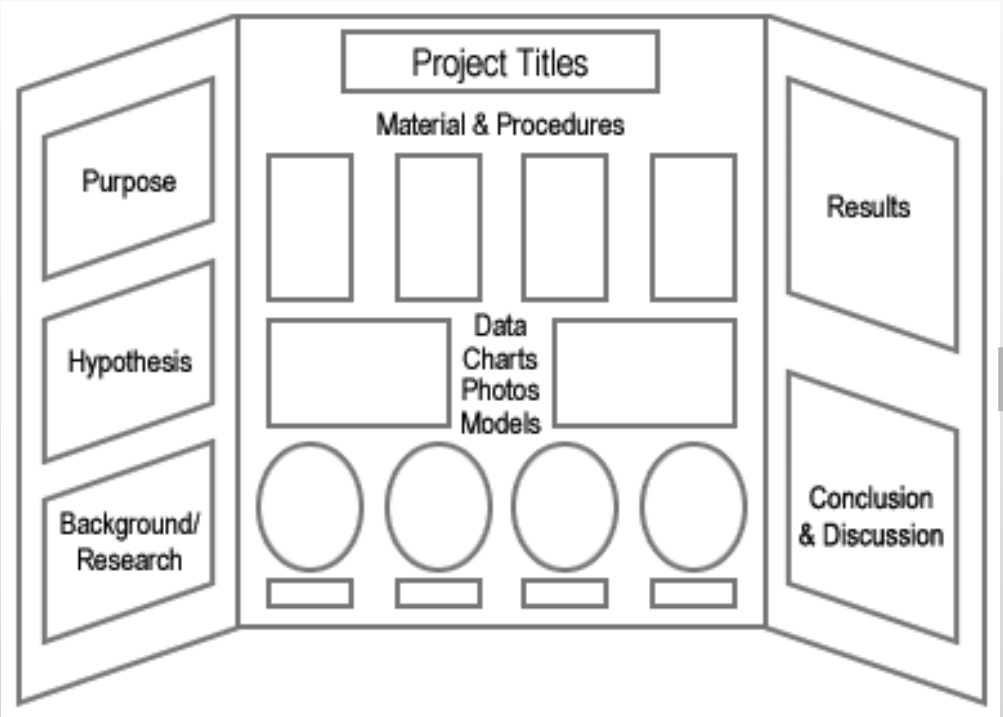
Journal of Emerging Investigators

Science publishing is not just for Ph.D.'s.

Students can do it too!

Sarah C. Fankhauser, Harvard Medical School

July, 2012



Environmental strains of P. aeruginosa are not highly resistant to rifampicin despite having sequence variability in the antibiotic target gene RNA polymerase.

John Doe[1], Jane Doe[2], members of the JEI Staff[1]

[1]Harvard Medical School, [2]Local High School

Summary:

Introduction:

Results:

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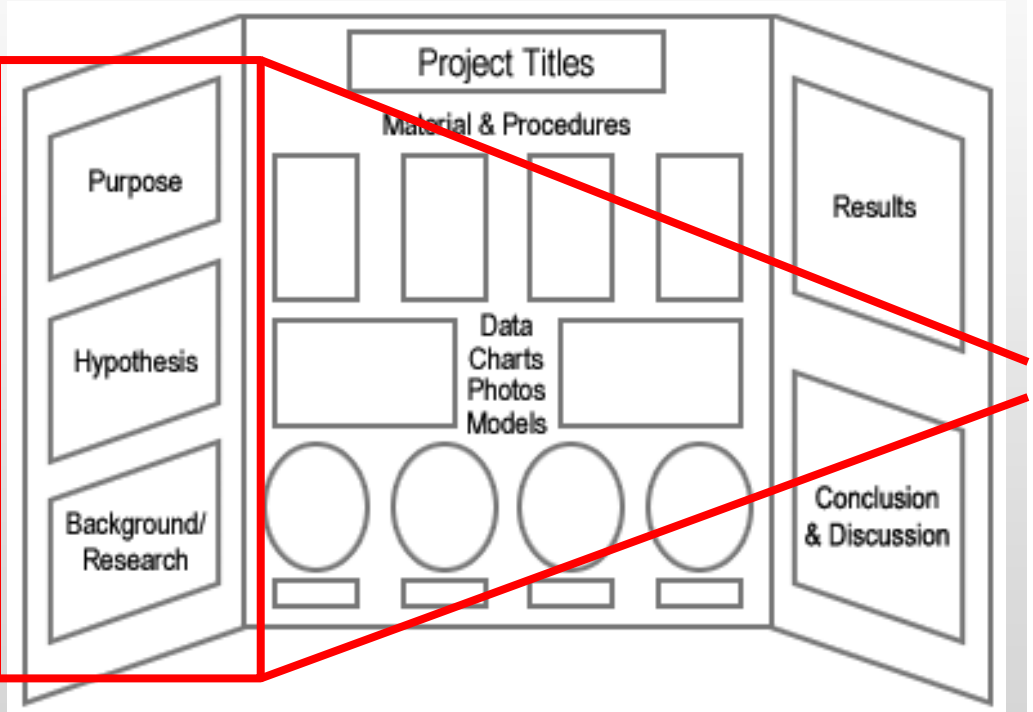

