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Antimicrobial resistance genes are enriched in aerosols near impacted urban surface waters in La Paz, Bolivia

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Abstract

Antibiotic resistance poses a major global health threat. Understanding emergence and dissemination of antibiotic resistance in environmental media is critical to the design of control strategies. Because antibiotic resistance genes (ARGs) may be aerosolized from contaminated point sources and disseminated more widely in localized environments, we assessed ARGs in aerosols in urban La Paz, Bolivia, where wastewater flows in engineered surface water channels through the densely populated urban core. We quantified key ARGs and a mobile integron (MI) via ddPCR and *E. coli* as a fecal indicator by culture over two years during both the rainy and dry seasons in sites near wastewater flows. ARG targets represented major antibiotic groups —tetracyclines (*tetA*), fluoroquinolines (*qnrB*), and beta-lactams (bla_{TEM})—and an MI (*intI1*) represented the potential for mobility of genetic material. Most air samples (82%) had detectable targets above the experimentally determined LOD: most commonly bla_{TEM} and *intI1* (68% and

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47% respectively) followed by *tetA* and *qnrB* (17% and 11% respectively). ARG and MI densities in positive air samples ranged from 1.3×10^1 to 6.6×10^4 gene copies/m^3 air. Additionally, we detected culturable *E. coli* in the air (52% of samples <1 km from impacted surface waters) with an average density of 11 CFU/m^3 in positive samples. We observed decreasing density of bla_{TEM} with increasing distance up to 150 m from impacted surface waters. To our knowledge this is the first study conducting absolute quantification of ARGs near uncontained urban wastewater flows. Environments in close proximity these impacted waters experience locally elevated concentrations of ARGs, a possible concern for the emergence and dissemination of antimicrobial resistance in cities with poor sanitation.

Keywords

bioaerosol; wastewater; *E. coli*; mobile integron; dissemination; antibiotic resistance

INTRODUCTION

Antibiotic resistance (AR) is a serious threat to global public health (World Health Organization, 2019). Increased antibiotic use coupled with poor antibiotic stewardship has contributed to the rapid development and dissemination of resistance in microbial communities and ultimately the emergence of AR as a global crisis (Davies and Davies, 2010; de J. Sosal et al., 2010; Nadimpalli et al., 2020; Pruden et al., 2006; Vikesland et al., 2017). Bacteria may inherit resistance, acquire resistance by horizontal gene transfer (Bennett, 2008) or may possess resistance genes conferring resistance indirectly (Allen et al., 2010). Genes that enable resistance to antimicrobials are known as antibiotic resistance genes (ARGs); genes that confer horizontal gene transfer ability or enable bacteria to take up resistance genes from the environment are known as mobile genetic elements (MGEs); and genes that are physically related to MGEs and enable the recombination and functional conversion of ARGs are known as mobile integrons (MIs) (Barraud et al., 2010; Ma et al., 2017; Mazel, 2006).

Although most studies have focused on AR development and dissemination in clinical settings (Schrag et al., 2004; Wellington et al., 2013), understanding the fate and transport of ARGs in environmental media is crucial to controlling AR, especially because the full extent of AR dispersion in the environment is still poorly characterized. Sabatino *et al.* recently detected AR residues in a highly diluted marine environment far from any waste sources, showcasing the potential for extensive AR circulation at large scales and subsequent introduction into previously susceptible microbial species and populations where rapid resistance development may occur (Sabatino et al., 2020). A noteworthy example of rapid resistance development is the class A enzyme of β-lactamase genes, Cefotaximase (CTX-M), which appears to have originated in the *Kluyvera* spp. bacteria that exist in both environmental media and the human gut (Cantón, 2009). From first identification in the 1980s to the 2000s, CTX-M had displaced other variants to become the dominant extended spectrum β-lactamase (ESBL) in countries where testing occurred (Cantón et al., 2012; Cantón and Coque, 2006), with environmental transmission implicated. Closely related in the same class and often co-occurring with CTX-M are the Temoneira (TEM) enzyme

and *Pseudomonas* extended resistance (PER) enzyme (Celenza et al., 2006; Shahid et al., 2011). The TEM enzyme, first discovered in *E. coli* isolated from a patient in Athens, