"Swine, Salad, Surfers and You: Is the Post-Antibiotic Era Upon Us?"

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Dr. Greenberg's research interests revolve around ways to mitigate the threat of antibiotic resistance. His laboratory focuses on the development of pathogen-specific antibiotics utilizing antisense molecules. This approach allows for a way to inhibit bacteria by silencing specific genes that are critical for growth. In addition, he is working to develop ways to better predict antibiotic resistance through the use of next-generation sequencing and innovative bioinformatic approaches.

Purpose and Overview: The purpose of this lecture is to review the impact that antibiotic resistance has on our patients and community. In addition we will discuss the major drivers of resistance in the hospital and broader environment as well as strategies that can be used to combat this worldwide crisis.

Educational Objectives:

1. Recognize that antibiotic resistance is a normal biological process.
2. Review the human and economic impact that antibiotic resistance has on the world.
3. Review common mechanisms of antibiotic resistance.
4. Review recent examples of the emergence of resistance mechanisms in different regions of the world.
5. Review majors drivers of resistance and various mitigation strategies.

Antibiotic resistance, a normal biological process. The discovery and subsequent clinical use of antibiotics is one of the great medical achievements of the past 100 years (Figure 1). There is no doubt that these drugs were not only lifesaving, but were critical for numerous medical advances which would have otherwise been difficult if not impossible. Treatment of the patient with malignancy, transplantation, chronic dialysis and implantable devices would all be at risk if we returned to the pre-antibiotic era. The emergence of the phrase "post-antibiotic era" is now discussed not as a hypothetical but as a looming possibility that could occur sooner than many would think possible (1).

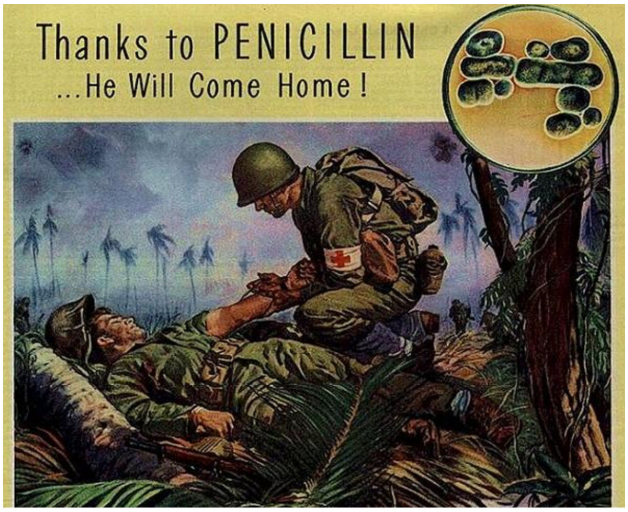


Figure 1. An advertisement by Schenley Laboratories Inc. By 1944, laboratories across the country were increasing penicillin production. Schenley's advertisement stated, "When the thunderous battles of this war have subsided to pages of silent print in a history book, the greatest news event of World War II may well be the discovery and development of penicillin." Credit: Research and Development Division, Schenley Laboratories Inc., Lawrenceburg, Indiana, USA.

The development of antibiotic resistance is a normal biological process. However, what we are currently witnessing worldwide is a rapid expansion of resistance, the origins of which are myriad. The emergence of resistance once an antibiotic is introduced into the population goes hand in hand with the history of antibiotic development and is frequently rapid (2). For example, the first reports of methicillin-resistant *Staphylococcus aureus* were reported shortly after the introduction of methicillin.

Antibiotic resistance: future economic and mortality consequences. In 2014, the Prime Minister of the UK asked the economist Jim O'Neill to study global antibiotic resistance and its potential future impact on health and the world economy. This two-year process resulted in a significant report that outlined both the threat and potential solutions (3). The report had a number of striking findings. Models suggested that by the year 2050, the annual mortality rate attributed to antimicrobial

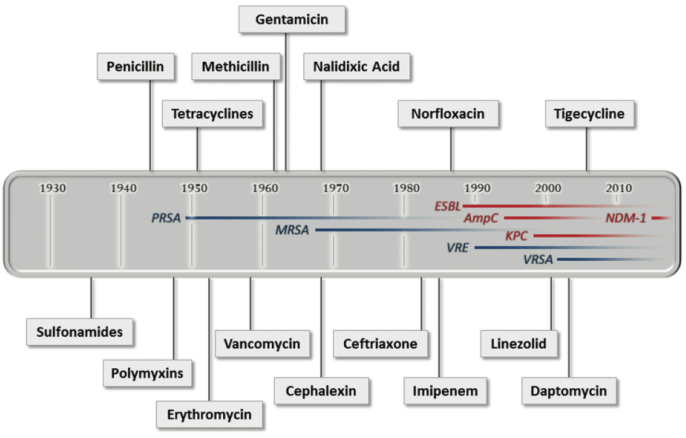


Figure 2. An antibiotic timeline depicting the years various antibiotics were introduced into clinical practice. The center of the timeline depicts the approximate time that various antibiotic resistance mechanisms emerged. Abbreviations: PRSA (penicillin-resistant *Staphylococcus aureus);* MRSA (methicillin-resistant *Staphylococcus aureus*); ESBL (extended-spectrum β-lactamase-producing *Enterobacteriaceae*); VRE (vancomycin-resistant *Enterococcus*); AmpC (AmpC-producing *Enterobacteriaceae*); KPC (*Klebsiella pneumoniae* carbapenemase-producing *Enterobacteriaceae*); VRSA (vancomycin-resistant *Staphylococcus aureus*); NDM-1 (New Delhi metallo β-lactamase-1-producing *Enterobacteriaceae*) (2).

resistance (AMR) would be 10,000,000 excess deaths per year. This would exceed the current yearly mortality rate due to cancer. The major geographic regions that will see disproportionate numbers of deaths will be in Asian and African countries although the U.S. will continue to be heavily affected. The report concluded that the aggregate number of deaths over the next 35 years would total 300,000,000 persons worldwide. This translates to a global loss in GDP of 2-3.5% with a global GDP loss of 60-100 trillion dollars (not including healthcare costs).

