Microbial Evolutionary Medicine – from theory to clinical practice

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Abstract:

Bacteria and other microbes play a crucial role in human health and disease. Medicine and clinical microbiology have traditionally attempted to identify the etiological agents that causes disease, and how to eliminate them. Yet this traditional paradigm is becoming inadequate for dealing with a changing disease landscape. Major challenges to human health are noncommunicable chronic diseases, often driven by altered immunity and inflammation, and persistent communicable infections whose agents harbor antibiotic resistance. It is increasingly recognized that microbe-microbe interactions, as well as human-microbe interactions are important. Here, we review the "Evolutionary Medicine" framework to study how microbial communities influence human health. This approach aims to predict and manipulate microbial influences on human health by integrating ecology, evolutionary biology, microbiology, bioinformatics and clinical expertise. We focus on the potential promise of evolutionary medicine to address three key challenges: 1) detecting microbial transmission; 2) predicting antimicrobial resistance; 3) understanding microbe-microbe and human-microbe interactions in health and disease, in the context of the microbiome.

Introduction

A diverse range of bacteria plays a crucial role in human health and disease. Opportunistic or specialist pathogens may colonize the urinary tract (1), the gut (2) or the lungs (3), while the gut microbiome composition affects nutrient absorption (4), and resilience to infection (5). Global antibiotic use is on the rise (6) and antibiotic resistant bacteria are now so widespread that the World Health Organization warns that the world is running out of functional treatments (7), while bacteria continue to evolve resistance to new drugs. This problem is caused by the extensive use of antibiotics in the clinic, as well as unregulated over-the-counter purchases, and therapeutic, prophylactic and growth-promoting use in agriculture. Another major challenge is disease attributed to the perturbation of the healthy microbiome, where changes due to a diet of processed food, altered hygiene practices, and antibiotic use is suggested to leave individuals vulnerable to opportunistic infections and prone to develop metabolic syndromes (8,9).

The emerging field of Evolutionary (or Darwinian) Medicine seeks to apply the approach of evolutionary biology to address challenges to human health, aiming to identify the ultimate causes of

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disease (10). This approach is particularly relevant for understanding our associated microbes: they evolve rapidly due to their large population sizes and short generation times (11), and some have a long history of association with their host (12). Given a short generation time, the scales of ecology and evolution overlap, and there is an inherent feed-back between ecology and evolution (13,14). When microbes are concerned, Evolutionary Medicine is therefore inseparable from Ecological Medicine. Theoretical and experimental biology have been instrumental in elucidating how microbial interactions shape the stability of microbial consortia (15,16), and how clinical interventions affect bacterial evolution and co-existence (17). Whilst such an approach is directly relevant from a medical perspective, the application of eco-evolutionary approaches to clinical systems has thus far been limited despite some success stories (Box 1).

Below we discuss three areas where such an eco-evolutionary approach to microbial-associated diseases may be beneficial. These were defined during the first Workshop on Microbial Darwinian Medicine, held in August 2017 at the Lorentz Center (Leiden, the Netherlands). These constitute key challenges where an interdisciplinary approach would be feasible and useful: 1) the timely detection of microbial transmission; 2) the prediction of antimicrobial resistance; and 3) the importance of microbial interactions in health and disease, in the context of the human microbiome. Lastly, we discuss challenges of the interdisciplinary approach needed to address the above-mentioned issues, exemplified by the sharing and utilization of high-throughput sequencing data for both basic research and clinical applications (Figure 1).

