

Phytotelm to assess bacterial multi-resistance to the antibiotics in a deep tropical forest

Stephanny Sanchez-Vargas

Laboratorio de Microbiología Marina (LaMMaR), Escuela de Ciencias Biológicas, Universidad Nacional, Costa Rica <https://orcid.org/0000-0002-0396-0598>

Paula Vargas-Jiménez

Laboratorio de Ecología Funcional y Ecosistemas Tropicales (LEFET), Escuela de Ciencias Biológicas, Universidad Nacional, Costa Rica <https://orcid.org/0000-0002-0762-2158>

Luis Vega-Corrales

Laboratorio de Microbiología Marina (LaMMaR), Escuela de Ciencias Biológicas, Universidad Nacional, Costa Rica <https://orcid.org/0000-0003-3389-4373>

Junior Pastor Pérez-Molina (✉ junior.perez.molina@una.cr)

Laboratorio de Ecología Funcional y Ecosistemas Tropicales (LEFET), Escuela de Ciencias Biológicas, Universidad Nacional, Costa Rica <https://orcid.org/0000-0002-3396-0599>

Short Report

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Abstract

Paradoxically, the use of antibiotics has resulted in the proliferation of resistant microorganisms in environments where they were not thought to be likely to appear. In this context, this research aimed to evaluate the phytotelmata as a matrix to detect the presence of bacterial strains with antibiotic resistance in the deep understory of tropical cloud forest and in a nearby rural town. The study was carried out in the Santa Elena Cloud Forest Reserve and the urban zone of the Santa Elena town, only 10 km. A compounded sample of phytotelm water was collected from 10–15 bromeliads and heliconia plants by site. Gram-negative bacterial strains found were evaluated to antibiotic susceptibility tests (13 antibiotics). In both sites, five strains presented multi-resistance to more than three antibiotics. Overall, antibiotics possessing cell wall synthesis inhibitor mechanisms were the most common with resistance for both sites followed by those acting as protein synthesis inhibitors. NMDS and PERMANOVA revealed that both environments had a similar response to antibiotic resistance ($P > 0.05$). The detection of bacterial multi-resistance to antibiotics in understory phytotelm water could become a quick tool to determine the degree of exposure of natural ecosystems to anthropogenic effects. This research opens the discussion of the potential dangers of the appearance of bacterial antibiotic multi-resistance in natural ecosystems, whose future impact is uncertain.

Introduction

The spread of antimicrobial resistance (AMR) has been considered one of the greatest threats to public health (Tyrrell et al., 2019). Resistance is constantly increasing because of the mismanagement of antimicrobial compounds (Lee & Lin, 2003), combined with the natural capacity of microorganisms to generate resistance (Milkman, 1990). AMR continues to be reported throughout the world, expanding into the natural environments due to the growth of urbanizations and vectors of spread such as birds (Modupe et al., 2021), bats (Cláudio et al., 2018), felines (Sacristán et al., 2020), fertilizers (Radu et al., 2021). Strains resistant to chloramphenicol were detected in the surface waters of isolated cave microbiomes in Mexico (Bhullar et al., 2012). The biodiversity of microorganisms that can be found in tropical forests is only surpassed by their ability to mutate, adapt, and transfer multi-resistance genes to any antibiotic. This situation may lead to a potentially dangerous scenario for the appearance of infectious diseases. Therefore, it is of utmost importance to provide information on the presence of AMR to contribute to a better understanding of their spread into natural surroundings and to help to combat this global challenge.

Water catchments stored in the natural cavities of plants (phytotelm) as bromeliads tanks are commonly used as bioindicators, because their physicochemical and geochemical characteristics, macro- and micro-organisms communities, and their interactions, make them appropriate matrices to evaluate ecological interactions, carbon and nitrogen cycle and environmental stresses (Benavides-Gordillo et al., 2019). Bacterial communities inside bromeliads tanks are unique and their bacterial abundance remains stable even during drought or increased rainfall events (Benavides-Gordillo et al., 2019; Rodríguez Pérez et al., 2018). To our knowledge, there is no study of AMR from phytotelm samples of the deep understory of a

tropical cloud forest. Consequently, we isolated bacterial strains from phytotelm and tested their susceptibility to antibiotics in order to assess these bacterial communities and how far antibiotics have reached inside the forest. This study aimed to evaluate the phytotelmata as a matrix to detect the presence of bacterial strains with antibiotic resistance from the deep understory in a tropical cloud forest and in a nearby rural town. Also, a scientometric analysis of the literature about bacteria antibiotic resistance in forests was done to evidence the interest of the investigation over the years.

