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Transfer and Suppression of Antibiotic Resistance Genes in the Water Cycle

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I. Summary

Antibiotic-resistant bacteria have become prominent in ecosystems due to the improper use of antibiotics. Various water sources and soils are being contaminated with antibiotic-resistant bacteria due to horizontal gene transfer. When water encounters pollutants through the water cycle, various types of water contamination can occur, and such contamination can spread through water circulation. If the antibiotic resistance genes (ARGs) move as such during the water cycle, there is a risk of transmitting antibiotic resistance through evaporation to bacteria that exist in distant water and soil. Therefore, the objectives of this study were to identify the effects of the water cycle on the movement of ARGs and to develop potential treatments to suppress the gene transfer. The results showed that the ARGs were indeed transferred to nearby fresh water and soil through the evaporation of water. In this process, the ARGs granting antibiotic resistance moved to nearby fresh water and soil, creating antibiotic-resistant *E. coli*. Consequently, the leaves of the plants that grew from the soil with the *E. coli* containing ARGs were found to be contaminated as well. In addition, this study found that the humic substances in soils near the urban residential area successfully suppressed the ARG transfer caused from evaporation. The significance of this study comes from its confirmation that ARGs can move through the water cycle and negatively affect the ecosystem by spreading antibiotic-resistant bacteria to water sources. In future research, we hope to study more about different treatment methods to alleviate the problem.

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III. List of Abbreviations and Acronyms

ARGs: antibiotic resistance genes

IV. Acknowledgements

We would like to express our special thanks to our science teacher, Dr. Coleman, who encouraged us to start this research project and supported us to complete this wonderful project. We would also like to thank all the professors and officials of the Korea Junior Water Prize for their insightful and helpful judging comments along with their support throughout the competition.

1. Introduction

Over the century, antibiotic resistance has emerged to become a critical issue worldwide in the public health area. Recently, antibiotic-resistant bacteria have been found to be prevalent in various water sources, including Lake Geneva [1, 2], the Yangtze River Delta, China [3] and Paldang Lake [4], a water source protection area in South Korea. The spread of antibiotic-resistant bacteria in the natural environment is a serious threat to human health as they cause existing antibiotics to lose effectiveness.

The discovery and use of antibiotics not only made it possible to treat various diseases caused by bacteria, but also led to an increase in the life expectancy of humankind and healthier lifestyles. However, bacteria resistant to various antibiotics have emerged due to their inappropriate use. As a representative example, methicillin-resistant staphylococcus aureus (MRSA) is resistant to various antibiotics, including methicillin. Currently, 700,000 people worldwide die each year due to antibiotic resistance, and the number is estimated to increase to 10 million by 2050 due to the emergence of super-bacteria [5].

One of the main reasons for the emergence of antibiotic-resistant bacteria in the natural environment is that antibiotic resistance genes (ARGs) are transferred through horizontal gene transfer: conjugation, transduction, and transformation. Progressive stress, such as exposure to antibiotics, leads to the selection of traits that are resistant to antibiotics. These genes are more easily transmitted because many ARGs are in plasmids (self-replicating DNA molecules) [6]. Previous studies confirmed that plasmids are one of the leading causes of the rapid spread of ARGs; in fact, the studies found that plasmids carried and transferred approximately 10 to 15 antibiotic resistance-inducing genes. During the transfer process, plasmids are replicated and are retained in both the donor and recipient cells. It was also found that the recipient cells could simultaneously acquire resistance to several types of antibiotics since all ARGs are transmitted together [7].

ARGs are known to spread into nearby soils or water such as sewage or hospital wastewater due to unnatural causes [8]. In addition, a previous study suggests that ARGs enter the atmosphere by water evaporation or through snow and wind and spread to long distances, ultimately causing air contamination [9, 10]. Water on the ground evaporates in the form of water vapor via solar energy and transpiration in plants, thereby forming clouds and returning to the ground as rain via the "water cycle." As water goes through the cycle and encounters contaminants, various types of water contamination occur. This means that the contaminants–including ARGs–can spread through such circulation.

There are existing studies that investigate treatment methods to block the transfer of ARGs. Although previous studies report that there are pharmaceutical substances that can decompose ARGs, there is a lack of eco-friendly methods to suppress ARGs.

The use of humic substances in soils can be considered as one treatment strategy. Soil has biological factors like microbes and inorganic factors, like humic substances, that degrade and adsorb contaminants [11]. Humic substances are generated from degrading organisms that exist in soil and are organic matter that exists in the soil environment; however, humic substances also have inorganic conditions in the biotic environment. It is presumed that the molecular weight, directivity, and dispersibility of humic substances remove organic pollutants by combining with and adsorbing onto them or turning them into harmless substances by degrading them into other substances [11].