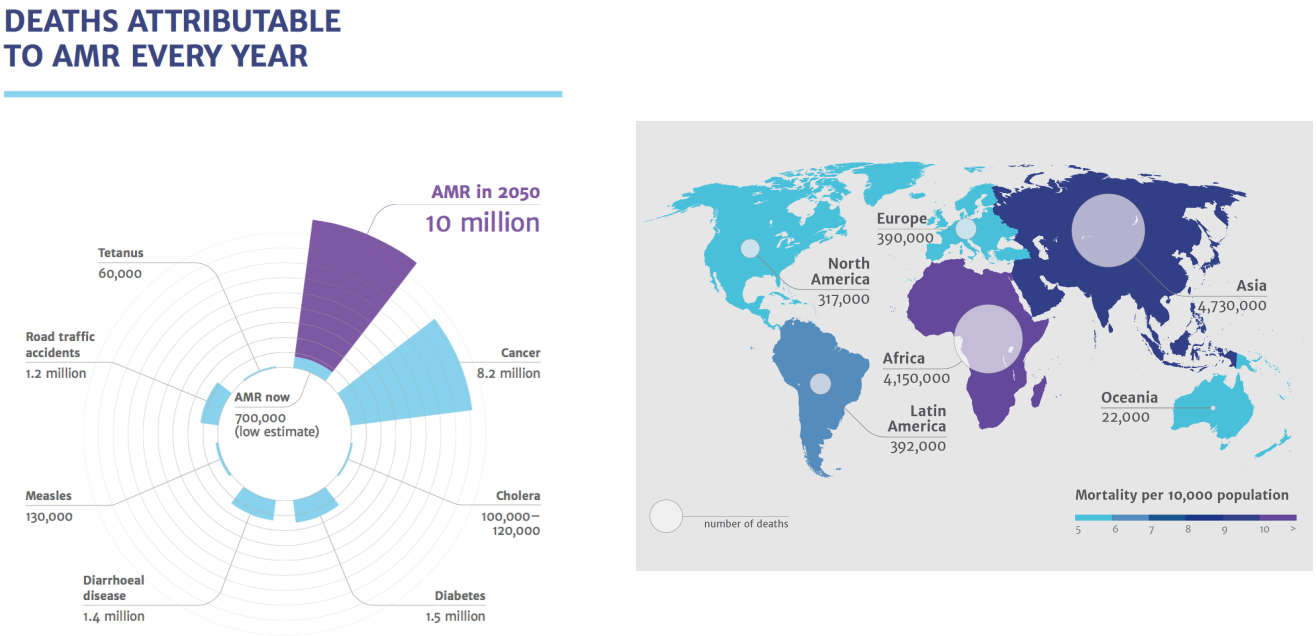


Figure 3. The future contribution of antimicrobial resistance (AMR) on worldwide mortality rates. The panel on the left depicts current leading causes of mortality worldwide (light blue). 700,000 excess deaths per year are currently attributed to AMR. In 2050, mortality directly attributable to AMR will exceed 10 million deaths per year. The panel on the right illustrates excess AMR mortality based on region of the world and displayed as mortality per 10,000 population (3).

Mechanisms of Antibiotic Resistance. There are a number of general mechanisms by which bacteria can become resistant (**Figure 4**)(4). An antibiotic can enter the bacterial cell and rapidly be pumped out of the cell through various efflux pumps. The antibiotic might be unable to enter the bacterial cell at all due to loss of various channels such as porins. Porin loss is one mechanism that is associated with carbapenem resistance in multiple pathogens. The antibiotic can successfully enter the cell and then be degraded by various enzymes. Examples of this would include the beta-lactamases. The target site of the antibiotic itself can be altered, such as with gyrase mutations and the quinolones. Finally, the antibiotic itself can be modified so it is no longer active, as is seen with aminoglycoside-modifying enzymes. One particularly dangerous scenario is when resistance determinants can be spread on mobile elements that can easily spread between different strains of

the same species or between different genera. The ability to rapidly spread resistance between various strains or genera is one of the most important drivers of some of the most notable emerging resistance stories over the past decade (5).