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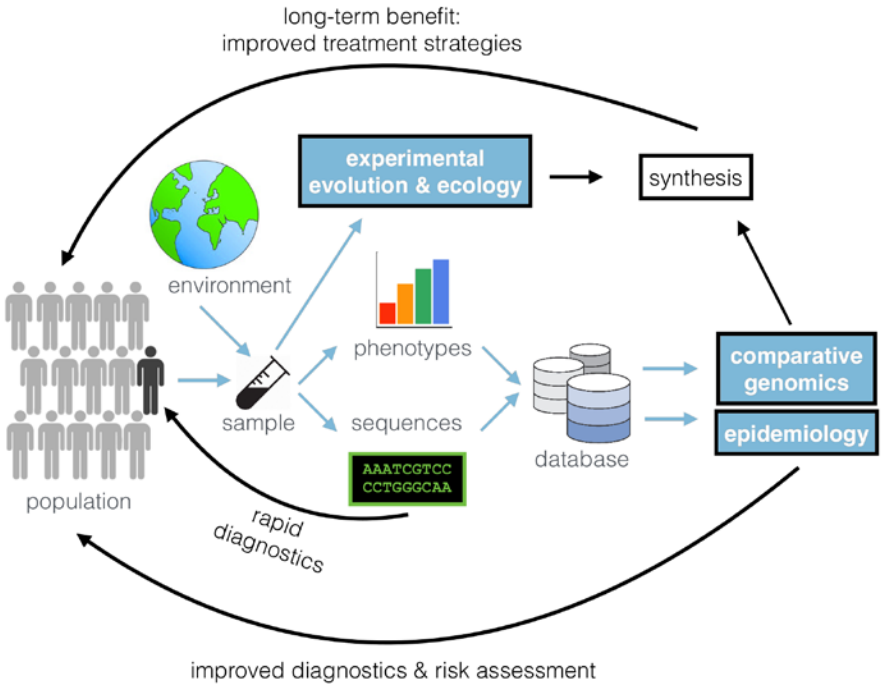

Figure 1. A vision of how the information flow in an interdisciplinary microbial evolutionary medicine approach could improve basic knowledge, public health, and patient care. A microbial sample collected from a diseased person, a healthy person or the environment can be sequenced and phenotyped, e.g. by assessing antibiotic susceptibility. Sequencing and annotation of isolates may serve as a rapid diagnostic to directly benefit the individual patient. On the longer term, compiled sequence and phenotypic data in an accessible database, with appropriate metadata, may represent a goldmine for subsequent analyses. Inferred epidemiological transmission patterns can be used for improved diagnostics and risk assessment in future cases. Comparative genomics and experimental ecology and evolution can help in the formulation of hypotheses of why a given clinical outcome occurred. This may in turn be experimentally verified with collaborative, interdisciplinary efforts. This has the potential to lead to improved treatment strategies and precautions, which feed back to benefit the population on the long term.

1. Detecting transmission

Tracking pathogen transmission via genome sequencing is possible because many pathogens are measurably evolving during an epidemic, and even within patients (18,19). Globalization, urbanization,

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changing climate and land-use patterns, and large-scale farming all contribute to the risk of epidemics caused by both viral and bacterial pathogens. While viral outbreaks are often detected quickly, epidemic spread of bacterial pathogens often goes unnoticed for some time. This is particularly the case when the causative agent is considered a common pathogen, such as transmissible *Pseudomonas aeruginosa* clones spreading between cystic fibrosis patients (3,20), and the ST131 *E. coli* clone, which was not discovered until 2008 when it had already spread globally (21,22). Through retrospective evolutionary investigation of bacterial pathogen population dynamics, we are now beginning to understand how these epidemics begin (23), which leads to the question of how these events could be detected earlier, when there is still time to prevent further transmission.

Faster sharing of genomic data and annotations (*e.g.* pathogenicity islands, plasmids that easily spread, and virulence gene markers) through public databases may support the tracking of ongoing epidemics, allowing scientific consortia of local and global scientists to contribute to the analyses. Currently, many studies on clinical isolates are focused on the core genome, which is the portion of alignable genomic regions common to a collection of isolates. This is primarily to ease analysis and comparisons across strains, as researchers in evolutionary genomics and epidemiologists traditionally use the core genome to infer phylogenies and transmission events. Yet, clinical microbiologists appreciate the clinical importance of mobile elements such as plasmids, phages and transposons in the spread of antibiotic resistance and virulence factors (24). Exemplary is the spread of the colistin resistance-conferring *mcr-1* gene that is not unique to a particular *E. coli* clone but appears to be widely spread on plasmids (25,26). Current technology that outputs short reads of DNA/RNA sequences makes assembly of plasmids and other mobile elements challenging, but not impossible, and long-read technologies may solve these problems entirely (27,28). Increasingly, genomic approaches are used to track plasmid dissemination (*e.g.* 29), and a cohesive view of both core and accessory genomic components is needed to fully understand pathogen evolution, transmission, and virulence (30). Looking beyond the core genome raises the question whether the microbe is merely a vector of disease, in cases where virulence and antibiotic resistance stem from transferable mobile elements (31,32). If we aim to understand and predict the transfer of disease it is crucial to know which genetic elements to monitor. This has implications for attempts to predict the outcome of infection in individual patients, or over the course of an outbreak of resistant bacteria.