Materials And Methods

Study area

The study was carried out at two sites, in the Santa Elena Cloud Forest Reserve (SECFR) and in a small urban zone of a nearby town with the same name, just about 10 km apart, in the Pacific-facing slopes of the Cordillera de Tilarán, Monteverde Conservation Area, Costa Rica. The first site (hereafter referred to as the forest) is known for the cloud forest, evergreen tropical mountains. SECFR is a mature forest at 1,600 m.a.s.l., composed of upper canopy trees (15–30m) such as *Cecropia polyphlebia*, *Heliocarpus appendiculatus*, and *Ocotea tonduzii*; in addition, a developed understory, dominated by abundant bromeliads fallen from the canopy and several *Heliconia* sp, among other herbaceous plants (Nadkarni et al., 1995). The year is characterized by a rainy season (May to October), a very windy transitional season (November to January) and dry (February to April). Moisture from dripping fog (horizontal precipitation) is frequent the year around. The second site corresponds to the rural urban area of Santa Elena (hereafter referred to as urban) at 1,300 m.a.s.l., founded in 1968, with an area of 53 km² and an approximate population of 5,000. This site is surrounded by farms and has dairy products production companies and a well developed hotel infrastructure.

Samples of water from the phytotelmata and bacterial antibiotic susceptibility tests

Phytotelm water was collected from 10–15 bromeliads and heliconia plants from the understory forest and urban zone. *In situ*, 1 mL of the compounded sample was placed in a broth culture to maintain grown microbial integrity and then transported to the laboratory for bacterial isolation. Gram-negative bacterial strains isolation was performed using Trypticase Soya Agar (TSA) (BD Difco™). The strains were incubated for 24 h at ambient temperature. The bacterial strains were selected by their morphological characteristics. The antimicrobial resistance was analyzed by the disk diffusion method with the following antibiotics (OXOID®): ampicillin (AMP 10µg), fosfomicin (FOS 50µg), cefotaxime (CTX 30µg), amoxicillin/clavulanic acid (AMC 30µg), florfenicol (FFC 30µg), chloramphenicol (C 30µg), gentamicin (CN 10µg), tetracycline (TE 30µg), oxytetracycline (OT 30µg), enrofloxacin (ENR 5µg), ofloxacin (OFX 5µg), ciprofloxacin (CIP 5µg), trimethoprim/Sulfamethoxazole (SXT 25µg), and vancomycin (VA 30µg) as a negative control. The resistance category (sensitive or resistant) was selected based on the Clinical Laboratory Standard Institute guidelines (Humphries et al., 2021).

Data analysis

Bacterial antibiotic resistance by type of antibiotic and inhibition mechanism for forest and the urban environment was plotted. The response of the presence or absence of bacterial antibiotic resistance from the forest and the urban environment was evaluated by non-metric multidimensional scaling (NMDS). Permutational multivariate analysis of variance (PERMANOVA) was used to compare forest and urban treatment and test the null hypothesis that the centroids and dispersion of the treatments as defined by measure space are equivalent for both treatments. All statistical analyses were performed with the programming language R version 4.1.0 with a level of significance of $\alpha = 0.05$. In addition, a scientometric analysis of the literature above bacterial antibiotic resistance in the forest was done. The database was obtained from PubMed.gov using the search descriptor "*antibiotic resistance*"[Title/Abstract] AND "*forest*" [Title/Abstract]. PubMed.gov does not represent the potential totality of studies; it is simply a sample to evidence the general trend in published articles. The number of scientific articles per year was plotted. A network analyses keyword greater than three co-occurrences was defined. A cluster analysis of the 50 most relevant words was computed using the Kullback-Leibler distance to compare the distribution of word co-occurrences with the overall distribution of words. Scientometric analysis of literature was performed with the help of VOSviewer software version 1.6.16.