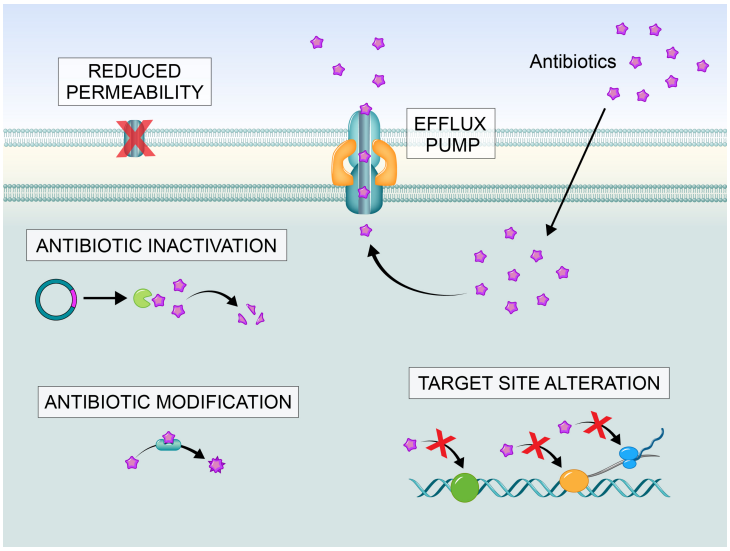


Figure 4. Common mechanisms of antibiotic resistance. Antibiotics can enter the cell and then rapidly be pumped out through efflux mechanisms. The antibiotic may be unable to enter the cell due to reduced permeability through loss of proteins such as porins. The antibiotic can be inactivated through either enzymatic inactivation or direct modification. Finally, the target site of the antibiotic can be altered resulting in loss of activity.

A story of swine: resistance in an interconnected world. In 2016, investigators in a veterinary school in China reported on an observed increase among *Escherichia coli* isolates that were resistant to colistin, a member of the polymyxin class of antibiotics. These detergent-like antibiotics have gone out of favor for clinical use given their toxicity. However, use has increased in recent years due to increasing rates of multidrug resistance. A series of studies lead to the discovery of a new gene called mobile colistin resistance gene or MCR-1 (6). This gene was the first documented example of colistin resistance that could be spread via a plasmid. An environmental study was performed looking for MCR-1 in *E. coli* isolates in pigs at slaughter as well as in retail meat in China (**Figure 5**). The percentage of MCR-1 positivity was found to be 14.4% in 2002 and

Figure 1: Map of China

	Year	Positive isolates (%)/number of isolates
Escherichia coli		
Pigs at slaughter	All	166 (20·6%)/804
Pigs at slaughter	2012	31 (14·4%)/216
Pigs at slaughter	2013	68 (25·4%)/268
Pigs at slaughter	2014	67 (20·9%)/320
Retail meat	All	78 (14·9%)/523
Chicken	2011	10 (4·9%)/206
Pork	2011	3 (6·3%)/48
Chicken	2013	4 (25·0%)/16
Pork	2013	11 (22·9%)/48
Chicken	2014	21 (28·0%)/75
Pork	2014	29 (22·3%)/130
Inpatient	2014	13 (1·4%)/902
Klebsiella pneumoniae		
Inpatient	2014	3 (0·7%)/420

Table 2: Prevalence of colistin resistance gene *mcr-1* by origin

Figure 5. MCR-1 spread through China. The left panel indicates regions where samples were taken and analyzed for the presence of the MCR-1 gene. The right panel indicates the percentage of isolates collected that were positive for the MCR-1 gene. Isolates were collected from live pigs, retail meat and human isolates (6).

increased to 20.9% in 2014. The overall rate of MCR-1 positivity in chicken or pork retail meat was 14.9%. Particularly worrisome was the finding that 13/902 (1.4%) of *E. coli* samples and 3/420 *Klebsiella pneumoniae* samples from humans in 2014 were found to be positive for MCR-1.

China is the world's largest producer of poultry and pigs (for pigs, they produce $1.134x10^{11}$ pounds per year). 10% of this production is for export out of the country. Currently, 12,000 tons of colistin are used per year in the agriculture sector with an estimated 16,500 tons to be used by 2021. MCR-1-related infections have now been reported worldwide, including in the U.S. (7-13) (**Figure 6**).

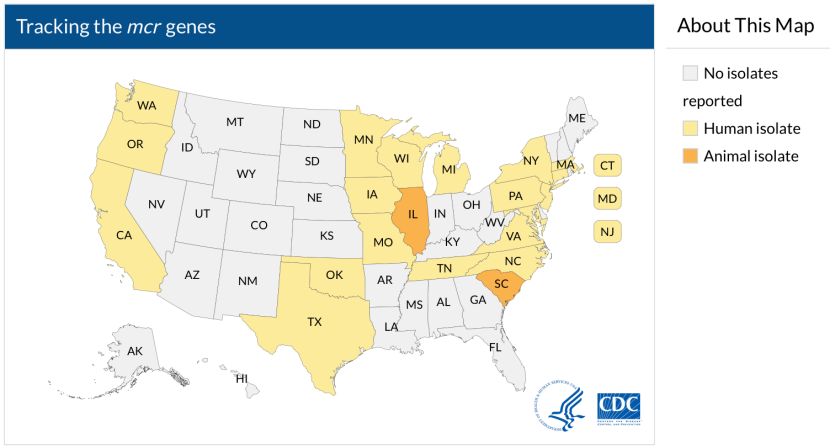


Figure 6. Current states reporting at least one confirmed human or animal isolate containing MCR-1 (Centers for Disease Control and Prevention) as of November 2, 2018.

A story of salad: it may be green, but it is definitely not healthy. There have been a number of outbreaks of bacterial diseases related to ingestion of contaminated produce (14,15). The vast majority of these are associated with gastrointestinal illness and usual pathogens include *E. coli*, *Salmonella*, *Shigella* and *Campylobacter*. Given a long history of bacterial infections associated with multiple varieties of produce, it is no surprise that in a world of rising resistance rates, items such as lettuce are at high risk of transmitting drug-resistance bacteria. Contamination of produce can occur both pre-harvest and post-harvest. A recent study illustrates the magnitude of the problem (16). Researchers tried to measure what was termed "the resistome" of produce, and specifically tried to use both culture-dependent and culture-independent methods to look for tetracycline resistance elements because tetracyclines are frequently used in the agriculture setting. Produce samples (n=24) were obtained from local supermarkets in Braunschweig or Magdeburg Germany in 2016 and 2017. Samples that were tested included mixed salad, cilantro and arugula. Both culture-dependent and molecular approaches were used to screen for resistance. 63 tetracycline-resistant isolates were identified (n=54 from cilantro, n=7 from arugula, n=2 from mixed salad). Most isolates showed resistance to at least one class of antibiotics. The *tet*(A) gene was identified in 59/63 isolates and most isolates contained mobile elements (plasmids) that harbored resistance genes. Importantly, culture-dependent methods seemed to outperform molecular detection of specific resistance genes in some cases.

In another recent study performed in the Netherlands, 1216 vegetables were obtained from various grocery stores between 2012 and 2013 and were analyzed for resistance to 3rd-generation cephalosporins (17). Samples included both conventionally and organically grown vegetables as well as from those originating from other European countries. Isolates included those that produced extended-spectrum beta-lactamases (ESBL) and were fecal-oral related bacteria (*Escherichia coli*, *Enterobacter* spp.), those that had inducible resistance and were fecal-oral related (AmpC-producing *Citrobacter freundii*, *Enterobacter* spp.), ESBL-producing environmental bacteria and AmpC-

Origin and number of 3GC-positive vegetable items.

Vegetable type (% positive items)	No. of positive items/no. of investigated items				
		Conventional		Organic	
	Total	NL	non-NL	NL	non-NL
Blanched celery (4.7%)	9/192	3/48	4/48	1/48	1/48
Bunched carrots (8.9%)	17/190	2/48	7/51	5/47	3/44
Butterhead lettuce (6.6%)	9/137	4/48	0/0	4/48	1/41
Chicory (0.0%)	0/96	0/48	0/0	0/48	0/0
Endive (3.7%)	7/188	3/48	0/48	2/48	2/44
Iceberg lettuce (2.6%)	5/193	2/48	0/48	3/49	0/48
Radish (5.0%)	6/120	1/48	0/0	5/48	0/24
Spring onion (10.0%)	10/100	0/4	6/48	4/48	0/0
Total (5.2%)	63/1216	15/340	17/243	24/384	7/249

Figure 7. The origin and number of third-generation cephalosporin-resistant *Enterobacteriaceae* isolates in European vegetables. The overall prevalence of resistant isolates was 5.2% (17).

producing environmental bacteria. Overall, 5.2% of all sampled vegetables were resistant to 3rd-generation cephalosporins (Figure 7). There was no statistical difference between country of origin or method of growth in the rate of drug-resistant bacteria that were found.

A story of surfing: a peaceful pastime no longer. The widespread use of antibiotics can lead to resistance in a variety of environmental niches. In one 2017 study, investigators aimed to link exposure to water to colonization with antibiotic resistant pathogens (18). This cross-sectional study aimed to compare those who had frequent water exposure (in this case surfers and body boarders) to those with little water exposure. 97 water sites from England and Wales were sampled in 2012 and culture was performed to isolate *E. coli* colonies that were resistant to 3rd-generation cephalosporins. Molecular assays were used to test for the presence of CTX-M beta-lactamases. 15% of the sites contained *E. coli* that were resistant to 3rd-generation cephalosporins and 11% were positive were CTX-M genes. Between April and October 2015 surfers and non-bathing controls were recruited and asked to submit a rectal swab and answer a short questionnaire. 143 surfers and 130 controls were included in the final analysis (Figure 8). 9.1% of surfers were found to be fecal carriers of cefotaxime-resistant *E. coli* compared to 3.1% of controls giving a risk ratio of 2.95. In addition, 6.3% of surfers were found to carry CTX-M genes compared to 1.5% of controls which was also statistically significant.

The number (%) of surfers and controls colonised by antibiotic-resistant *E. coli*.

	Surfers (N = 143)	Controls (N = 130)	Risk ratio (95% CI)	*p* value
Carriage of cefotaxime-resistant *E. coli*	13 (9.1%)	4 (3.1%)	2.95 (1.05 to 8.32)	0.040
Carriage of $bla_{CTX\text{-}M}$-bearing *E. coli*	9 (6.3%)	2 (1.5%)	4.09 (1.02 to 16.4)	0.046

Figure 8. Rates of 3rd-generation cephalosporin resistance in UK surfers versus those with little coastal water exposure. Surfers were 3 times as likely as controls to be colonized by cefotaxime-resistant *E. coli*.

CDC Emerging Threats and Frequent Mechanisms of Resistant Transmission. In 2013, the Center for Disease Control and Prevention published a report on antibiotic threats in the United States. This report outlined the major pathogens that pose an urgent or serious threat to public health due to their rapidly-increasing rates of antibiotic resistance (19). In addition, this document nicely illustrated some of the major transmission dynamics of antibiotic resistance that put humans at risk (Figure 9). Individuals who receive antibiotics will develop resistant bacteria in their gut. Although they may not develop clinical disease, these individuals can spread these potential pathogens both at home or in the community. If the individual gets care at a health care facility, resistant bacteria

can spread to other patients who then return into the community. In addition, **animal exposure in animals** leads to resistant bacteria in their guts. Drug-resistant bacteria can remain on meat and can then spread to humans if improperly handled. Also, fertilizer or water runoff containing animal feces can spread resistant bacteria onto other food-related cops which human then ingest.

