Are Kanamycin and Erythromycin Antibiotic Resistant Bacteria and Multi-Drug Resistant Bacteria present in disinfected waste water samples from Central Valley Water Reclamation Facility?

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Abstract

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Research regarding Antibiotic Resistant Bacteria (ARB) and Multi-Drug Resistant Bacteria (MDR) has been gaining major importance worldwide. As antibiotic resistance increases in bacteria, more antibiotics are losing their effectiveness towards infectious treatment. As antibiotic resistance passes from bacterium to bacterium, it can result in widespread bacterial contamination. Researchers have previously studied the effects of ARBs/MDRs on public health regarding water contamination. Researchers have tested water samples such as waste water, hospital water, surface water, and industrial processed water. ARBs and MDRs have been found all over the world ranging from tiny villages to high-tech societies. If antibiotic resistance to infectious treatment progresses, our advanced world may find itself in a time where today’s sensational drugs never existed (Andrews et al. 2005). In the present investigation, waste water samples were collected from the Central Valley Waste Water Reclamation Facility (Salt Lake City, UT, USA). Bacterial growth plates were made with Luria Bertani Broth (LB), Agar, and were exposed to two different types of antibiotics (Kanamycin and Erythromycin). This was done in order to conclude whether ARBs and/or MDRs were present in the waste water sample. The following research question was formulated: Are Kanamycin and Erythromycin Antibiotic Resistant Bacteria and Multi-Drug Resistant Bacteria present in disinfected waste water samples from Central Valley Water Reclamation Facility? The results suggest that Erythromycin resistant bacteria were present in the waste water samples. No Kanamycin or Kanamycin/Erythromycin resistant bacteria grew on the plates. These observations conclude that there was a presence of ARBs but there was no presence of MDRs.
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Introduction

Antibiotic resistance is the ability to resist the effectiveness of an antibiotic. Antibiotic resistance arises when microorganisms are able to change in a way that allows them to blot out or reduce the effect of agents that have been created in order to cure or prevent certain infections (Cunha et al. 2017). When bacteria further survive and multiple, they can cause a danger to societal health. In relation to Antibiotic Resistant Bacteria (ARB), Multi-Drug Resistance Bacteria (MDR) are bacteria that impede the usage of multiple antibiotics (“World Health Organization” 2016).

Furthermore, many ARBs and MDRs stem from drinking water. Water is a very vital component to human life and is the most common natural deposit on Earth. “Seventy-five percent of the Earth's surface, seventy percent of the human body, and seventy-eight percent of the blood consist of water.” (et al Diner, Yigittekin) Water is essential for activities such as reproduction, circulation, nourishment, and respiration. Since water is an integral part of life, its pollution is perilous to all life forms (Rails et al. 1998). Wastewater is water that has been used in homes, industrial processes, and in businesses. After wastewater is treated, the treated water is then released into oceans and lakes. Water from lakes and oceans or surface water is used for drinking water. If treated wastewater has a presence of ARBs/MDRs and the water from oceans and lakes is not properly treated, a life threatening health issue will arise. Therefore, water contamination due to ARBs and MDRs must be properly treated.

Antibiotic and Multi-Drug Resistance can occur in four distinct ways (Amador et al. 2015). Intrinsic Resistance allows a bacterium to resist the activity of an antibiotic through the
bacterium’s structural or functional characteristics. For example, some bacteria are not surrounded by a cell wall. An antibiotic like Penicillin that averts the advancing of a cell wall, can’t harm a bacterium that doesn’t build a cell wall in the first place (Charpentier et al. 2000).

Acquired Resistance occurs when a bacterium obtains the ability to resist an antibiotic that it was previously susceptible to. This occurs in two ways: a change in genetic information or by receiving DNA from a bacterium that was previously resistant to the antibiotic (Tuomanen et al. 2000). Mutations are rare and spontaneous changes that occur in a Bacterium’s genetic material. Mutations enable bacteria to produce antibiotic-degrading enzymes and the ability to deny the entry of the antibiotics in their cells (Vidyasagar et al. 2016). Bacteria can spread their genetic information through natural selection. Natural selection allows bacteria with resistance toward certain antibiotics to better survive and adapt to their environments. Thus, when a certain antibiotic is used upon bacteria, the ones that are not resistant will die and the ones that are resistant will continue to survive and reproduce through binary fission. (Hawkey et al. 1998).