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A better understanding of the environmental niches and genetic variation of opportunistic pathogens is also required for the detection of virulence determinants. Do disease-causing strains represent a random sample from an environmental or host reservoir, or do they harbour specific characteristics that facilitate human colonization and infection? A comparison of *Vibrio cholerae* genomes from environmental and clinical sources revealed that a combination of specific core-genome SNPs, already present in the environment, was a prerequisite for the acquisition of mobile elements encoding key virulence factors that affect colonization and virulence in the human host (33). Likewise, sampling from animal hosts can show linkage between animal and human reservoirs (34–36). Additionally, various studies looking at bacteria residing in the urinary tract have shown that what are traditionally thought of as 'pathogens' can actually be present in both patients and controls (37,38). In this case, more extensive sequencing in conjunction with laboratory studies may shed light on why pathogenicity only occurs in certain people, and whether this is due to the genetics of the 'pathogens' or the different niches provided by genetically or physiologically different human hosts.

2. Predicting the evolution of resistance

Due to the wide use of antibiotics in the community and in agriculture, and the proximity of humans and livestock, resistance spreads rampantly back and forth from livestock to waste water and likely humans, for example in small family farms in Vietnam (39,40) and industrial-sized farms in China (41). The spread of resistance back and forth between livestock and humans seems to be socio-economically dependent as this appears to happen less in the Netherlands (42). While antibiotic resistance is a widespread phenomenon, and also found in organisms never exposed to man-made antibiotics (43–45), resistance is not distributed equally across all environments (46).

There is a need to study the role of bacterial genetic background in determining the likelihood of resistance evolution, via both *de novo* mutations and the uptake of mobile elements conferring resistance. Genetic background plays a key role in shaping the evolution of resistance to antibiotics by point mutation (47–49), and can also impact the evolution of resistance by horizontal gene transfer (50). For example, clinical isolates of the nosocomial pathogens *Enterococcus faecalis* and *E. faecium* lack CRISPR-cas systems, making them more prone to accept foreign DNA and thus more likely to acquire antibiotic resistance genes (51,52). The most recombinogenic strains of the human pathogen *Streptococcus pneumoniae* are also the most likely to become antibiotic resistant (53). Fitness barriers

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may prohibit the transfer or functioning of mobile elements in the once they arrive in a new host genome. Such costs can be caused by the regulatory inefficiency experienced once new elements are incorporated (54), or biochemical incompatibilities (55). To identify clones that are antibiotic resistant and adapted to the host environment, the inter-dependent nature of resistance and fitness should be recognized.

Approaches aimed at identifying resistance should not only focus on specific resistance genes, but should also consider mutations in non-coding sequences (e.g. promoters and intergenic regions) and in coding sequences (30). In *E.coli* ST131, the uptake of mobile elements involved in resistance was found to lead to selection for compensatory mutations in the genome (56). Extraintestinal pathogenic ST131 clones may further be ecologically separated in different niches, as drug susceptible ST131 clones incorporate different phages or plasmids compared with drug resistant ST131. Developing tools for predicting which strains of a pathogen have a high risk of evolving resistance may be a daunting task, but could help to guide the use of antibiotics in clinical settings. Developing such insight requires richly annotated genome and mobilome data, in publicly accessible databases that include antimicrobial susceptibility metadata.

In the laboratory, microbes are capable of quickly adapting to high concentrations of antibiotics (57). However, for experimental work on resistance evolution to be clinically relevant, we need to address the question of whether resistance *in vivo* evolves under strong or weak selection pressure. The antibiotic concentrations used in experiments may well be different from what is experienced in patients, with unequal distribution of antibiotics across tissues and in biofilms that protect bacteria (58,59). The strength of selection in the host environment is basically unknown but it may affect the type of resistance mutations that arise, and the rate at which these mutations can be acquired. A weak pressure has been found to be more likely to select for a broader range of resistance mutations at little to no cost to the bacteria that carry them (60). Cross-resistance, in which mutations confer resistance to multiple antibiotics, was more likely to evolve under strong selective pressures. However, under some conditions, collateral sensitivity can occur. In such a case mutations that confer resistance to one drug can induce susceptibility to another drug (61). Cycling of antibiotics in the clinic has been suggested, to use collateral sensitivity to limit the development of resistance to each of the cycled drugs (62,63), yet studies show mixed results of effectiveness of the cycling strategies (64,65). Additionally,

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discrepancies between the experimental conditions where drugs are developed, and the clinical setting where they are used, may cause the failure of drugs during pre-clinical trials, even if they could actually perform well in a patient (66).

There is a general expectation that fitness trade-offs in different environments are a barrier to the spread of resistance (67). For instance, clinical isolates of *E. faecium* have a markedly larger genome than non-clinical isolates, in part because they carry a large pathogenicity island and other mobile genetic elements (52,68). In other species, resistance against fluoroquinolones and aminoglycosides can have a deleterious effect on either mobility or growth in the absence of the antibiotics (69–72). Trade-offs may thus lead to a fitness burden in the absence of the antibiotics. This may effectively select against the dissemination of resistant pathogens outside of the clinic (73). Yet, not all antibiotic-resistant bacteria suffer from such a fitness burden in the absence of antibiotics (74). Some conditions, such as heavy metal-rich environments, can even co-select for resistance (75). Understanding for which resistance mechanisms – and in which environmental contexts – fitness trade-offs limit the spread of antibiotic resistance will be a major future challenge of evolutionary medicine (Box 1).

3. Microbial interactions and the eco-evolutionary dynamics of the human microbiome

When considering the adaptive potential of opportunistic pathogens, we need to take into account intra- and interspecies interactions, *i.e.* the social environment in which bacteria evolve and interact. Bacterial behaviors that affect virulence often involve the production of extracellular *public good* molecules (76). These compounds are produced and shared within the population in a cooperative manner. Public goods are, however, by definition exploitable, as non-producing freeloaders may reap the benefit of their use, without paying the cost of their production (77). Therefore, intra-species bacterial interactions can drive changes in production of virulence factors during an infection (78). Further, pathogen diversity and order of arrival can affect disease severity. In urinary tract infections, for example, *E. faecalis* is able to facilitate the invasion of otherwise avirulent *E. coli* in an animal colonization model, and can even impact disease development after it is cleared (79). The stochastic nature of arrival may therefore play a role in the ecology, as not all bacteria are able to colonize the host in any order (80). Additionally, interactions between different bacterial species derived from polymicrobial urinary tract infections affect ecological stability and antibiotic tolerance *in vitro* (16). Indeed, in chronic urinary tract infection amongst the elderly with sub-acute symptoms, polymicrobial infections are the norm

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(81). An additional layer of complexity in this system can come from phages, that affect the growth of the microbial population and may thus mediate microbe-microbe interactions (82). Ecology and evolution thus both play an important role in the outcome of infections, as microbial interactions may affect pathogen colonization and survival.

Microbe-microbe and host-microbe interactions are not just pairwise but take place in the context of often diverse and complex host-associated communities called microbiomes. The composition, structure and stability of the healthy microbiome is impacted by human genetics, diet and other environmental factors (83,84). Gut microbiome research is particularly focused on identifying taxa that contribute to health and disease. For instance, the abundance of an *Akkermansia* species was observed to be reduced in hosts with metabolic disorders (85,86), but also, an increased abundance was observed in persons with Alzheimer's disease and ulcerative colitis (87,88). However, such bacterial species often act in concert with other microbiome members (87), and *Akkermansia* has been found to interact with other gut bacteria in metabolic networks (88). The contribution of members of the gut microbiome to health and disease may thus depend on the context of their surrounding microbiome ecosystem.

The degree of co-evolution between mammals and their microbiomes is debated, but phylogenetic studies show that several gut bacteria have been vertically inherited over millions of years of evolution and have co-speciated with mammals (12). The absence of some of these bacteria is associated with inflammatory bowel disease in humans (12). Several studies have shown that humans have experienced an accelerated depletion of gut bacterial biodiversity in recent times, in particular populations embracing "westernized" lifestyles and diets (89–91). It is suspected that processed foods, the use of antibiotics and overly hygienic environments are responsible for the disappearance of our ancestral gut symbionts, which could drive the rise of non-communicable diseases worldwide (9).