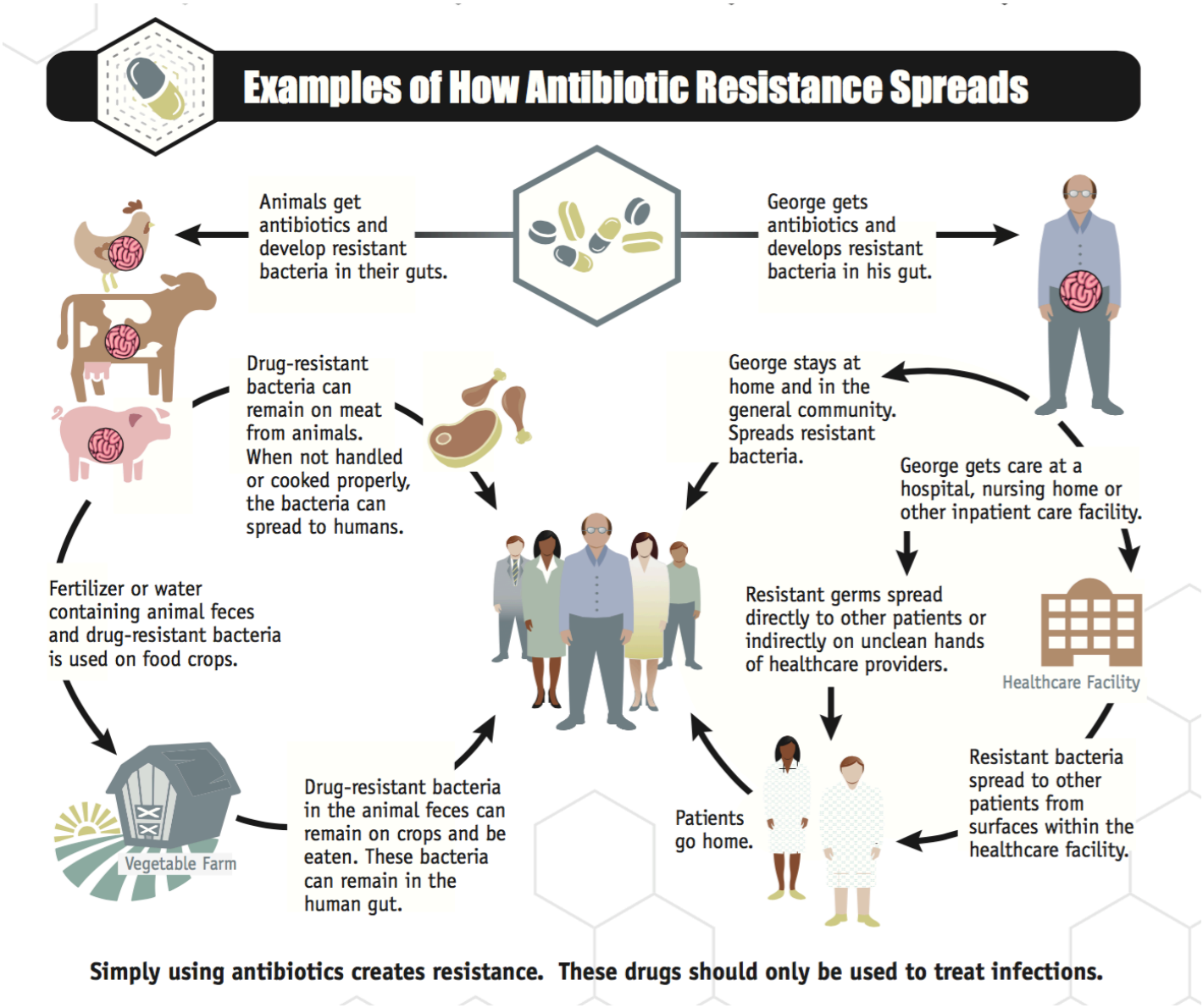


Figure 9. Frequent routes of transmission and acquisition of antibiotic-resistant bacteria among humans and animals (CDC 2013). Both human-to-human and food-to-human cycles play important roles in spreading antibiotic resistant pathogens.

This is not just an Asia problem. Developed countries to various degrees are seeing the rapid emergence of antibiotic resistance depending on the organism and antibiotic in question. A recent report from the European Centre for Disease Prevention and Control illustrates the problem that various countries in Europe are facing (20). As an example, the percentage of *Klebsiella pneumoniae*

isolates that had combined resistance to fluoroquinolones, third-generation cephalosporins and aminoglycosides in 2017 ranged from 0% in Iceland to greater than 57% in Slovakia (Figure 10). Over 13 European Union countries showed increasing trends of resistance between 2014-2017.