In our previous study, we investigated an eco-friendly approach: whether a soil component can decompose residual antibiotics in the soil using four different soils (mountain soils, organic soils, urban residential soils containing heavy metals, and horticultural soils) [12]. We found that the activity of antibiotics was inhibited by the humic substances found in urban residential soil. The analysis of the chemical composition of the soil types found that the urban residential soil had the smallest amount of organic matter (OM), including humic substances (Table 1). Moreover, the urban residential soil had at least a 6 times higher content of Ca among exchangeable cations compared to the other soils and 3 times higher content of salinity (EC) as well. This result shows that the urban residential soil has high calcium content that is known to degrade and promote organic matter. Therefore, even though urban residential soil contains relatively low organic matter compared to other soils, it can be inferred that humic substances, which are organic substances that adsorb or decompose ARGs, are present in urban residential soils due to the high content of calcium that promotes organic matter decomposition.

<Table 1> Analysis of the soil chemical composition

Soil type	pH	OM (g/kg)	AV P_2O_5 (mg/kg)	Exchangeable Cation (cmol+/kg)			EC (ds/m)
				K	Ca	Mg	
1	5.3	92.3/1	0.02/1	1.10	6.30	3.40	3.0
2	6.1	254.2/1	1.38/1	0.91	1.21	2.93	0.3
3	6.3	121.3/1	1.23/1	0.89	1.02	3.10	0.4
4	6.8	103.9/1	1.41/1	1.12	1.11	3.21	0.3

1: Urban residential soil 2: Organic soil 3: Garden soil 4: Mountain soil
pH (acidity), OM (organic matter), AV P_2O_5 (available phosphate), Exchangeable Cation [K(potassium), Ca (calcium), Mg(magnesium)], EC (salinity)

Therefore, this study aims to investigate whether the occurrence and spread of antibiotic-resistant bacteria is caused by the water cycle. Closely observing the gene transfer of antibiotic-resistant bacteria will lead to a deeper understanding of the gene transfer process, which will eventually contribute to the development of a possible treatment to suppress the spread of ARGs.

2. Objectives of the Study

There are two objectives of the study: (1) to identify how ARGs move around the water cycle (Study 1) and (2) to research eco-friendly methods to decompose ARGs (Study 2). Study 1 focuses on the effect of the water cycle on the movement of ARGs and how the ARGs get transferred to nearby fresh water and soil through water evaporation. It also focuses on how the transferred ARGs pollute the soil and the plants nearby. Study 2 attempts to determine whether humic substances in the soil can decompose the transferred ARGs.

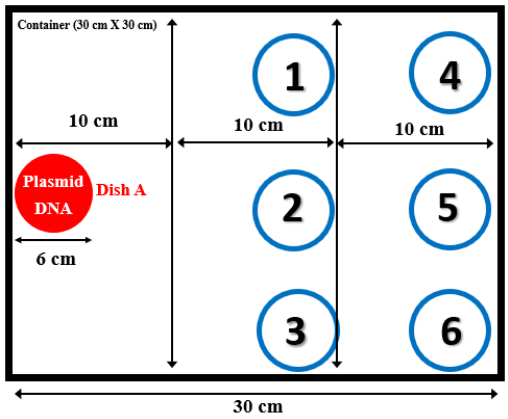
3. Research Methods and Procedures

3.1. Study 1: Transfer of ARGs

3.1.1. ARGs in the Water Cycle

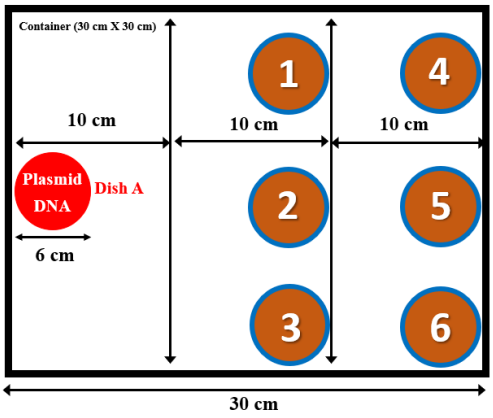
To determine whether ARGs can be transferred to nearby fresh water through the water cycle, the researchers prepared seven 6 cm X 6 cm plastic Petri dishes. First, one Petri dish was placed on the left-hand side and six additional Petri dishes were placed 10~20 cm away. 5 ml of fresh water was added into Dish A, the Petri dish on the left, and 100 $\mu\ell$ of a solution containing ARGs was inoculated.