The accelerated evolution of ARBs and MDRs, is occurring globally. Thus, menacing the efficacy of antibiotics which have revolutionized medicine and saved billions of lives. Bacterial infections have once again become a major threat towards the population (Tamu et al. 2017). In the United States alone, over 2,000,000 people are infected with ARBs/MDRs each year. Antibiotic resistance has been frequently reported in treated drinking water, river water, and waste water (Yang et al. 2015). Globally, more than 3.4 million people die each year, as a result of waterborne diseases. Thus, making water contamination the leading cause of death and disease worldwide. (W.H.O. Berman et al. 2009) Due to its abundance in nutrients and antimicrobial agents, wastewater is deemed the most favorable environment for both the
survival and transfer of antibiotic resistance. Therefore, active wastewater treatment is vital for reducing water contamination.

In a literature review by Thomas Schwartz (2003), the detection of ARBs and their resistance genes in wastewater, surface water, and drinking water biofilms were tested (Schwartz et al. 2003). The Vanomycin-resistant enterococci was distinguished using various microbiology methods. The article reported the Vanomycin-resistant enterococci was detected in both wastewater and drinking water biofilms. Thus, indicating wastewater contamination of the drinking water distribution. A considerable number of evidence reinforces the detection of ARBs and MDRs in wastewater and drinking water sources. Another article supporting Schwartz studied the ampicillin resistant-Enterobacteriaceae in six water sampling sites (Amador et al. 2015). The bacterial strains were both isolated and characterized (Fernandes et al. 2015). In conclusion, a higher concentration of ARBs and MDRs were found in the wastewater sampling sites compared to the other water samples (Duarte et al. 2015).

The research question drafted in this experiment is as follows: Are Kanamycin and Erythromycin Antibiotic Resistant Bacteria and Multi-Drug Resistant Bacteria present in disinfected waste water samples from Central Valley Water Reclamation Facility? Knowing that wastewater contamination of drinking water is a growing concern, my motive behind this investigation was to find out whether a local water facility contained a strain of antibiotic or multi-drug resistant bacteria in its treated wastewater. Firstly, I will analyze the methods used to experiment with ARBs and MDRs. Secondly, I will analyze the results of the data regarding the overall growth of ARBs and MDRs. Finally, I will discuss and further investigate the growth of the ARBs and MDRs, and their effect on public health.
Equipment/Methods and Variables

1. Central Valley Water Reclamation Facility is one of the largest water facilities in Utah. The targeted water facility needs to have an extensive impact on the population, therefore the present facility was selected for this experiment. The same waste water effluent was used for each LB Agar plate. This is important because different water facilities may contain wastewaters with different types of ARBs and MDRs. In order to collect the waste water, the manager of Central Valley Water had to be notified in advance. Close toed shoes, gloves, and a mouth mask had to be worn. A water holder jug was filled up with disinfected waste water, and then the water was poured into the clear plastic bottle.

2. The two types of antibiotics tested in this experiment was Kanamycin and Erythromycin. Kanamycin and Erythromycin are in the top ten most used antibiotic classes. Kanamycin treats severe infections regarding the abdomen and urinary tract (CDC 2017). Erythromycin is used to treat bacterial infections such as bronchitis, pneumonia, whopping cough, and diphtheria. Both of these antibiotics are used to treat waterborne diseases such as UTIs and throat infections, therefore they were chosen to test upon (Health 24 2013).

3. In order to test for ARBs/MDRs, the waste water effluent was plated on LB Agar plates. Luria Bertani (LB) is a nutrient rich growth medium for bacteria. Agar is the jelly-like substance that makes the base for bacterial growth (Sanders et al. 2012). Eight grams of LB, four grams of Agar flakes, and 400 ml of DI water were mixed together in a 1000 ml
flask. The same ratio of antibiotic to LB Agar to DI water was used. This is important because if there was more antibiotic compared to LB Agar and water, the bacteria would not grow due to starvation. If there was more LB Agar and not enough antibiotic and water, the bacteria may overgrow and the effect of the antibiotic would not have been seen.