Sample Paper: Environmental strains of P. aeruginosa are not highly resistant to rifampicin despite having sequence variability in the antibiotic target gene RNA polymerase.

John Doe, Jane Doe, members of the JEI Staff
Harvard Medical School, Local High School

Summary

Antibiotic resistance of bacterial pathogens is an increasing public health concern. Patients with normally routine hospital-acquired infections are now at serious risk due to insufficient antibiotics that result in the death of thousands worldwide. One bacterial species that is partially responsible for these deaths is P. aeruginosa. As an opportunistic pathogen, P. aeruginosa normally does not cause infections, and is found in the normal environment. While many clinical P. aeruginosa strains are resistant to several antibiotics there have been no previous studies examining the

Introduction

antibiotic resistant species is Pseudomonas aeruginosa. Some P. aeuriginosa antibiotic resistant strains are able to avoid toxicity by synthesizing an efflux pump that simply pumps the compound out of the bacteria (5). Other resistant strains have mutations in their DNA that prevent the antibiotics from working (6-7). For example, many of these bacterial strains have single point mutations (one base pair changes) that alter the amino acids coded by the DNA. These single amino acid changes can alter the antibiotic target and thus prevent the antibiotic from binding and working (7). One question that remains is: how prevalent are mutations in specific antibiotic targets in environmental bacterial populations? We hypothesized that all environmental P. aeuriginosa strains isolated would be sensitive to the antibiotic rifampicin, since they have never been exposed to the drug previously and thus have not evolved resistance. Rifampicin works by binding to one subunit of the bacterial RNA polymerase (RNAP) and inhibiting its essential function of transcribing DNA to RNA (8). Mutations at the DNA level, however, can result in a mutant polymerase which is resistant to

http://www.trsef.org/how-do-i-do-a-science-fair-project/

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INTRODUCTION

Science Fair/ lab report

- Purpose
- Hypothesis
- Background research

Science Article

- Purpose
  - Explain the topic.
  - Why is this topic important?
- Background
  - What is already known?
  - What is unknown? What questions are you interested in?
- Hypothesis
  - What is your hypothesis and how did you come up with it?
- Brief summary
  - What are the general results that you found?

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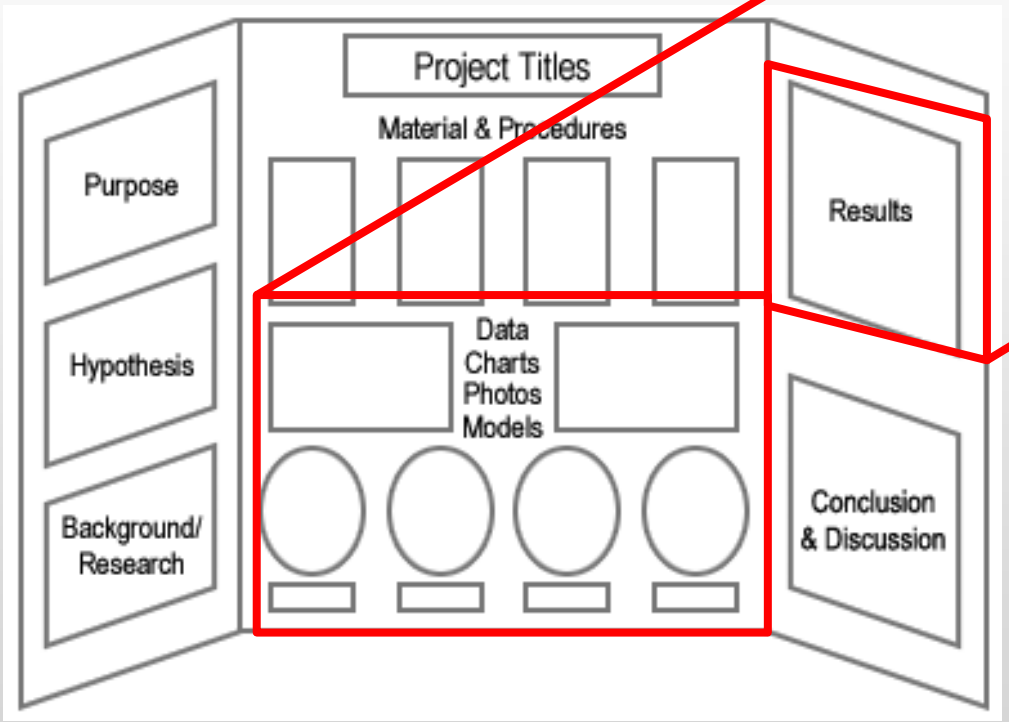