Then, 5 ml of fresh water was also added to the other six Petri dishes (Dishes 1~6) separated from Dish A. All dishes were placed in a 30 cm X 30 cm airtight container and stored at room temperature of 23 degrees Celsius. Every 24 hours, a solution containing the ARGs was inoculated into Dish A. At the same time, the experiment was also conducted with control groups using samples not inoculated with ARGs (Fig. 1).



<Fig. 1> A device designed to determine if the ARGs do transfer to nearby fresh water through the water cycle

Next, to determine whether the ARGs were transferred to nearby soil through the water cycle, 4 g of garden soil and 5 ml of triple distilled water were added into each of the six Petri dishes. Then, the same procedure as the previous experiment determining gene movement to nearby fresh water was followed in this experiment to determine gene movement to nearby soil (Fig. 2).



<Fig. 2> A device designed to determine if the ARGs do transfer to the nearby soils through the water cycle

After seven days, the contents in Dishes 1~6 were extracted, and the presence of ARGs in each Petri dish was determined using electrophoresis (Fig.3). Their presence in water

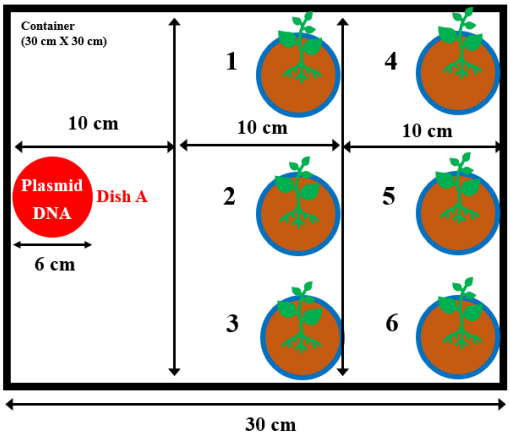
droplets obtained via evaporation cannot be determined using standard electrophoresis; therefore, polymerase chain reaction (PCR) was used (Fig. 4). In addition, after ARGs were electrically neutralized, DNA transfer through freshwater evaporation was investigated.

<Fig. 3> Electrophoresis **<Fig. 4> PCR (Polymerase Chain Reaction)**

3.1.2. Soil and Plant Contamination of ARGs

This experiment was conducted to confirm whether the ARGs transferred through the evaporation of fresh water caused the existing *E. coli* in the soil to become antibiotic-resistant *E. coli*. 5 ml of water collected from various freshwater sources was added into a 6 cm X 6 cm plastic Petri dish, and 100 ㎕ of a solution containing the ARGs was inoculated. The solution containing the ARGs was added to the plastic Petri dish prepared at intervals of 24 hours. This procedure was repeated for one week. After seven days, 1 g of soil was collected from a plastic Petri dish. The collected soil was placed in a 15 ml conical tube containing 5 ml of sterile triple distilled water, and the tube was sufficiently shaken to mix its content. 100 ㎕ of the ampicillin solution (50㎍/100㎕) was added into a culture medium. The culture medium was rubbed using a sterile spreader and cultured to confirm whether ampicillin-resistant *E. coli* could be detected.

Another experiment was conducted to find out whether the plants grown in the soil with transferred ARGs were contaminated with antibiotic-resistant bacteria. 16 cabbage seeds were planted using tweezers in a plastic Petri dish containing the soil with ampicillin-resistant *E. coli*. The seeds were then grown at room temperature for 12 hours in the light and 12 hours in the dark for a week. After a week, the leaves of the grown cabbage were collected, placed in a 1.5 ml microtube containing 1 ml of liquid culture medium, and stored for 30 minutes. Then, 100 ㎕ of the ampicillin solution (50 ㎍/100 ㎕) was added to a culture medium in which only *E. coli* could be cultured. The culture medium was rubbed using a sterile spreader and then cultured to confirm whether ampicillin-resistant *E. coli* could be detected (Fig. 5).



<Fig. 5> A device designed to confirm soil and plant contamination of the ARGs transferred through the water cycle

3.2. Study 2: Suppression of ARGs

Our previous study [12] reported that humic substances found in urban residential soils decomposed ARGs. Based on this finding, Study 2 investigates whether these humic substances can suppress the transfer of ARGs caused by water evaporation.

In order to investigate this property, samples of urban residential soil with the ability to degrade ARGs were extracted. 1 g of urban residential soil samples was mixed with 10 ml of triple distilled water. The suspension was then passed through 0.2 ㎛ pore-size filters. Through this process, foreign substances including bacteria were removed from the soil extract, and the filtrate was stored in the refrigerator until its use.