4. After mixing Agar, LB, and DI water, the flask was placed in the autoclave in order to sterilize the mixture. The mixture was autoclaved for 15 minutes at a slow pace. Once the agar was autoclaved, it was put into a bath bowl to cool down, otherwise the liquid would solidify.

5. Addition of the antibiotics was done under a hood in order to reduce exposure to toxins and bacteria. The hood and the area around the hood should always be cleaned with ethanol before and after the process to reduce contamination. All the plates and flasks were labeled in order to eliminate human error. A total of ten plates were plated with waste water effluent. Two of the plates contained LB Agar and were spiked with 250 μl of Kanamycin; these two plates tested for bacteria that were resistant only to Kanamycin. The other two plates contained LB Agar and 250 μl of Erythromycin; these two plates tested for bacteria that were resistant only to Erythromycin. The last six plates contained LB Agar along with a total of 750 μl of both Kanamycin and Erythromycin; these plates tested for bacteria that were multi-drug resistant.

6. The LB Agar mixture in each plate had to cool and solidify before the waste water effluent could be plated. To check for solidification, the plates were slightly rotated to see if any liquid movement was present.
7. Waste water effluent was plated on the LB Agar plates in two different amounts. One plate contained 50 µl of effluent and the other contained 100 µl. Two different amounts were used to increase the concentration of bacterial colonies. Each plate was placed on the rotor, and each amount of water effluent was pipetted onto the plates.

8. A spreader was used to spread the effluent throughout the plate. The spreader was first sterilized over a Bunsen burner, and then cooled for 20 seconds before being used. The spreader was slightly placed onto the effluent and the effluent was spread slowly by spinning the rotor.

9. The plates were then placed in an incubator upside down at 37°C for 24 hours, to accelerate bacterial growth. The same incubation temperature and incubation period was used for all plates. This is important because a higher or lower incubation temperature/period may cause more or less bacterial growth. 37°C is the optimum temperature for many types of bacterial strains. Bacterial colonies were then further analyzed. Methods are used from Erin R. Sanders (2012), and as per suggested by Dr. Goel.

The hypothesis formulated in this experiment is the following: There should not be any ARB or MDR growth due to the wastewater having undergone disinfection. Since disinfection is a process in which the number of microorganisms are reduced, the water should be fairly immaculate. Disinfection is the tertiary step of waste water treatment, therefore all water is treated with UV radiation and chlorination. UV radiation and chlorination both have a long term history of effectiveness in killing bacteria. Thus, ARBs and MDRs should have no presence.
The independent and dependent variables are as follows:

a. The independent variable is the addition of either Erythromycin, Kanamycin, or both Kana and Erythromycin to the LB Agar plates.

b. The dependent variable is the growth of ARBs and/or MDRs on each of the LB Agar/Antibiotic plates.

**Results**

The results of plating the waste water effluent revealed the presence of ARBs with respect to Erythromycin. Three bacterial colonies had grown on the LB Agar/Erythromycin 100 μl plate, and two bacterial colonies had grown on the LB Agar/Erythromycin 50 μl plate. On the 100 μl Erythromycin plate, the smallest and medium sized colony had grown on the outer edge of the plate. The largest colony had grown two inches away from the edge of the plate. The largest and medium sized colony had grown an inch away from one another, whereas the smallest colony was on the opposite side of the plate. Some water evaporation had occurred during incubation.

As can be seen in the bar graph below, the 50 μl plate of Erythromycin had less colonies when compared to the 100 μl Erythromycin plate. This is because there was a lower amount of waste water effluent present in the 50 μl plate.

As seen in the table below, no bacterial colonies were present on the 100 μl or the 50 μl Kanamycin plate, and no water evaporation occurred during incubation. A few air bubbles were present on the 100 μl Kanamycin plate, but there was no bacterial colony growth. No bacterial
colonies were present on the 3 100 µl plates and on the 3 50 µl Kana/Erythro plates. Also no water evaporation had occurred during incubation. As seen in the bar graph below, the colony concentration is accurate because no colonies grew on any of the Kanamycin and Kana/Erythro plates, with respect to waste water effluent amount.