The microbiome composition may affect the propensity for non-communicable diseases through the immune system, as a diverse and stable microbiome is suggested to be a key contributor to its maturation. Early life events that affect the development of the microbiome ecosystem may therefore be of crucial importance as these events also shape the development of the immune system (92). Early life perturbation of the microbiome is exemplified by the treatment with antibiotics at a young age, which has been shown to be associated with an increased risk of developing both asthma and obesity

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later in life (93,94). A prospective cohort study also showed that an adequate maturation of the gut microbiome in the first year of life was critical for protecting children against asthma at age 5 years, especially for children born to asthmatic mothers (95).

Knowledge of ecosystem dynamics may inform gut microbiome treatments, for instance by reducing the occurrence of available niches for pathogens. As *Clostridium difficile* colonization is facilitated by a low diversity of the gut microbiome (87), repopulating the system towards a healthy, diverse state may cure such infections (96). Fecal microbial transplants (FMT) were found to be about 85% effective at treating recurrent *Clostridium difficile* infections in such a manner (97), holding great promises for the future design of microbiome-based therapeutics. It is still unclear exactly which components of the transplant lead to success (98), but the effect of an FMT can be partly predicted based on the microbiome composition of donor and recipient (99).

To harvest information on the eco-evolutionary dynamics from microbiome data with the aim to develop clinical interventions, we must take into account the temporal feedback between the host and the microbiome, as the microbiome composition fluctuates. These fluctuations can be regulated endogenously by circadian clocks (100) and are subjected to both seasonal cycles (101) as well as jet-lag (102). Because the immune system also has endogenous rhythmicity (103), patterns in the microbiome and immune system could interact to shape the temporal dynamics of disease. Diet and other host behaviors can also lead to temporal fluctuations in the microbiome (83,101,104). This may have implications for sampling strategies used in investigations of the microbiome, such as the collection of data series over time, the timing of sampling, and the initiation of treatments against disease.

Development of novel eco-evolutionary models to discern the short-term (ecological) and long-term (evolutionary) feedback between the host and the microbiome may facilitate an understanding of their role in health and disease (12,15,105). Recent work also highlights the importance of considering the potential of the host to control the microbiome composition, e.g. through oxygen regulation, and the resulting "dysbiosis" if control is lost (105,106). To facilitate the accessibility of data to study these objectives, the Human Microbiome Project (https://hmpdacc.org/), the American Gut project (http://americangut.org/) and the Global Microbiome Conservancy

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(http://microbiomeconservancy.org/), among others, are providing sequencing data from different body sites and different human populations. Such investigations may pave the way for future microbiome disease interventions (107). We argue that an interdisciplinary approach will help build a theoretical framework to infer causation from observed correlations between gut bacteria and disease, and importantly how we may be able to manipulate them for health benefits.

Challenges to the use of high-throughput sequencing in the clinic

Recent advances in sequencing tools and bioinformatics have helped us understand which bacteria are where, how their genomes evolve over time, and how pathogens and antibiotic resistance determinants are transmitted (Figure 1). Applying evolutionary theory to these data might enable us to prioritize possible approaches in the clinic. At present, making decisions about the best course of treatment for a patient based on their personal (meta)genomic data is not feasible. In principle, whole genome and metagenomic sequencing directly from clinical samples hold promise for eventually speeding up clinical diagnoses, selecting appropriate treatments, and epidemiological inferences; however, there are still many challenges in the translation of results to clinical practice (19). In addition to costs, we identified the main issue to be one of scale at the level of time available and certainty required. Whole genome sequencing (WGS) may be incredibly powerful for studying epidemics at the population level over a longer time period, such as identifying transmission of MRSA (108). It is, however, not yet competitive with traditional culture-based and PCR assays at the individual patient level, where fast diagnosis and appropriate treatment plans are key. This is due to the challenges in extracting high quality pathogen DNA directly from human samples as well as the additional cost and time needed to analyze the sequencing data, in particular when there is not yet a clear link between genotypes and phenotypes of clinical interest (*e.g.* antibiotic resistance) for many species (109). The small degree of uncertainty acceptable in diagnostics, compared with epidemiology, limits the current implementation of this new technology. There is thus a strong need for theoretical and technological development, as well as interdisciplinary collaborations to fill this knowledge gap.