Results

characterize several strains. We used Cetrimide selective agar to isolate unique environmental strains (10). We took samples from 50 locations around the Boston area including playgrounds, soil, and plants. From these 50 sample locations we were able to grow 30 P. aeuriginosa strains. In addition, we grew two laboratory strains in parallel as controls: one strain (R) is resistant to rifampicin and the other strain (S) is sensitive to rifampicin. We confirmed that these 30 strains were in fact P. aeruginosa through the use of bacterial strain identification strips. Furthermore, all strains smelled similar to the two control strains which have a grape scent, a common feature of growing P. aeruginosa on agar plates (11).

Most strains of P. aeruginosa are sensitive to rifampicin

We next wanted to test the sensitivity of these strains to the antibiotic rifampicin. Rifiampicin is an RNA polymerase inhibitor that is known to inhibit the growth of P. aeuroginosa. We first grew all 30 strains, along with our two control strains, in liquid culture in the presence and absence of varying concentrations of rifampicin. We then used the optical density (or the cloudiness) of these cultures as a readout of growth over time. In liquid culture 28 of the 30 strains and our S strain grew dramatically slower in the presence of antibiotic when compared to growth with no antibiotic present. The remaining two environmental strains and the R lab strain grew identically whether or not rifampicin was present in the culture media.

We next wanted to test whether the sensitivity to the antibiotic seen in liquid cultures was also observed when grown on agar plates. We first spread a lawn of each bacterial strain on two "blank" agar plates

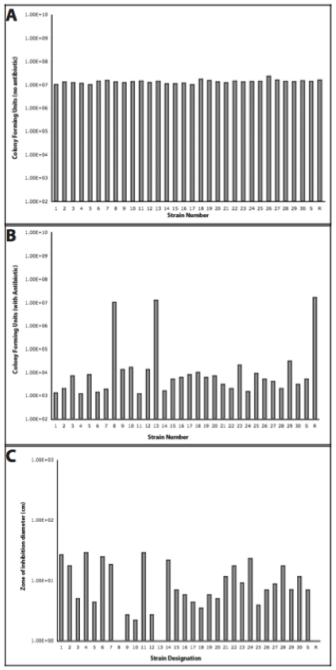

Figure 1. (A) Novel P. aeruginosa strains isolated from the environment grow similarly in media lacking antibiotics. All 30 isolated strains as well as two reference strains (R and S) were grown in LB broth lacking antibiotics for 24 hours. The cultures were then serially diluted onto plates lacking antibiotics and colony-forming units were calculated. Shown is a representative of two independent experiments. **(B)** Most novel P. aeruginosa strains are sensitive to rifampicin in liquid culture. All 30 isolated strains as well as two reference strains (R and S) were grown in LB broth containing rifampicin for 24 hours. The

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RESULTS

Science Fair/ lab report

- Data charts/ graphs
- Variables
- Summary of data

Science Article

- Explanation of questions to test the hypothesis.
- Rationale for each experiment
  - How does each experiment address an aspect of the hypothesis? What are the variables?
- Explanation of data
  - Refer to data charts/graphs and provide summary of results.
- Data charts/graphs