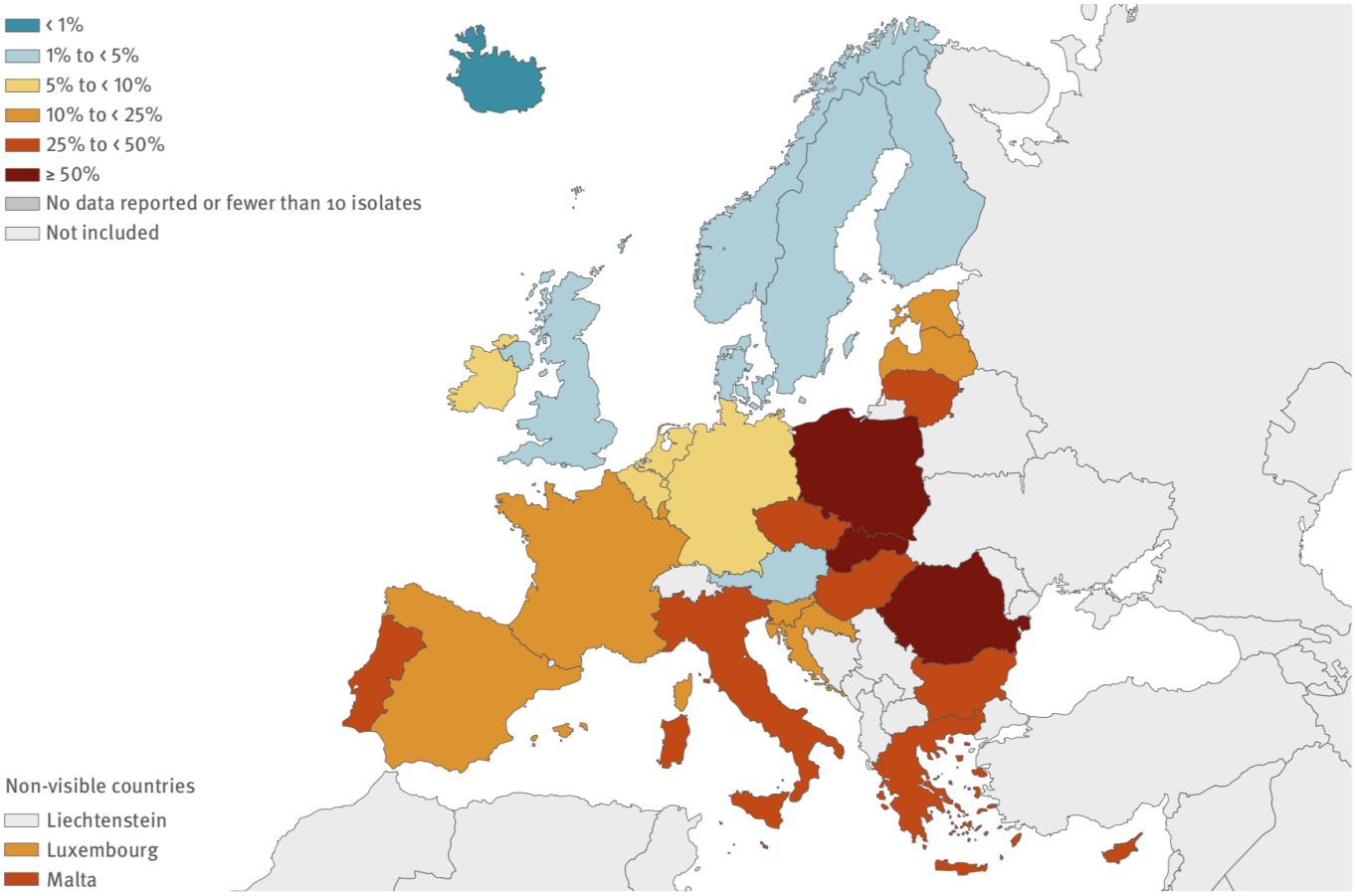


Figure 10. Distribution of multidrug-resistant *Klebsiella pneumoniae* among European countries in 2017. Isolates were invasive and had combined resistance to fluoroquinolones, third-generation cephalosporins and aminoglycosides. Rates of MDR *K. pneumoniae* varied greatly from 0% in Iceland to >57% in Slovakia.

This trend is not unique to just members of the *Enterobacteraciae*. MDR *Pseudomonas aeruginosa* is also frequent (Figure 11) ranging from 0% in Iceland to >59% in Romania. For example, if you were hospitalized while on vacation in Greece and developed a *P. aeruginosa* infection, you would have an ~1 in 3 chance of having that isolate be resistant to >3 groups of the most active antibiotics used for this pathogen.

In the United States, levels of antibiotic resistance vary by state, pathogen and class of antibiotic. The CDC is one tool that is helpful in getting data on levels of resistance. In addition, The Center For Diseases Dynamics, Economics & Policy (CDDEP, resistancemap.cddep.org) has a variety of useful tools for visualizing levels of AMR in multiple countries. Examples of this are shown in Figure 12. The rate of carbapenem resistance in *P. aeruginosa* isolates range from 5% to over 16% in 2016.

Resistance rates can also be analyzed across different categories of antibiotics. As an example, the rate of methicillin-resistant *Staphylococcus aureus* (MRSA) varied by region from over 30% in the Mountain region to over 50% in the East South Central region of the U.S.

