# Behind the Antibiotic Resistance Curtain

A novel device and Illumina sequencing enable researchers to identify the multiple pathways *Escherichia coli* uses to evolve and outwit three different antibiotics.

## Introduction

According to the World Health Organization, most pathogenic species in existence today have developed resistance to one or more antimicrobials. The rapid increase in antibiotic resistance of *Escherichia coli* is particularly troublesome since it is such a prevalent microbe. *E. coli* is an essential component of the digestive tract flora of healthy humans and even most animals. Outside of that realm, it can cause severe illness and sometimes death, largely because of a growing resistance to the antibiotics used to combat it. In fact, multidrug resistance in *E. coli* has increased from 7.2% during the 1950s to 63.6% during the 2000s[1].

Recent studies suggest that the overuse of antibiotics drives not only antibiotic resistance, but also supports the selection of highly evolvable bacteria that mutate in record time[2]. Scientists investigating the development of antibiotic resistance have used next-generation sequencing (NGS) to identify the mutations that turn antibiotic-sensitive bacteria into resistant bacteria. Still in question are the specific pathways bacteria use to evolve and achieve resistance. Understanding this process requires a system that closely mimics bacterial evolution in the human body in response to the presence of antibiotic.

Researchers in Roy Kishony's laboratory in the Department of Systems Biology at Harvard Medical School developed a novel microbial selection device, called the morbidostat, to identify bacterial resistance pathways. Led by Erdal Toprak, Ph.D., the team used the device and Illumina sequencing to analyze the evolution of *E. coli* resistance under selection with chloramphenicol, doxycycline, and trimethoprim[3].

iCommunity spoke with Dr. Toprak to learn about the advantages of the morbidostat and Illumina sequencing in tracking bacterial resistance evolution.

Erdal Toprak, Ph.D. was in Dr. Kishony's lab at Harvard Medical School and led the team performing bacterial resistance research with the morbidostat device. He is currently a member of the Engineering and Natural Sciences faculty at Sabanci University.

*Q: What were the limitations of previous drug resistance experiments that prompted you to conduct this study?*

**Erdal Toprak (ET):** We've had two approaches to studying bacterial resistance, clinical studies and *in vitro* studies. In clinical studies, patient sample cultures are isolated, the genomes of resistant bacteria are compared to those of wild-types that are sensitive to drugs, and the resistance mutations identified. The process provides you with the beginning and end points, but provides little information about how the resistance evolved and the adaptive pathways used.

People have tried to simulate those pathways in the laboratory, culturing cells, plating and incubating them with high levels of antibiotic, identifying resistant colonies, and then sequencing those resistant mutants to understand the changes that have occurred. Once again, we have data on the beginning and end points of bacterial evolution, but no pathway data.

*Q: Do those approaches typically obtain the same results?*

**ET:** You'd like to see the same results either way you do it. Unfortunately, there are inconsistencies. Patient isolates typically have multiple mutations that combine to make them super resistant to drugs. However, the results of *in vitro* experiments typically identify only one or two mutations. There might be some mutations in common, but there's usually not a complete match of the results.

Performing mutant selection experiments in the lab is very difficult because of the minute adjustments of drug concentration that are necessary. If you use too much drug, then nothing will grow. If you use too little, then you won't generate resistant bacteria. It's a very sensitive and touchy experiment that's hard to reproduce.

*Q: Describe the morbidostat device. How does it enable microbial selection?*

**ET:** We wanted to see the evolution of drug resistance in real-time. Rather than using traditional agar plates and incubation techniques, we used computers and optical detection devices to follow the evolution. The idea was simple, although the device is complex. Everything is automated through a robotics system that can test up to 15 strains at once.

The morbidostat can count cells every second and calculate the growth rate every 5 minutes, as opposed to normal lab experiments where you're calculating growth rate every 12 hours or so. It continuously adjusts antibiotic concentration to maintain nearly a constant growth inhibition of a microbial population. The computer monitors the fitness of the bacteria in high resolution, assessing the number of cells in the culture and their fitness. If the number of cells is too much and they are growing too fast, the computer increases the drug concentration. We don't kill the bacteria, just inhibit their growth by 50%. As antibiotic-resistant bacteria evolve and start

multiplying, the computer detects it. When they reach a certain threshold, the drug concentration is increased and the cycle begins again.