Results And Discussion

Sixteen gram-negative bacterial strains were isolated, nine and seven from the forest and urban site, respectively. Despite the small number of bacterial strains isolated, only one was susceptible to all antibiotics and the other sixteen strains were resistant to at least one antibiotic, where five strains were multi-resistant to different antibiotics (> 3) in the forest as well in the urban area (Fig. 1A, B). Congruently, NMDS and PERMANOVA revealed that both environments had a similar response to antibiotic resistance ($F_{(1,13)} = 1.02$, $P > 0.05$, Fig. 1C). We hypothesize that the antibiotics were probably moved from the cattle areas around (~ 50 km) or the capital San José-Costa Rica (more than 100 km distance) by the cloudiness formed by the trade winds that cross the Tilarán Mountain Range of Costa Rica, which precipitates in the forest. Zhu et al., (2021) founded antimicrobial resistance genes (ARG's) transported by air pollution; Xie et al., (2018) demonstrated the AGRs dissemination via fine particulate matter (PM 2.5) contains in the atmosphere; and Segawa et al. (2013), revealed the presence of resistance genes due to clinical tests in polar snow and glaciers (where humans have not intervened), the researchers argue that these were spread by bacteria through the air. These kinds of explanations are difficult to demonstrate in our case. However, AMR could be founded in the forests due to different dissemination pathways that could carry ARG's, resistant bacteria, or antibiotic molecules. Finally, we cannot set aside the bacterial natural ability to generate resistance. Phytotelm is a complex and small ecological niche with several communities' interactions, where bacteria are going to produce their antimicrobial compounds to compete for available space and nutrients, which leads to a natural resistance generation. In addition, bacteria can mutate, adapt, and transfer multi-resistance genes, which may enhance the mechanisms of AMR gene transfection in this small niche (von Wintersdorff et al., 2016).

Overall, the cell wall synthesis inhibitor mechanism (AMC, CTX, FOS, and AMP) was the most common type of antibiotic resistance for both sites, closely followed by those based on protein synthesis (C and FFC) (Fig. 3). However, protein (OT and T) and DNA synthesis inhibitions (OFC and CIP) were the mechanisms showing the highest bacterial sensitivity. Some hypotheses emerge from these findings: i) the bacteria were probably exposed to similar or the same antibiotics with mechanisms of bacterial inhibition, and/or ii) that these are the antibiotics that suffer less degradation during transport, prolonging their exposure time. Reverse response to antibiotics that showed greater bacterial sensitivity, where little bacterial exposure and/or rapid degradation of the antibiotic during transport, would be expected. Both hypotheses assume that bacteria acquire antibiotic resistance by exposure, disregarding possible natural resistance.

The potential dangers of the emergence of antibiotic resistance in natural ecosystems are uncertain. Efforts to determine the impact of antibiotic resistance on the human population estimated that deaths from antimicrobial resistance could rise from about 700,000 deaths a year to about 10 million deaths per year by 2050 (O'Neill, 2014), despite criticism from Kraker et al., (2016) and NOAH (2016). Although antibiotic resistance is a natural process, the use of antimicrobials has increased significantly in recent decades, by exposing bacteria to greater numbers and increased concentration, which increases their chances of developing resistance. The issue is worryingly critical due to the limited number of new antibiotics in development to replace those that become useless, *i.e.* from the economic point of view, the supply of new medicines has been depleting while the demand is growing given the accelerated increase in multi-resistant microorganisms.

Regarding a scientometric analysis of literature, a total of 71 articles were found to coincide with the established search made in PubMed.gov. Within the limited number of articles found, a clear rapid increase in the last five years evidences a sudden change in the interest if studies about the presence of antibiotic resistance in forests (Fig. 2A). In this search, the first article was in 1993 on the ecology of aquatic bacteria and multiple antibiotic resistance (Ogan & Nwiika, 1993), but it was not until 2018–2021 that the interest had this upturn in studies. Among the most recurrent concepts linked to forests has been terms like genes, soil microbiology, environmental monitoring, agriculture, land use, rivers, antibiotic resistance genes, and microbiota (Fig. 2B). This is the first study to attempt to prove the presence of bacterial strains with some degree of antibiotic resistance in phytotelm from the deep understory of a tropical cloud forest.

Conclusions

Phytotelm could be used as a convenient and practical matrix for detecting AMR dissemination. Additionally, the capacity of their bacterial communities to remain stable and resist fluctuations in the water volume, offer the possibility to use these water catchments as a matrix to evaluate the environmental changes occurring along time, contrary to other samples commonly used such as animal feces. Moreover, it is a fast tool where monitoring of large extensions of protected forest areas could be carried out simultaneously.