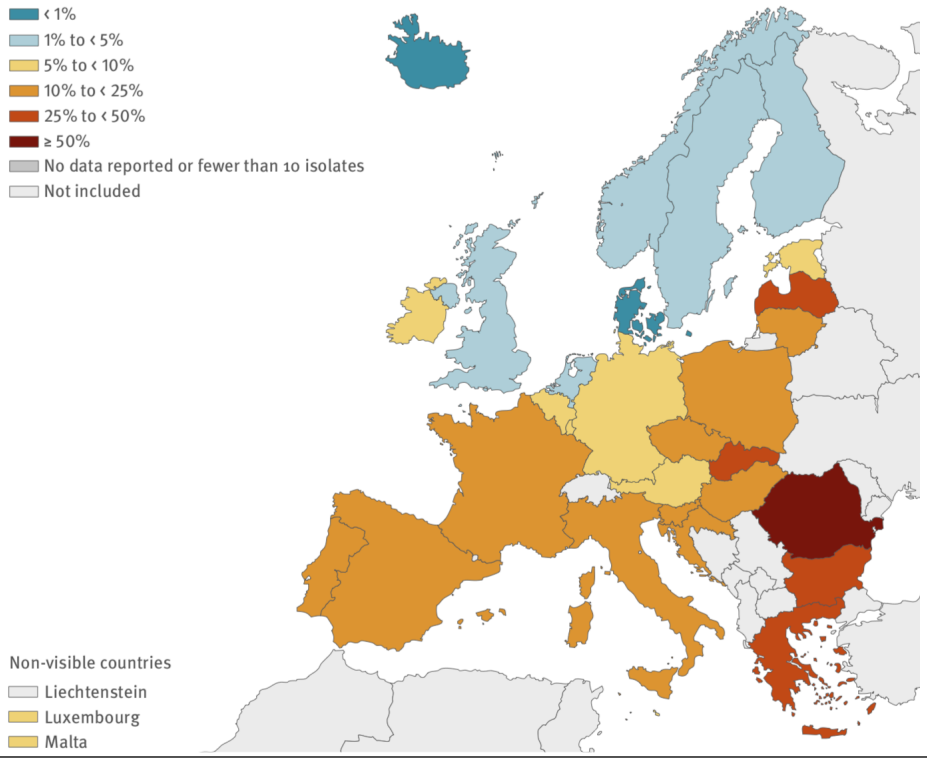


Figure 11. Distribution of multidrug-resistant *Pseudomonas aeruginosa* among European countries in 2017. Invasive isolates had combined resistance to three or more antimicrobial groups among piperacillin +/- tazobactam, ceftazidime, fluoroquinolones, aminoglycosides and carbapenems. Rates of MDR *P. aeruginosa* varied greatly from 0% in Iceland to >57% in Slovakia.

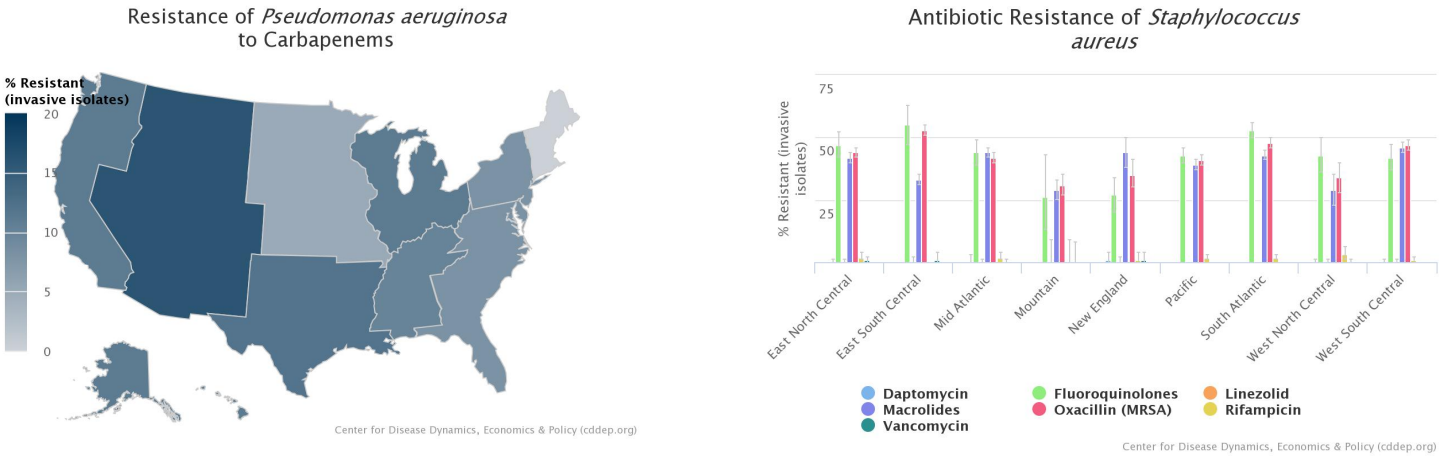


Figure 12. Examples of MDR data in the United States. The left panel displays in map form the % of carbapenem-resistant *P. aeruginosa* by region. Over 15% of isolates in the Mountain region are resistant to carbapenems as of 2016. The right panel displays the % of resistant *S. aureus* by both drug and region. As of 2016, some regions of the U.S. (such as the East South Central region) have methicillin-resistant *Staphylococcus aureus* rates greater than 50% (resistancemap.cddep.org).

The way forward. Given the interconnected world that we live in, the response to preventing a "post-antibiotic" world from becoming a reality must involve multiple strategic approaches at the local, federal and international level (Figure 13).

