIID to expand the literature data base and to augment the isolate database. We will be convening several ROAR investigators and advisors at the 2008 ASM Annual Meeting to refine a research agenda and plan to apply to NIH for a continuation of several aspects of the ROAR project. An article is in preparation for the ASM *Microbe* publication in late 2008 to highlight the resources of ROAR and to generate interest in the field.

E. PUBLICATIONS

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13 genera, isolated from healthy Portuguese children. Antimicrob Agents Chemother. 2004 Sep;48(9):3451-6 PMID: 15328110

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9. White DG, Hudson C, Maurer JJ, Ayers S, Zhao S, Lee MD, Bolton L, Foley T, Sherwood J. Characterization of chloramphenicol and florfenicol resistance in Escherichia coli associated with bovine diarrhea. J Clin Microbiol. 2000 Dec;38(12):4593-8 PMID: 11101601 (from ROAR 1 project)

10. Payne, D. B., B. Beall, P.A. Coan, R.E. Gertz, and S. K. Hollingshead. Persistence of *Streptococcus pneumoniae* clones over two decades, murM alleles, and the gradual development of penicillin resistance in *Streptococcus pneumoniae*, *in revision for J. Clinical Microbiology*

11. Hollingshead, S. K., Northrup, M., Coan, P., Patel, R., Hardy, D., Amabile-Cuervas, J., Robinson, D. A.,and Kilian, M. K. A characterization of oral streptococci as a reservoir for fluoroquinolone resistance and cell wall changes contributing to penicillin resistance for Streptococcus pneumoniae*. In preparation*

12. McNamara, S., Srinivasan U, Zhang L, Marrs C, Whittam T, Foxman B. Comparison of probe hybridization array typing to multilocus seqeunce typing for pathogenic *E. coli*. *In preparation.*

F. PROJECT-GENERATED RESOURCES

See Aim 1 under B. STUDIES AND RESULTS.