Further research is needed to determine whether AMR is derived from the anthropogenic influence or by natural mechanisms of bacteria AMR generation. Even though bacterial antibiotic susceptibility tests with the disk's diffusion technique is a low-cost, easy, and fast method that allows identifying not only the resistant but also the multi-resistant bacteria. However, this method left unattended the ARGs and resistance in non-cultivable bacteria, but can be supplemented with the detection of antibiotic compounds traces in the phytotelmata. Finally, it is important to consider how the ecology and physicochemical factors can influence the AMR depending on the geographical location where samples are taken.

Declarations

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Authors' contributions

JPP-M and LV-C designed the experiment. PV-J conducted the fieldwork. SS-V performed bacterial antibiotic susceptibility tests. JPP-M performed the statistical analysis. All authors helped interpret the results. JPP-M led the writing of the manuscript, with input from all authors.

Data availability

Data are available from the corresponding author upon request.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Benavides-Gordillo, S., Farjalla, V. F., González, A. L., & Romero, G. Q. (2019). Changes in rainfall level and litter stoichiometry affect aquatic community and ecosystem processes in bromeliad phytotelmata. *Freshwater Biology*, *64*(8), 1357–1368. <https://doi.org/10.1111/fwb.13310>

Bhullar, K., Waglechner, N., Pawlowski, A., Koteva, K., Banks, E. D., Johnston, M. D., Barton, H. A., & Wright, G. D. (2012). Antibiotic resistance is prevalent in an isolated cave microbiome. *PLoS One*, *7*(4), e34953.

<https://doi.org/10.1371/journal.pone.0034953>

Cláudio, V. C., Gonzalez, I., Barbosa, G., Rocha, V., Moratelli, R., & Rassy, F. (2018). Bacteria richness and antibiotic-resistance in bats from a protected area in the Atlantic Forest of Southeastern Brazil. *PLOS One*, *13*(9), e0203411. <https://doi.org/10.1371/journal.pone.0203411>

Humphries, R., Bobenchik, A. M., Hindler, J. A., Schuetz, A. N., & McAdam, A. J. (2021). Overview of Changes to the Clinical and Laboratory Standards Institute *Performance Standards for Antimicrobial Susceptibility Testing*, M100, 31st Edition. *Journal of Clinical Microbiology*, *59*(12), e00213-21. <https://doi.org/10.1128/JCM.00213-21>

Kraker, M. E. A., Stewardson, A. J., & Harbarth, S. (2016). Will 10 Million People Die a Year due to Antimicrobial Resistance by 2050? *PLOS Medicine*, *13*(11), e1002184. <https://doi.org/10.1371/journal.pmed.1002184>

Lee, P. R., & Lin, C. (2003). The Antibiotic Paradox: How the Misuse of Antibiotics Destroys Their Curative Powers (review). *Perspectives in Biology and Medicine*, *46*(4), 603–604. <https://doi.org/10.1353/pbm.2003.0088>

Milkman, R. (1990). Recombination: Gene Transfer in the Environment. *Science*, *247*(4940), 350–351. <https://doi.org/10.1126/science.247.4940.350>

Modupe, S. L., Yaa, N.-B., Henaku, O. E., Ohya, K., Masato, S., Opare, O. J., & Baboreka, K. B. (2021). Protected but not from Contamination: Antimicrobial Resistance Profiles of Bacteria from Birds in a Ghanaian Forest Protected Area. *Environmental Health Insights*, *15*, 117863022110176. <https://doi.org/10.1177/11786302211017687>

Nadkarni, N. M., Matelson, T. J., & Haber, W. A. (1995). Structural characteristics and floristic composition of a Neotropical cloud forest, Monteverde, Costa Rica. *Journal of Tropical Ecology*, *11*(4), 481–495. <https://doi.org/10.1017/S0266467400009020>

NOAH. (2016). NOAH responds to the O'Neill review. *Veterinary Record*, *179*(6), 132–132. <https://doi.org/10.1136/vr.i4266>

O'Neill, J. (2014). *Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations*. The Review on Antimicrobial Resistance (AMR). [https://amr-review.org/sites/default/files/AMR Review Paper - Tackling a crisis for the health and wealth of nations_1.pdf](https://amr-review.org/sites/default/files/AMR%20Review%20Paper%20-%20Tackling%20a%20crisis%20for%20the%20health%20and%20wealth%20of%20nations_1.pdf)

Ogan, M. T., & Nwiika, D. E. (1993). Studies on the ecology of aquatic bacteria of the lower Niger Delta: multiple antibiotic resistance among the standard plate count organisms. *J. Appl. Bacteriol.*, *74*(5), 595–602.