*Q. How is the morbidostat different from continuous culture systems such as chemostats?*

**ET:** Like chemostats, the morbidostat feeds a culture with fresh medium at a constant rate, achieving a steady-state or fixed-growth inhibition when the culture's growth rate is equal to the fixed dilution rate. In a chemostat, nutrient limitation provides the feedback to achieve a steady state. In contrast, the growth rate in the morbidostat is set by adjusting the antibiotic level to make the bacteria perpetually "unhappy", creating a stressful environment. The computer's control algorithm doesn't elevate drug concentration in a predefined way to maintain a fixed growth inhibition. Instead, it automatically adjusts drug concentration according to the actual rate at which resistance evolves.

*Q: Using the morbidostat, you analyzed the resistance evolution of 15* E. coli *populations in response to three different antibiotics. What did the results show?*

**ET:** To test the reproducibility of the evolutionary pathways, five isogenic populations of drug-sensitive *E. coli* (MG1655) were evolved in parallel using the morbidostat under inhibition by chloramphenicol, doxycycline, and trimethoprim. We exposed the bacteria to the three antibiotics separately, so we had bacterial resistance evolution results for each antibiotic.

Close to 400 bacterial generations evolved after about three weeks using the morbidostat device. The resistance levels increased dramatically, with all parallel populations showing similar qualitative and quantitative phenotypic changes. The resistance for chloramphenicol and doxycycline increased smoothly over time, whereas trimethoprim resistance increased in a stepwise fashion.

*Q: You performed whole-genome sequencing to identify SNPs in the different bacterial populations. What technology did you use?*

**ET:** We used the Illumina Genome Analyzer™ system to perform whole-genome sequencing. It took us less than two days to prepare libraries for 20 samples. We submitted them to the Partners Center for Personalized Medicine for sequencing and in less than a week we received the sequence data. It was an extremely fast turnaround.

Our lab had always used Sanger sequencing and was performing Illumina sequencing for the first time. To check the accuracy of the results, we performed Sanger sequencing on the samples to see if the Genome Analyzer identified the same SNPs. Illumina sequencing identified 90–95% of them, which made us very confident in the sequencing results.

*Q: What did the Genome Analyzer whole-genome sequencing data tell you about these populations?*

**ET:** On the final day of the experiment, we selected an isogenic clone for each evolved population and sequenced its genome using the Genome Analyzer. Of the 47 SNPs found with whole-genome sequencing and confirmed by Sanger sequencing, the majority resulted in amino acid alterations or were localized to gene promoters.

*Q: Did you see SNP patterns unique to the bacterial resistance generated to each antibiotic?*

**ET:** Chloramphenicol and doxycycline are ribosomal inhibitors and the mutations in response to these two drugs occurred mostly in genes encoding membrane proteins and factors involved in transcription or translation. Trimethoprim inhibits folic acid biosynthesis by binding to dihydrofolate reductase (DHFR). Most of the mutations that evolved under this antibiotic were found in the DHFR gene, with almost all of the encoded amino acid substitutions appearing repeatedly in several of the evolved populations.

*Q: Were you surprised by some of the mutations and pathways that you identified for the chloramphenicol and doxycycline challenged populations?*

**ET:** The sequencing yielded several unexpected results. The evolution of the five different chloramphenicol-challenged *E. coli* populations had almost identical phenotypic changes. The same was true of the doxycycline-challenged populations. Despite the fact that both drugs target ribosomes, we didn't see any ribosomal gene mutations in either of the populations. That was surprising since ribosomal mutations had previously been isolated in selection experiments performed on agar plates. This might reflect a cost in growth rate or negative epistatic interactions with other mutations fixing in these cultures.

> "It took us less than two days to prepare libraries for 20 samples... and in less than a week we received the sequence data. It was an extremely fast turnaround."

The chloramphenicol- and doxycycline-challenged populations also exhibited marked differences in mutation patterns. There was very little overlap in membrane protein mutations, although the mutations appeared in genes with similar functions, including the efflux protein genes *acr*, *cmr*, and *mar* that are known to confer resistance to these antibiotics. Differences in mutation patterns were also evident in gene mutations involved in transcription and translation.

For both of these drugs, all of the populations reached the same level of resistance, even though they acquired different sets of mutations. There were clearly multiple alternative pathways to circumvent chloramphenicol- or doxycycline-induced protein synthesis stress, with each requiring a small number of mutations in a diverse set of genes. The waiting time for mutations to appear in these populations is likely to be short due to the large target size for possible mutations, consistent with the smooth phenotypic changes in these populations.

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*Q: Could any of the new mutations you identified be useful as targets for new* E. coli *therapeutics?*

**ET:** Absolutely. We have a list of new genes involved in chloramphenicol and doxycycline resistance that we are currently characterizing.