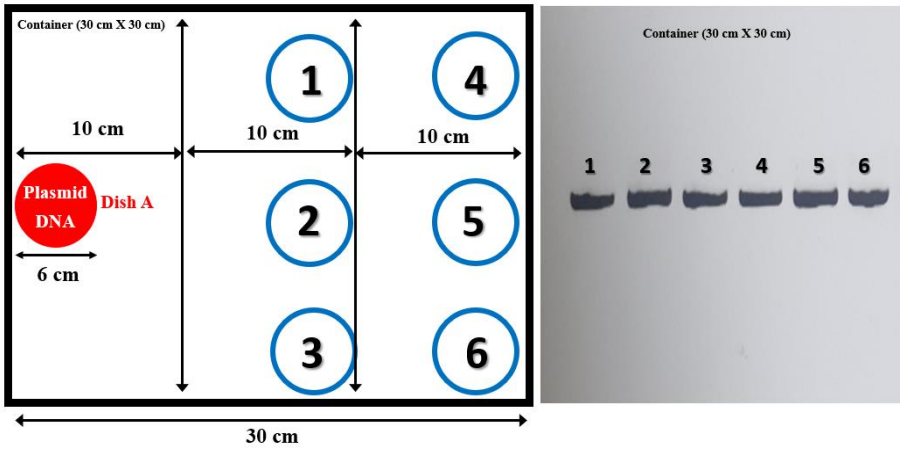
Then, the ARGs were inoculated into the urban residential soil extract to study if the ARGs in the soil were able to move to other sources through water evaporation. The same experiment was performed using fresh water inoculated with ARGs without the urban residential soil extract as a control group.

4. Results and Discussion

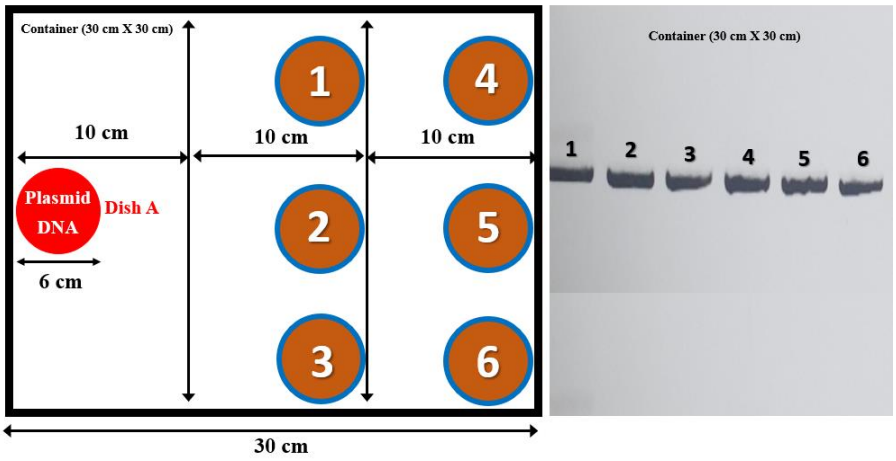
4.1. Do ARGs Transfer through the Water Cycle?

The results showed that the ARGs were detected in the water and soil of the two sets of six Petri dishes at least 10~20 cm away from Petri dish A, where the solution containing the ARGs were inoculated. In contrast, in the control experiment where the ARGs were not

inoculated, the ARGs were not detected in the evaporated fresh water and in all Petri dishes. The results suggested that the ARGs were transferred to nearby fresh water (Fig. 6) and soil (Fig. 7) through evaporated water vapor.