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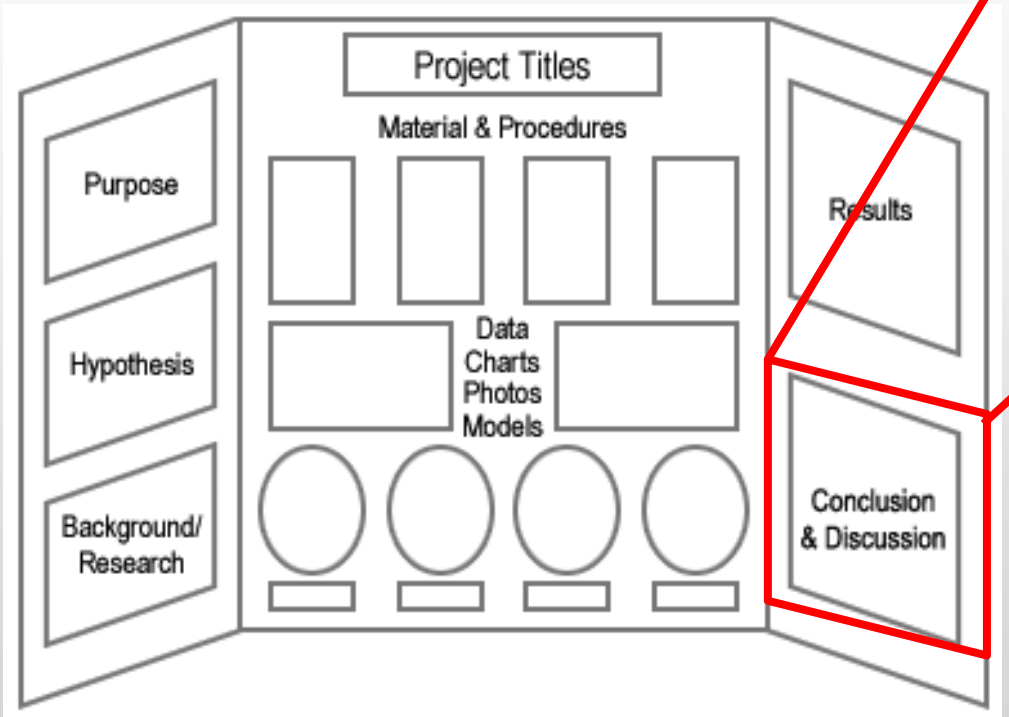

Discussion

broad range of bacterial pathogens that
ugs has recently resulted in the
emergence of bacteria that are resistant to many or all forms of

current antibiotic therapy. Presently, the majority of these antibiotic resistant strains can be found in hospital settings, where the use of antibiotics is high. In this study we wanted to determine the frequency of antibiotic resistance in environmental bacterial populations that have not been exposed to antibiotics. We chose to isolate several strains of the bacterium Pseudomonas aeruginosa from various locations around the Boston area. P. aeurginosa is an opportunistic bacterial pathogen that is notoriously antibiotic resistant. By examining environmental strains of a clinically relevant species, we hoped to gain insight into the underlying mechanisms of antibiotic resistance. From fifty unique sampling locations, we were able to isolate 30 different strains of P. aeurginosa using selective agar media. We re-isolated all 30 strains as clonal populations for this study individually for future use. While using these types of selective media is not always 100% effective for identification, we have the advantage that P. aeurginosa strains smell subtly of grapes as well as confirmation through a bacterial identification striP. We were able to use these attributes as secondary tests to confirm that the strains growing were indeed P. aeruginosa. While 30 strains of a single bacterial species is by no means an exhaustive examination of environmental bacteria, these experiments lay the groundwork for expanding these studies to other environmental species.

We were curious whether these unique strains would be sensitive to the antibiotic rifampicin. We hypothesized that since these strains had never been exposed to antibiotics in a clinical setting, they would all be sensitive to this drug. To our surprise, we were able to identify two different strains that were resistant to this antibiotic in both liquid culture and when grown on agar plates. This means that even in environmental settings there may be an underlying antibiotic resistant population of bacteria. This is an important finding for clinics, where giving a single antibiotic treatment may not yield the desired result. This suggests that giving multiple antibiotics as a cocktail may be a more effective strategy in order to eliminate these naturally occurring antibiotic resistant strains. However, these results confirmed our original prediction that the majority of environmental strains would be sensitive to antibiotics.