*Q: Did the observed similarities between the chloramphenicol- and doxycycline-resistant bacteria confer cross-resistance? What about with trimethoprim?*

**ET:** When the five *E. coli* populations became chloramphenicol-resistant, we challenged them with doxycycline, and then trimethoprim. The chloramphenicol-resistant *E. coli* were doxycycline-resistant, demonstrating cross-resistant evolution had occurred. The two drugs have a similar target, which explains how the bacteria could evolve resistance to a drug that hadn't yet been introduced. Trimethoprim has a different target and we saw no evidence of cross-resistance evolution.

*Q: Could these results impact* E. coli *treatment decisions?*

**ET:** These cross-resistance profiles definitely inform clinical choices. If you begin treating *E. coli* infections with chloramphenicol or doxycycline, resistance will build to both of them and your next antibiotic option will be limited. I am not a medical doctor, but I believe that providing a large and reliable data set for cross resistance might be very handy for clinicians.

*Q: How did the mutations and pathways of the trimethoprim-challenged* E. coli *populations differ from the other bacterial resistance profiles?*

**ET:** For trimethoprim, the resistance change occurred in discrete steps. Illumina sequencing identified only four mutations in the genome and all of them were in the DHFR enzyme gene sequence. These included mutations in the promoter and amino acid substitutions in P21L, L28R, A26T, I94L, and W30R/G. Each of the five isogenic clones had very similar mutation traces, with resistance increasing in four steps via two main pathways.

It appears evolutionary path constraints limit the number of pathways that can make the bacteria highly resistant to trimethoprim. That tells pharmaceutical scientists that if they can cut those pathways, they can reduce bacterial resistance to the drug. That's very valuable information, and it is the first time that ordered adaptive pathways leading to high levels of antibiotic resistance have been seen in bacteria. Typically, such sequential fixation of mutations had been seen only in virus populations. We wouldn't have been able to identify the pathways without the morbidostat.

*Q: Could this information be valuable in developing other drugs that target the DHFR enzyme gene?*

**ET:** DHFR is also a target for anti-malaria and cancer drugs and this information will be very helpful in determining how to fine tune these drugs to be more effective. One very common drug resistance mechanism occurs once the drug binds to the target, with a mutation in the DHFR gene decreasing the binding strength of the drug to the target. Now that we've identified the kinds of mutations that bacteria generate when challenged with a DHFR-targeted drug, pharmaceutical scientists can modify the chemical composition of those drugs, circumventing those mutations and enabling the drug to create a strong bond with the enzyme to efficiently compromise the pathogen's ability to thrive.

*Q: What did your study demonstrate about the evolution of drug resistance?*

**ET:** We now know how drug resistance evolves, the mutations, pathways, and biochemical changes that transform antibiotic sensitive bacteria into antibiotic-resistant bacteria. If we know what kind of changes will happen throughout the drug treatment, then we can take further actions to stop those that thwart the effectiveness of the antibiotic. For example, the drug amount can be increased or decreased, or additional drugs can be administered.

> "I found that I could sequence an entire genome for the price of using Sanger to sequence 40 genes."

*Q: Could this type of testing be used in real-time with patients?*

**ET:** It could be very useful in a patient care setting, and we've started performing these types of analyses. If you provide me with an *E. coli* sample of unknown resistance strength, in about eight hours I can characterize its resistance using the morbidostat.

*Q: The morbidostat provides a window into evolution in real-time. How could the morbidostat be used for other studies?*

**ET:** I think it will be a very strong application, especially in pharmaceutical research. If you are a researcher developing a new drug and you want to see how fast bacteria will acquire resistance to it, the morbidostat would be the best tool to use.

*Q: What's the next step in your research?*

**ET:** This first study was a large-scale cloning experiment. The next step for us is to perform these experiments using other antibiotics in other bacteria. I have a single-molecule biophysics background and am trying to combine morbidostat with a custom microfluidics system so we can characterize cells at the single-cell level.

*Q: What will the single-cell measurements provide you that the large-scale cloning does not?*

**ET:** In our first experiment, the morbidostat is making calculations based on the data received from an optical density measurement device. If there are rare mutants or if they have different morphologies, we can't see them. With a microscope, we could see what they look like and the physiological state they are in. We'd have much more information about the physiology of the cell and would be able to perform the analysis in real-time as well.

*Q: Will you be using whole-genome sequencing in these experiments?*

**ET:** Absolutely. When I started this project, I barely knew what sequencing was. I had always thought it was a very long process and would be very costly. I found that I could sequence an entire genome for the price of using Sanger to sequence 40 genes. That makes it a very cost effective way to obtain genomic information. What's great is you can isolate the DNA once, sequence a very small portion of it, and keep the rest for other analyses.

## References


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