**Number of Bacterial Colonies Present in Plates after Incubation**

<table>
<thead>
<tr>
<th>Amount of Waste Water Effluent Present (µl)</th>
<th>Type of Antibiotic Present</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kanamycin</td>
</tr>
<tr>
<td>100 µl</td>
<td>0</td>
</tr>
<tr>
<td>50 µl</td>
<td>0</td>
</tr>
</tbody>
</table>

![Number of Bacterial Colonies Present in Plates](chart.png)
Analysis and Conclusion

The presence of ARBs is indeed characterized in the growth of bacterial colonies on the Erythromycin plate. Both of the 50 $\mu$l and 100 $\mu$l plates had bacterial growth, which confirms that Erythromycin resistant bacteria are present in the treated waste water samples from the Central Valley Water Reclamation Facility. Since there was no bacterial growth on the 100 $\mu$l and 50 $\mu$l Kanamycin plates, no Kanamycin resistant bacteria were present in the waste water samples. There was no presence of MDRs regarding resistance towards both Kanamycin and Erythromycin, because no bacterial colonies grew on the six Kana/Erythro plates. Although the waste water effluent had undergone disinfection through UV radiation and chlorination, bacterial colonies still arose. This clearly contradicts the first half of the formulated hypothesis, that there would be no presence of ARBs in the waste water effluent due to disinfection. The growth of Erythromycin resistant bacteria proves this. The results do support the second half of the formulated hypothesis, that no MDRs will be present in the waste water effluent samples. This is because there was no bacterial growth on the Kana/Erythro plates.

The first limitation to the experiment was that bacterial characterization was not done due to its long processing and short time frame availability. An improvement to this would be to analyze bacterial characterization as phase II of this experiment. The second limitation was the testing of only two types of antibiotics. Therefore, there could have been a larger variety of ARBs and MDRs present in the effluent that were not analyzed. An improvement to this would be to use five to ten different types of antibiotics, so that multiple ARBs and MDRs can be
analyzed. The last limitation to this experiment was the number of times the waste water was collected. The waste water was only at one time of day, so it did not give an accurate representation of bacteria. To improve upon this limitation, multiple samples of waste water should be collected at different times.

The experiment was successful in confirming previous findings in published research paper conclusions regarding ARBs being found in treated waste water samples (Dincer et al. 2017). Although the Central Valley waste water samples had been treated using UV radiation and chlorination, bacterial growth still transpired. This ensures that even disinfected waste water contamination can be dangerous to health.

If treated waste water from Central Valley enters surface water, and the surface water is not cautiously treated, drinking water will have a presence of ARBs. Hence, making bacterial infections due to drinking contaminated water more challenging to treat. If accurate prevention measures are not taken towards water contamination, various illnesses such as dysentery, UTI, diarrhea, Cholera, and Typhoid Fever will increase (CDC 2014). A recent high profile report estimates that, by 2050, 10 million people will die each year due to ARBs, if accurate prevention methods are not taken (Kraker, Stewardson, Harbath et al. 2016)

Prevention measures can be taken towards both reducing water contamination and antibiotic resistance. Although drinking water is treated through four essential steps, using more extensive disinfectants such as ozone and boiling can better treat contaminated water (Groundwater Foundation 2017). The most impactful way to reduce antibiotic resistance at its root, is to not overuse and misuse antibiotics. The best way to decrease this is to only take antibiotics when the doctor prescribes them, and to not take antibiotics for quick relief symptoms (CDC 2014).
Conclusively, a number of bacteria have been classified into groups such as urgent, serious, and concerning threats by the CDC. Many of these have placed a financial and clinical burden on the U.S. health care system and patients (Anon et al. 2017). Therefore, organized efforts to further implement new policies, renew research efforts, and seek steps to administer the crisis is necessary (Ventola et al. 2015). These organized efforts will benefit public health as a whole.