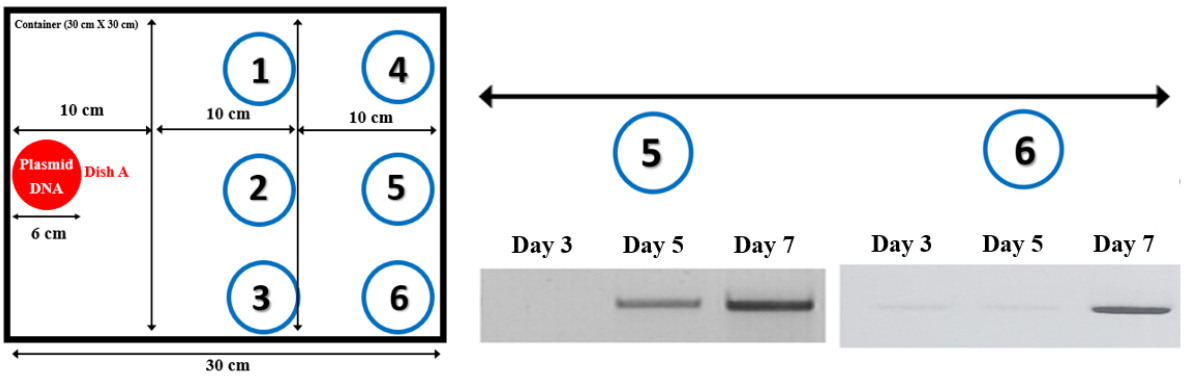


<Fig. 6> The electrophoresis result that confirmed ARGs movement to nearby water



<Fig. 7> The electrophoresis result that confirmed ARGs movement to nearby soil

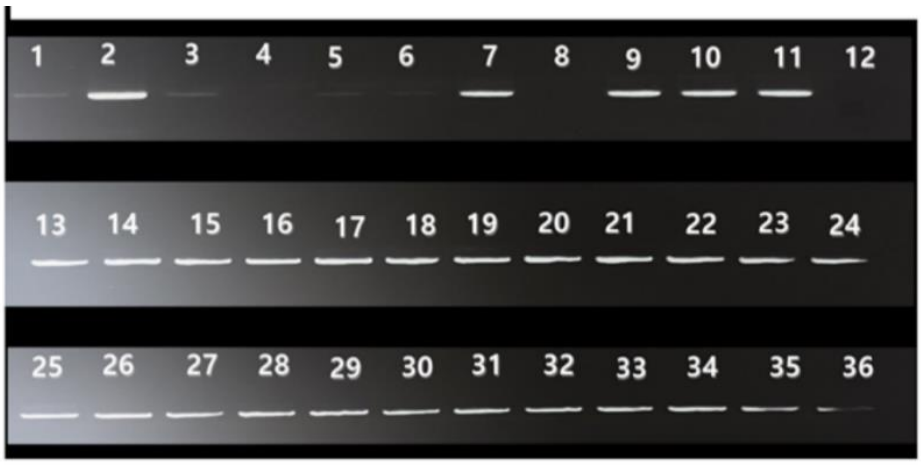
In this study, the dates when the ARGs were identified were examined. As a result, the ARGs were found in the water of the Petri dishes 10~20 cm away from Petri dish A containing the solution with the gene after approximately three days on average (Fig. 8).



< Fig. 8> The electrophoresis result that showed the transfer of ARGs per day

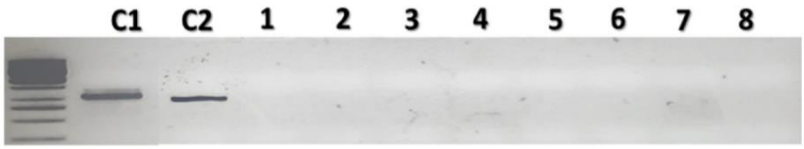
However, when checking the presence of the ARGs in the water vapor condensed on the inner surface of the airtight container through an electrophoresis test, the ARGs were not detected. This may be due to the especially low concentration of the ARGs in the condensed water vapor. Therefore, the presence of ARGs was checked using PCR, which allows gene amplification.

36 water droplets condensed through water evaporation during the experiment were collected, and the presence of ARGs was determined using PCR. The results showed that the ARGs were detected in 33 out of the 36 water droplets (not detected in water droplets 4, 8, and 12). This reveals that because the amount of ARGs in the droplets was too low, ARGs were not detected using standard methods. However, they were detected via gene amplification using PCR. The results can be seen as conclusive evidence that the ARGs were transferred via water evaporation (Fig. 9).



<Fig. 9> PCR results confirming the presence of ARGs in the vapor of the evaporated fresh water

Moreover, to clearly determine whether the ARGs were transferred through water evaporation, an experiment was conducted to remove their charge, or electrostatic attraction. The results showed that, after this change, the ARGs were not detected in the evaporated fresh water. This significant result supports the theory that the electrostatic attraction of water and the electrostatic attraction of ARGs cause the two to bind to each other and that the gene was transferred through the evaporation process due to such electrostatic attractions (Fig. 10).