Even though the majority of the strains have the same phenotype (sensitivity to rifampicin), we wondered whether these strains had identical DNA sequences in the target of this drug, RNA polymerase. We hypothesized that there would be no differences between these strains at the DNA sequence level especially considering the essential nature of RNA polymerase. In order to test this we used Cel1 digests that allow for the sensitive detection of mismatches between DNA fragments without the need of DNA sequencing. We used PCR to amplify the RNA polymerase gene from all 30 strains and made heteroduplexes of each product with the RNAP gene from a laboratory strain. Interestingly, all 30 strains showed varying point mutations throughout this gene product, regardless of whether the strain was resistant or sensitive to rifampicin. However, a major limitation to the Cel1 digest approach is that this technique does not identify the exact location of the mutation within the gene. Even so, these results suggest that even in the environment, bacterial strains are developing new mutations in their DNA sequence. The majority of these mutations, however, are silent mutations that have no functional impact on gene products, in this case RNA polymerase. Furthermore, in two of these cases this low-level mutation frequency allowed for the development of antibiotic resistant strains even though they had not been exposed to rifampicin in a clinical setting. This important finding means that bacterial species are constantly mutating and evolving while our treatment drugs (antibiotics) have remained static for the last decade. This study paves the way for future more expansive studies across many different bacterial species that will help us gain more insight into the underlying mechanisms of acquiring antibiotic resistance.

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DISCUSSION

Science Fair/ lab report

- Summary of data

- Conclusions

- Future experiments

Science Article

- Restate hypothesis
- Summary of results
- Interpretation of results
  - What are your conclusions?
- How do the conclusions agree/disagree with the hypothesis?
- Significance of conclusions?
- Future directions, remaining questions.

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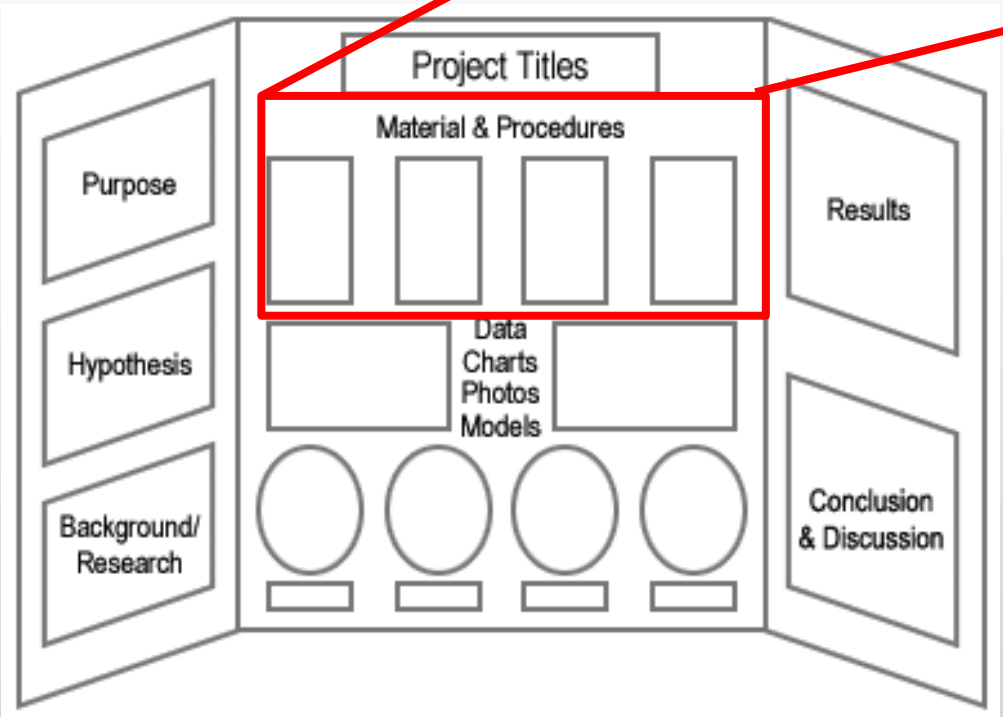

Methods

osa strains were collected from multiple locations in the
s were cultured from these swabs by inoculating Cetrimide
in single colonies. Cetrimide selectively inhibits the growth of
e production of a P. aeruginosa-specific blue-green pigment,
facilitating the identification of the desired strains. For rifampicin-sensitivity assays, environmental and laboratory P. aeruginosa strains were grown in Luria-Bertani (LB) rich medium for both liquid-culture and agar plate-based assays. When required, P. aeruginosa strains were grown in the presence of rifampicin.