To further investigate this experiment, bacterial colony isolation and characterization for the Erythromycin-resistant bacteria can be done. Bacterial isolation will allow to grow a pure culture of Erythromycin-resistant bacteria in order to further characterize the bacteria. Characterizing the bacteria will name and classify the bacteria into groups. This will give a more detailed result, and allow to take more specific safety precautions depending upon which type of bacteria has been classified.
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Appendix

Hood Area: bacterial plating is done here
50/100 μl LB Agar/Kanamycin/Erythromycin plates
Outside of Incubator
Inside of Incubator
Largest Bacterial colony

Specks on lid are water evaporation

100 µl Erythromycin bacterial growth plate
50 µl and 100 µl Erythromycin plates with bacterial growth
Erythromycin Powder
Kanamycin Powder
LB Powder
Agar Powder
More Specific Methods, Procedures, and Materials

Materials and Apparatus for Waste Water Sampling
1. Gloves
2. Closed toe shoes
3. Face masks
4. 1000 ml clear plastic bottle

Materials and Apparatus for LB Agar
1. 8 grams of Luria Bertani Broth Powder
2. 4 grams of Agar Flakes
3. 400 ml DI water
4. 1000 ml flask
5. Graduated Cylinder
6. Autoclave
7. Autoclave Tape
8. Scale
9. Plastic holding cup
10. Spatula

Materials and Apparatus for Antibiotic/LB Agar Plates
1. 10 agar plates
2. LB Agar
3. Bath Bowl
4. Hood
5. 20-200 µl Pipette and pipette tips
6. 250 microliters of Kanamycin (antibiotic)
7. 250 microliter of Erythromycin (antibiotic)
8. ethanol
9. 3 flasks
10. graduated cylinder
11. Bunsen burner
12. Kimwipes
13. Central Valley Waste Water Samples
14. Steel Spreader
15. Rotor
16. Incubator
Experimental Method for Waste Water Effluent Collection
1. Talk to manager of Central Valley waste water facility and ask about waste water sample collection regarding antibiotic resistant bacteria experimentation.
2. Follow waste water collection protocol.
   a. Wear gloves and close toe shoes.
   b. Wear mouth mask.
3. Go to the end of disinfection process and fill water holder jug with waste water.
4. Pour waste water in water holder jug into 1000 ml clear plastic bottle.
5. Repeat steps 3 and 4 till 1000 ml clear plastic bottle is full.
6. Discard all protective gear into biohazard trash bin.

Experimental Method for making LB Agar
1. Calculate amount of LB and Agar needed to make 10 agar plates. (calculations shown in table below)
2. Place plastic holding cup on scale and press TARE to zero out measurements.
3. Using a spatula measure out 8 grams of LB and 4 grams of agar into plastic holding cup.
4. Fill graduated cylinder with 400 ml DI water (purified/filtered water).
5. Pour DI water, LB powder, and agar flakes into 1000 ml flask.
6. Loosely close the flask’s lid, and put a piece of autoclave tape on the front of the flask.
7. Autoclave flask for 15 minutes at a slow pace.

Experimental Method for plating Antibiotic/LB Agar/Waste Water Effluent
1. Place autoclaved LB Agar flask in bath bowl to cool down to 55 degrees Celsius.
2. Turn on hood, light, and Bunsen burner.
3. Clean hood area and chair with ethanol and kimwipes to sterilize area.
4. Calculate amount of Erythromycin, Kanamycin, and LB Agar needed to put into each agar plate.
5. Label all 10 plates (2 are labeled Erythromycin, 2 are labeled Kanamycin, and 6 are labeled Kana and Erythro) and the 3 flasks (1 is labeled kana, 1 is labeled Erythro, and the last is labeled Kana/Erythro).
6. Using graduated cylinder to measure, pour 50 ml LB Agar into Kanamycin and Erythromycin flasks and 150 ml LB Agar into Kana/Erythro flask.
8. Rotate all three flasks in a circular motion to mix the LB Agar with the Antibiotics.
9. Take the lids off of all plates and place the lids aside.
13. Allow liquid in each plate to cool and solidify for 10-20 minutes.
14. Check whether the plates have solidified. To check for solidification slightly rotate the plate to see if any liquid movement is present. If there is still a presence of liquid, leave the plates to cool. If there is no liquid present, put the lids back on the plates and place them upside down for the time being.
15. Flip plates back over and remove the lids.
16. Place the first plate onto the rotor.
17. Pipette 50 $\mu l$ of waste water effluent in the center of one of the LB Agar/Kanamycin plates.
18. Discard pipette tip.
19. Dip spreader into ethanol and place over Bunsen burner to sterilize.
20. Wait for the spreader to cool down for 20 seconds before spreading the waste water effluent on the plate. To check if spreader has cooled, gently touch edge of spreader to the agar plate and see if agar melts. If gel melts allow spreader to cool more, if gel does not melt continue with the next steps.
21. Slightly place the spreader on the effluent and slowly spread the effluent by spinning the rotor.
22. Make sure all of the waste water effluent has spread over the gel.
23. Place spreader into ethanol.
24. Close the lid of the plate and place it upside down.
25. Repeat steps 16-24 for the one 100 $\mu l$ LB Agar/Kanamycin plate, one 50 and one 100 $\mu l$ LB Agar/Erythromycin plate, and the three 50 and three 100 $\mu l$ LB Agar/Kanamycin/Erythromycin plates.
26. Place all 10 plates in the incubator upside down for 24 hours at 37°C.
27. Analyze ARB growth.
More Specific Calculations for LB Agar, Kanamycin, and Erythromycin