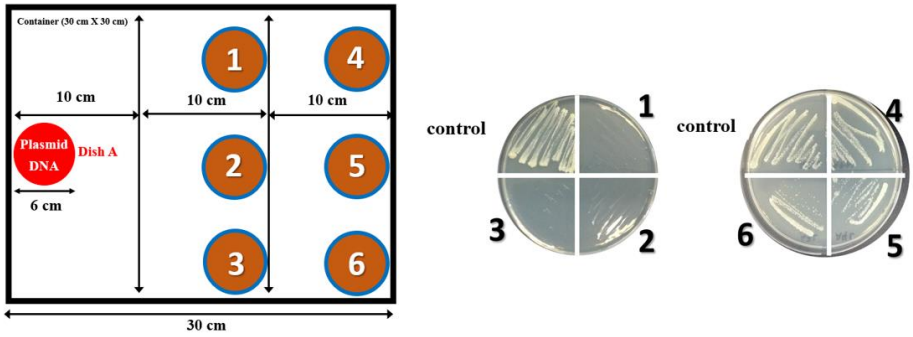
C1 & C2: PCR result of water droplets evaporated from fresh water treated with the ARGs

1-8: PCR result of water droplets evaporated from fresh water treated with the ARGs whose electrostatic charges were neutralized.

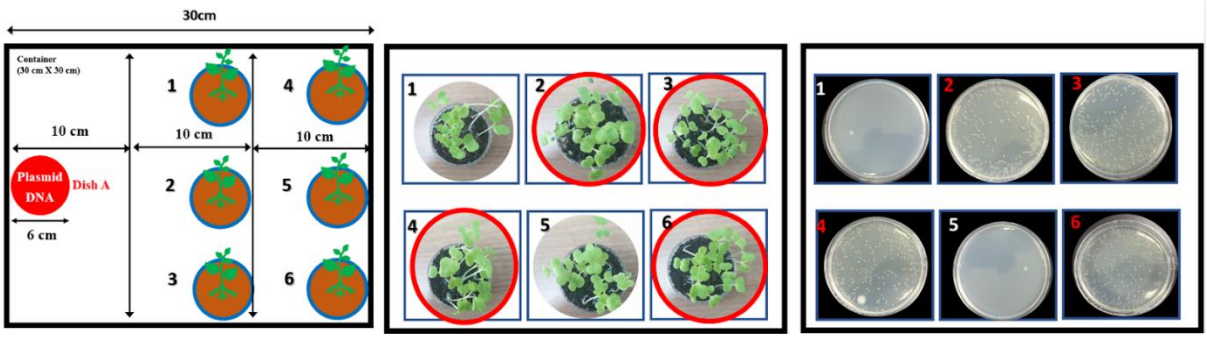
<Fig. 10> Study results confirming that the vapor of evaporated fresh water was transferred due to the electrostatic attraction between water and the ARGs

4.2. Are Soil and Plants Contaminated by ARGs?

Previous experiments demonstrated that ARGs were transferred to distant fresh water and soils during water evaporation and condensation. Here, the result demonstrated that genes transferred in this way can transform typical *E. coli* in the soil into antibiotic-resistant *E. coli* (Fig. 11). Plant leaves grown in the soil with ARGs that were transferred from freshwater evaporation were contaminated with antibiotic-resistant *E. coli* (Fig. 12. marked with red circles).



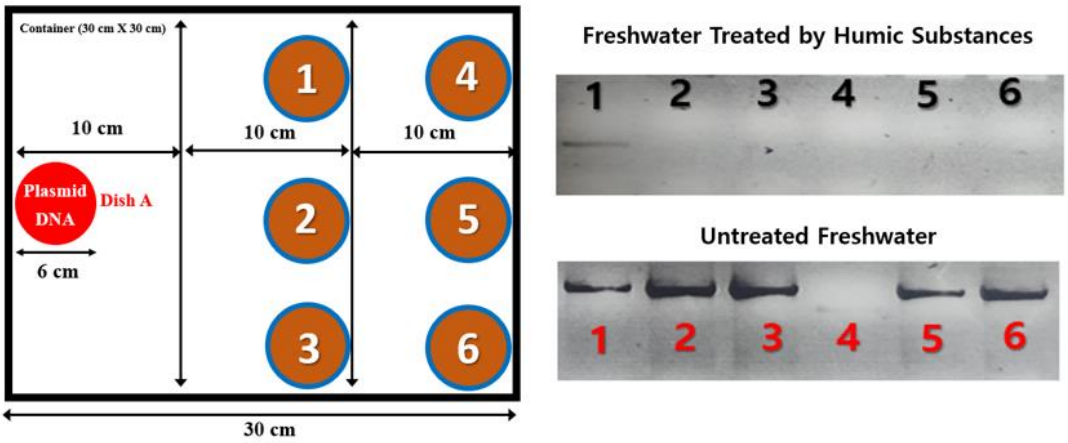
<Fig. 11> Generation of antibiotic-resistant *E. coli* due to the ARGs transferred to the nearby soils through freshwater evaporation