Radu, E., Woegerbauer, M., Rab, G., Oismüller, M., Strauss, P., Hufnagl, P., Gottsberger, R. A., Krampe, J., Weyermaier, K., & Kreuzinger, N. (2021). Resilience of agricultural soils to antibiotic resistance genes

introduced by agricultural management practices. *Science of The Total Environment*, 756, 143699. <https://doi.org/10.1016/j.scitotenv.2020.143699>

Rodríguez Pérez, H., Borrel, G., Leroy, C., Carrias, J.-F., Corbara, B., Srivastava, D. S., & Céréghino, R. (2018). Simulated drought regimes reveal community resilience and hydrological thresholds for altered decomposition. *Oecologia*, 187(1), 267–279. <https://doi.org/10.1007/s00442-018-4123-5>

Sacristán, I., Esperón, F., Acuña, F., Aguilar, E., García, S., López, M. J., Cevidanes, A., Neves, E., Cabello, J., Hidalgo-Hermoso, E., Poulin, E., Millán, J., & Napolitano, C. (2020). Antibiotic resistance genes as landscape anthropization indicators: Using a wild felid as sentinel in Chile. *Science of The Total Environment*, 703, 134900. <https://doi.org/10.1016/j.scitotenv.2019.134900>

Segawa, T., Takeuchi, N., Rivera, A., Yamada, A., Yoshimura, Y., Barcaza, G., Shinbori, K., Motoyama, H., Kohshima, S., & Ushida, K. (2013). Distribution of antibiotic resistance genes in glacier environments. *Environmental Microbiology Reports*, 5(1), 127–134. <https://doi.org/10.1111/1758-2229.12011>

Tyrrell, C., Burgess, C. M., Brennan, F. P., & Walsh, F. (2019). Antibiotic resistance in grass and soil. *Biochemical Society Transactions*, 47(1), 477–486. <https://doi.org/10.1042/BST20180552>

von Wintersdorff, C. J. H., Penders, J., van Niekerk, J. M., Mills, N. D., Majumder, S., van Alphen, L. B., Savelkoul, P. H. M., & Wolfs, P. F. G. (2016). Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. *Frontiers in Microbiology*, 7. <https://doi.org/10.3389/fmicb.2016.00173>

Xie, J., Jin, L., Luo, X., Zhao, Z., & Li, X. (2018). Seasonal Disparities in Airborne Bacteria and Associated Antibiotic Resistance Genes in PM 2.5 between Urban and Rural Sites. *Environmental Science & Technology Letters*, 5(2), 74–79. <https://doi.org/10.1021/acs.estlett.7b00561>

Zhu, G., Wang, X., Yang, T., Su, J., Qin, Y., Wang, S., Gillings, M., Wang, C., Ju, F., Lan, B., Liu, C., Li, H., Long, X.-E., Wang, X., Jetten, M. S. M., Wang, Z., & Zhu, Y.-G. (2021). Air pollution could drive global dissemination of antibiotic resistance genes. *The ISME Journal*, 15(1), 270–281. <https://doi.org/10.1038/s41396-020-00780-2>

Figures

Figure 1

A) Test of gram-negative bacterial antibiotic resistance categorized by type antibiotic and inhibition mechanism for forest and urban sites. The size of the circles shows the diameter of the bacterial growth inhibition halo. Bacteria with three or more resistant antibiotics are designated as multi-resistant. B) Illustration of two antibiograms. C) Non-metric multi-dimensional scaling (NMDS, presence/absence) of

bacterial antibiotic resistance in phytotelmata for forest and urban sites. Antibiotic: AMC—Amoxicillin/Clavulanic Acid, AMP—Ampicillin, C—Chloramphenicol, CIP—Ciprofloxacin, CN—Gentamicin, CTX—Cefotaxime, ENR—Enrofloxacin, FFC—Florfenicol, FOS—Fosfomycin, OFX—Ofloxacin, OT—Oxytetracycline, SXT—Trimethoprim/Sulfamethoxazole, and TE—Tetracycline.

Figure 2

A) The number of scientific articles about bacterial antibiotic resistance in forests, 1993–2020 ($n = 71$). Word cloud is the analysis of the frequency of the keywords of all the articles with co-occurrences greater than three words. B) Network analyses keywords of all the scientific articles with co-occurrences greater than three words.