Primers and PCR amplification of P. aeruginosa rpoB

We used the P. aeruginosa PAO1 genome sequence (GenBank accession number AE004091.2) to design forward and reverse primers to amplify the gene encoding the protein target for rifampicin, a subunit of RNA polymerase known as β (encoded by the gene rpoB). The sequence of the forward primer is 5' ATGGCTTACTCATACACTG 3'; that of the reverse primer is 5' TTATTCGGTTTCCAGTTCG 3'. We used a high-fidelity DNA polymerase (PhusionTM, Finnzymes) for the PCR amplification of the rpoB gene in the 30 environmental and two control P. aeruginosa strains to ensure that no mutations were introduced during the amplification process.

Cel1 Preparation

We prepared Cel1 enzyme directly from celery juice as described in REF. Essentially we juiced 4 lbs? of celery to produce 1.5 liters of celery juice. To each liter of celery juice we added 50ml of 2M Tris, pH 7.7, and 1ml of .1M PMSF (a protease inhibitor). The solution was centrifuged at 15,000 g for 20 min at 4 C. The supernatant was saved; we added 144 g of (NH4)2SO4 to each liter of supernatant. This solution was mixed gently for 1 hour at 4 C. We centrifuged the solution at 15,000 g for 45 minutes at 4 C. We added 390 g of (NH4)2SO4 to each liter of supernatant. This solution was mixed gently overnight at 4 C. The solution was then centrifuged at 15,000 g for 90 minutes at 4 C. The supernatant was discarded and the pellet, which contained the protein-precipitate, was saved at 4 C. We thoroughly resuspended the protein pellet in 150 mls of Buffer B: .1M Tris, pH 7.7, 0.5M KCl, 0.01% Triton X-100, and 100uM PMSF. We extensively dialyzed the protein solution in to 5 L of Buffer B (with 5-6 exchanges). The dialyzed protein was aliquoted and stored at -80 C.

Cel1 Digest

Heteroduplexes were formed by mixing wildtype PCR products and mutant PCR products at a 1:1 ratio; the samples were then heated at 98 C for 5 minutes to denature the strands and then slowly cooled to room

temperature to re-anneal the strands. The digests were performed with 5 ul of the heteroduplex (or homoduplex control) mixed with 5 ul of the Cel1 digestion solution. The Cel1 digestion solution was made up of a 1/10 dilution of 10x Cel1 digestion buffer (100 mM HEPES (pH7.5); 100 mM MgSO4; 0.02% (w/v) Triton X-100; and 200 ng/ml BSA) in H2O with Xng of Cel1 enzyme.

What I've been doing is making 100 uL of a Cel1 mix that contains 20uL 10x buffer, 1 ul of a 1:10 dilution of the Cel1 aliquots in the -80, and 79 ul of H2O.

For digestions, I take 5 ul of the heteroduplex (or homoduplex control) and mix it with 5 ul of the Cel1 mix, and run Cel1Dig program.

Finally, I add 10 ul of a stop solution containing 75 mM EDTA (ph 8), and 2.5 M NaCl, and run the results on a gel.

Rifampicin sensitivity assays

To assess the sensitivity of the environmentally-isolated and laboratory strains of P. aeruginosa in liquid culture, we inoculated culture tubes containing LB broth in the absence of rifampicin with a single colony of each strain and incubated overnight at 37°C to allow the cultures to become saturated. We then made a 1:100

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METHODS

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- List of materials

- List of procedures

Science Article

- No lists
- Methods broken up into individual sections (if many experiments were performed)
- Explanation of procedure in paragraph form
  - Description of unique materials

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REFERENCES

Science Fair/ lab report

- List of references at end of document

Science Article

- References placed at end of article.
  - In order in which they are cited in the article.
- Citations are expected throughout the paper.
  - Introduction should be especially highly referenced.

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TITLE/ ABSTRACT

Science Fair/ lab report

- Catchy title
- Abstract: shortened introduction

Science Article

- Descriptive title
- Abstract: brief summary of hypothesis, experiments and **results**. Less than 250 words.

Overview of the Scientific Review Process