<Fig. 12> Contamination of antibiotic-resistant bacteria in plants grown in soil contaminated with antibiotic-resistant *E. coli* due to the gene transfer through evaporation of fresh water

4.3. Can Humic Substances Suppress the Transfer of ARGs?

Finally, we compared whether humic substances obtained from urban residential soils can suppress the transfer of ARGs in fresh water treated with humic substances. The results showed that the gene transfer to the soils on other Petri dishes through evaporation was suppressed in the case of fresh water containing humic substances. Therefore, the humic substances in the urban residential soils may degrade the ARGs or suppress evaporation of the gene with water by binding with them during the evaporation process (Fig. 13).

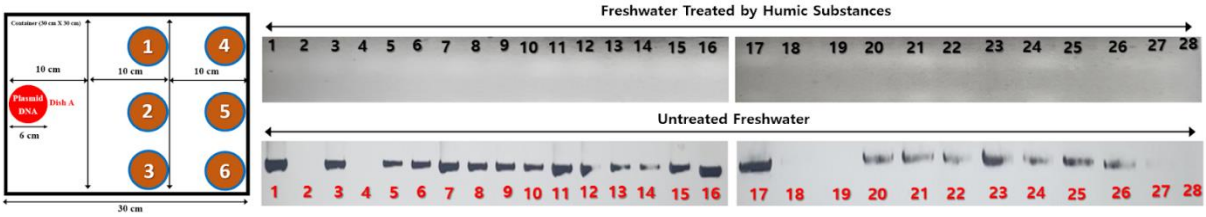


up: Fresh water contained humic substances obtained from urban residential soils and the ARGs

down: Fresh water contained only the ARGs

<Fig. 13> Confirmation of the transfer of ARGs through the evaporation of fresh water containing humic substances in the urban residential soils.

In addition, when the presence of ARGs was confirmed after collecting the water condensed in the experimental device during the evaporation process using PCR, the gene was not detected in the freshwater condition treated with humic substances obtained from urban residential soils (Fig. 14). Hence, this further confirmed that humic substances were able to suppress the movement of ARGs through the water cycle.



up: Detection of the ARGs in water droplets evaporated from fresh water containing humic substances obtained from urban residential soils and ARGs using PCR

down: Detection of the ARGs in water droplets evaporated from fresh water containing only the ARGs using PCR

<Fig. 14> Confirmation of the presence of the ARGs in the water vapor from fresh water containing humic substances obtained from urban residential soils.

5. Conclusion and Expected Impact

5.1. Conclusion

This study investigated whether ARGs were transferred through the water cycle and contaminated soils and plants. The results showed that the ARGs were introduced into the air through water evaporation and transferred to distant fresh water and soils. Specifically, this study proved that the gene is transferred during the water cycle, as PCR studies found that the water vapor contained the ARGs. These results are in line with the results of previous studies showing that ARGs enter the atmosphere through evaporation and cause environmental contamination to distant places [10, 13].

Furthermore, we also removed the charges (i.e. electrostatic attraction) of ARGs found in fresh water to study how ARGs were being transferred. Our results demonstrated that the ARGs were not contained in the evaporated fresh water. Instead, we found that the water molecules and the ARGs bonded together due to electrostatic attraction, which explained how ARGs were transferred during the evaporation process.

During this gene transfer process, *E. coli* resistant to antibiotics was generated. In

addition, it was confirmed that the leaves of the plants grown in soil contaminated with antibiotic-resistant *E. coli* were also contaminated with *E. coli* that contained the ARGs. This may be due to surface appendages called competence pili in bacteria that promote DNA uptake during the first step of natural transformation [14]; *E. coli* in the soil absorbs the ARGs existing in the soil, thus acquiring antibiotic resistance.

Finally, it was found that the humic substances in the soil around urban residences suppressed gene transfer caused by water evaporation. These results support the previous study [12] reporting that humic substances obtained from urban residential soils could suppress antibiotic-resistant bacteria. Soil analysis results showed that the content of calcium, which promotes organic matter decomposition, was higher in the urban residential soils than in that of other soils. This characteristic of urban residential soils is presumed to be the cause of the effective suppression of gene transfer. However, the content of organic matter containing humic substances in urban residential soils was relatively lower than that of other soils. Therefore, the humic substances that remove the ARGs via absorption are not proportional to the amount of organic matter.