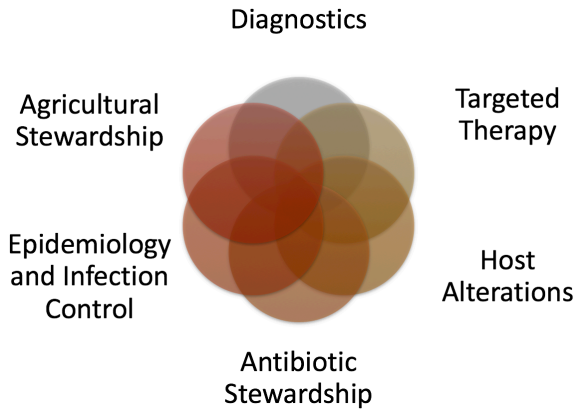
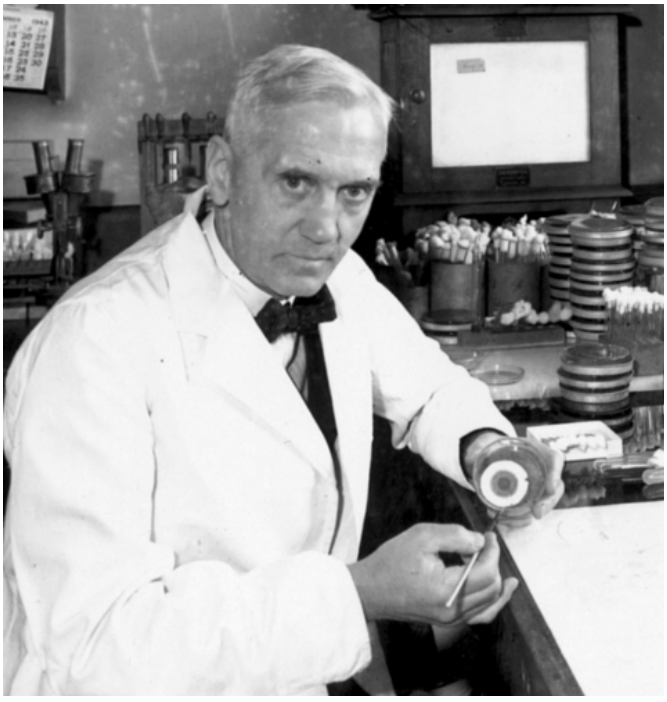


Figure 13. Strategies for combating antibiotic resistance. Addressing the antibiotic resistance crisis will require a multi-modality approach that focuses both on the control and exposure of antibiotics in the environment as well as new ways to treat and diagnose infections.

Not only are new antibiotics urgently needed, innovative paradigms for targeted pathogen therapy instead of broad-spectrum acting agents could provide effective antibacterial efficacy while minimizing potential resistance development. Examples of this approach include phage therapy (21,22) and antisense molecules which we have been developing in the lab (23-27). A major reason why there historically has been a broad embrace of developing broad spectrum antibiotics have been the challenges in diagnosing Infectious Diseases. Similar to the methods used by Alexander Fleming (Figure 14) in the 1940s, clinical microbiology laboratories still heavily rely on first cultivating a bacterium followed by testing to see to which antibiotics the bacterium is sensitive to. In many cases, this entire process can take days. Rapid diagnostics that can both speed up identification and antibiotic susceptibility testing could aid in a more judicious use of more narrow spectrum antibiotics. There have been a number of advances in this area, including the use of next-generation sequencing technologies (NGS) which we and others have been using to predict antibiotic resistance (28-30).



In one recent study from our group, we utilized NGS and new bioinformatic algorithms to try and predict the antibiotic resistance phenotype of bacteremia isolates from cancer patients at MD Anderson (28). Through both automated as well as manually curated approaches, we predicted whether the presence or absence of various antibiotic resistance genes (ARGs) or mutations in these genes could predict susceptibility or resistance to four commonly used antibiotics: cefepime, piperacillin-tazobactam, meropenem and ceftazidime in four frequently encountered pathogens (*K. pneumoniae*, *E. coli*, *P. aeruginosa*, *Enterobacter cloacae*)(). When using broth microdilution as the goal-standard test, the NGS approach had sensitivity (87% vs 82%) and

Figure 14. Alexander Fleming at the bench. Clinical microbiology laboratories continue to heavily rely on cultivation of bacteria for both identification as well as determination of antibiotic susceptibility testing.

specificity that was comparable to standard phenotypic tests (98% vs 95%) respectfully (Figure 15).

Diagnostic Performance	WGS	Clinical Microbiology	*P* Value
Sensitivity (95% CI)	0.87 (.81–.92)	0.82 (.76–.88)	.36[a]
Specificity (95% CI)	0.98 (.96–.999)	0.95 (.92–.98)	.07[a]
Positive predictive value (95% CI)	0.97 (.94–.999)	0.92 (.88–.97)	.025[b]
Negative predictive value (95% CI)	0.91 (.88–.95)	0.88 (.84–.92)	.24[b]

Abbreviations: CI, confidence interval; WGS, whole-genome sequencing.

[a]McNemar test.

[b]Score statistic derived from marginal regression model.

Figure 15. Diagnostic performance of whole-genome sequencing versus clinical microbiology data using broth microdilution as the gold standard. NGS and bioinformatic algorithms were compared to performance by the clinical microbiology lab using 3rd party broth microdilution as a gold standard. Both sensitivity and specificity were comparable to the hospital clinical microbiology lab that utilized phenotypic screening (28).

There is no doubt that a major driver of antibiotic resistance is the indiscriminate use of antibiotics in agriculture for both infection prevention and growth promotion. A number of countries have begun to curb or ban certain antibiotics in agriculture, including the U.S. In 2017, the FDA made it illegal to use antibiotics in animals for use as a growth promoter. However, they may still be used for infection prevention. It will likely require more stringent control measures in order to curb the trajectory of resistance.

Antibiotic stewardship remains a critical component for controlling resistance, particularly at the local and hospital level. Antibiotics are one of the few drugs that we tend to limit the use of, particularly when a new drug arrives on the market. This has had the dramatic effect of multiple companies getting out of the antibiotic development business as making antibiotics is currently not economically favorable. There have been, and will continue to be a number of incentives at the governmental level to spur the development of new antibiotics that will be needed in the future. It is likely that the treatment of infectious diseases, particularly those caused by bacterial pathogens, will require constant ongoing efforts to try and stay ahead of inevitable resistance emergence.

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