A clear exception is found for slow-growing pathogens such as *Mycobacterium tuberculosis* (TB), where a variety of resistance-conferring mutations can be identified more rapidly by WGS than by culture-based drug sensitivity assays (110–113). Even in this situation, communication of genomic data to clinicians is still a challenge. Genomic literacy may be a goal in medical training, thereby increasing

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the understanding of when WGS is the appropriate tool for solving a problem. The first evidence-based guidelines for presenting microbial genomics data to clinicians, who often have only a few minutes to evaluate the data and make a decision on a course of treatment, were recently published. A design study approach, combining user interviews, surveys, and testing of various prototypes for graphically presenting WGS-derived species identification, antibiotic resistance, and epidemiological data, was used to design a two-page report that met end-user needs (114). To increase the accessibility of WGS data it would be highly relevant to incorporate clinically desirable user-interfaces in future clinical WGS analysis software and databases. These databases may additionally be equipped with warnings on the detection of specific resistance mutations in submitted genomes, or alerts of possible transmission if a specific clone has been found elsewhere, thereby benefiting global detection and information exchange.

The path to interdisciplinarity

To further the understanding of the causal explanations for disease, more collaboration is needed between clinicians and basic researchers. Large amounts of sequencing data are already available and large collections of clinical isolates are stored in freezers with few available resources to study them. A database for matching strain collections and scientists, with questions, specific hypotheses and funds, may facilitate such interdisciplinary investigations.

To achieve such interdisciplinary research, funding agencies must also play a role. Funders should require open data sharing and incentivize new collaborations, and academic institutions should not discriminate against researchers who share extensive authorships with other groups when it comes to hiring and promotion. Open and immediate sharing of WGS data, and of metadata including clinically relevant phenotypes is important, along with depositing manuscripts on pre-print servers such as on BioRxiv. To improve reproducibility across studies funders and journals should encourage higher standards in data submissions, including standardized metadata to allow reuse of data for comparative studies (115). The bundling of human-related meta-data (*e.g.* microbiome sequencing, co-morbidities and diet data) may raise issues related to privacy. For example, microbiome data can be used to identify individuals (116). We believe, however, that the benefits of open data sharing, with appropriate checks and balances, clearly outweigh the potential risks.

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The hope for the future of the field of microbial evolutionary medicine is to establish a common ground between clinicians, epidemiologists, bioinformaticians, public health officials, and cell-, micro-, and eco-evolutionary biologists to tackle the extensive interdisciplinary challenges that lie ahead. A major aim is to be able to detect antibiotic resistance and virulence based on genomic signatures, and predict the development and spread of antibiotic resistance. For this, large advances are being made in relatively simple systems, exemplified by TB, which can serve as a test case for more complex systems (Box 1). Eventually we will be able to predict the health of the host based on the ecology of the personal microbiome, in concert with the genetics of the individual patient, as well as assess the risk of invasion of pathogens in complex systems, such as the gut. All these challenges cannot be solved by single disciplines in isolation. The path to applying evolutionary theory to improve patient care may seem discouragingly long at times (117,118). Yet initiatives such as the incorporation of evolutionary medicine in biological and medical curricula in universities throughout the world (http://www.evmeded.org/) may serve as encouragement.

Box 1. Successes and open questions for Microbial Evolutionary Medicine

Successes:

1) Exploiting bacterial evolution (molecular clock) to trace transmission events at the scale of hospitals and continents (19)

2) Using signatures of strong positive natural selection on antibiotic resistance mutations to identify potentially causal (or diagnostic) resistance mutations (*e.g.* 119)

3) Identifying evolutionary tradeoffs that limit the acquisition of resistance genes (*e.g.* 51,120)

4) Identifying the role of intra- and interspecies microbial interactions in pathogen adaptation (*e.g.* 16,78)

5) The discovery that gut microbes with a long evolutionary history of co-speciation with mammals tend to be depleted in human disease states, suggesting that ancient associates may tend to be beneficial to health (*e.g.* 12),.

Open questions/challenges:

1) How does evolution and natural selection of microbes in the environment (or non-human hosts)

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impact their ability to colonize and cause disease in humans?

2) Can we design pathogen treatment strategies that minimize the evolution of resistance?

3) Can we design treatment strategies that favour beneficial microbes, and prophylactic treatments that disfavor the invasion of pathogens?

4) Can we exploit fitness tradeoffs to reduce the spread of antibiotic resistance and other undesirable microbial traits?

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