**LB Agar Calculations:**

Standard Amount of LB used to make plates: 20 grams/liter

Standard Amount of Agar used to make plates: 1% of total volume

Only 400 ml of LB Agar is needed therefore using proportions calculate how many grams of LB and Agar is needed.

\[
\frac{20 \, g}{1 \, l} \times \frac{1 \, l}{1000 \, ml} \times 400 \, ml = 8 \, grams \, of \, LB
\]

\[
400 \, ml \times \frac{1 \, g}{100 \, ml} = 4 \, grams \, of \, Agar
\]

**Kanamycin and Erythromycin Calculations:**

What volume of Kanamycin/Erythromycin must be used to spike LB/Agar?

Stock Concentration: 10 mg/ml

Standard antibiotic concentration: 50 \( \mu g/ml \)

Since each plate holds 25 ml of solution and each antibiotic will have 2 plates, the final volume will be 50 ml. (2*25)

Using \( C_1V_1 = C_2V_2 \) calculate initial volume for Kanamycin and Erythromycin.

\[
\frac{10 \, mg}{1 \, ml} \times x = \frac{0.05 \, mg}{1 \, ml} \times 50 \, ml
\]

\[
\Rightarrow x = 250 \, \mu l \, of \, Kanamycin
\]

\[
10 \, mg \times x = \frac{0.05 \, mg}{1 \, ml} \times 50 \, ml
\]

\[
\Rightarrow x = 250 \, \mu l \, of \, Erythromycin
\]

**Calculations for Kanamycin/Erythromycin Mix:**

What volume of Kanamycin/Erythromycin must be mixed together?

Stock Concentration: 10 mg/ml

Standard antibiotic concentration: 50 \( \mu g/ml \)

Since each plate holds 25 ml of solution and 6 plates will have a mix of kana and Erythro, the final volume will 150 ml. (6*25)

Using \( C_1V_1 = C_2V_2 \) calculate initial volume for Kanamycin and Erythromycin.

\[
\frac{10 \, mg}{1 \, ml} \times x = \frac{0.05 \, mg}{1 \, ml} \times 150 \, ml
\]

\[
\Rightarrow x = 750 \, \mu l \, of \, Kana/Erythro
\]

Kanamycin Volume: 375 \( \mu l \)

Erythromycin: 375 \( \mu l \)
October 25, 2017

To Whom it May Concern.

I write to confirm that Aarushi Rohaj has completed over 100 hours of research work in my Environmental Engineering and Microbiology Lab. Aarushi’s project goal was to collect waste water samples from Central Valley Water Facility in Utah and analyze whether Kanamycin and/or Erythromycin ARBs and/or MDRs were present in the waste water samples. Aarushi worked alongside graduate student, Ahmed Karimi, to make LB Agar and plate the ARBs and MDRs. Aarushi further analyzed the growth of bacteria on the plates.

Aarushi completed her work sincerely and the work has great impact on Public Health.

Sincerely

Dr. Ramesh Goel,
Professor and Graduate Director
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