5.2. Expected Impact and Future Study Plan

This study suggests that humic substances could be an eco-friendly method to suppress the generation and activity of antibiotic-resistant bacteria introduced into water and soils through various routes in the ecosystem. Based on the results of this study, humic substances can be used as a method to purify bath water or wash water contaminated with antibiotic-resistant bacteria. In the case of third-world countries where water scarcity and water pollution are of critical concern, clean drinking water is provided at a certain level with the help of various organizations; however, there are many difficulties in supplying enough clean water to support daily activities. In addition, children living in third-world countries are easily exposed to diseases while washing or bathing in contaminated water. Therefore, the use of humic substances is exceptionally significant, for it can not only solve such contamination problems but also be an environmental-friendly solution. However, the exact principle of the mechanism is still unclear. It is recommended for future research to focus on analyzing humic substances in the urban residential soils from various aspects.

6. References

[1] Czekalski, N., Sigdel, R., Birtel, J., Matthews, B., Bürgmann, H., Does human activity impact the natural antibiotic resistance background? Abundance of antibiotic resistance genes in 21 Swiss lakes. Environ. Int. 81, 45-55, 2015.

[2] Thevenon, F., Adatte, T., Wildi, W., Pote, J. Antibiotic resistant bacteria/genes dissemination in lacustrine sediments highly increased following cultural eutrophication of Lake Geneva (Switzerland). *Chemosphere*, 2012, 86, 468-476.

[3] Guo, X., Li, J., Yang, F., Yang, J., Yin, D. Prevalence of sulfonamide and tetracycline resistance genes in drinking water treatment plants in the Yangtze River Delta, China. *Science of the Total Environment*, 2014, 493, 626-631.

[4] Kim, D. W. Development of Integrated Watershed Management Strategy Considering Pollutants Runoff Characteristics : Case Study of Paldang Lake Watershed. (Doctoral Dissertation). University of Seoul, 2014, Seoul, Korea.

[5] O'Neill J. Antimicrobial resistance: tackling a crisis for the health and wealth of nations. *Review on Antimicrobial Resistance*, 2014, 20, 1–16.

[6] Stevenson C., Hall J. P., Harrison E., Wood A., Brockhurst M. A. Gene mobility promotes the spread of resistance in bacterial populations. *ISME Journal*, 2017, 11, 1930.

[7] Hancock, S. J., Phan, M-Duy, Luo, Zhenyao, Lo, Alvin W., Peters, Kate M., Nhu, Nguyen Thi Khanh, Forde, Brian M., Whitfield, Jason, Yang, Ji, Strugnell, Richard A., Paterson, David L., Walsh, Timothy R., Kobe, Bostjan, Beatson, Scott A., Schembri, Mark A. Comprehensive analysis of IncC plasmid conjugation identifies a crucial role for the transcriptional regulator AcaB. *Nature Microbiology*, 2020, 5(11) 1340-1348.

[8] Tang X. J., Lou C. L., Wang S. X., Lu Y. H., Liu M., Hashmi M. Z., et al. Effects of long-term manure applications on the occurrence of antibiotics and antibiotic resistance genes (ARGs) in paddy soils: evidence from four field experiments in south of China. *Soil Biol Biochemistry*. 2015, 90, 179–87.

[9] Tripathi V., Cytryn E. Impact of anthropogenic activities on the dissemination of antibiotic resistance across ecological boundaries. *Essays Biochemistry*. 2017, 61, 11–21.

[10] Zhu, G., Wang, X., Yang, T., Su, J., Qin, Y., Wang, S. et al. Air pollution could drive global dissemination of antibiotic resistance genes. *ISME Journal*, 2021, 15, 270–281.

[11] Cho, H. H. Effects of dissolved matters and anionic surfactant on the solubility of hydrophobic organic contaminants. (Master's thesis). 2000, Ewha Women's University, Seoul, Korea.

[12] Kim, A. H., Lee, S. D. Selection and Utilization of Soil Materials to Eliminate Residual Antibiotic Contamination of Soil. *IJHSR, under review*.

[13] Tripathi V, Cytryn E. Impact of anthropogenic activities on the dissemination of antibiotic resistance across ecological boundaries. *Essays Biochemistry*. 2017, 61, 11–21.

[14] Ellison, C. K, Dalia, T. N, Ceballos, A. V, Wang, J. C-Y, Bias, N, Brun, Y. V, Dalia, A. B. Retraction of DNA-bound type IV competence pili initiates DNA uptake during natural transformation in vibrio cholerae. *Nature Microbiology*, 2018, 3, 773-780.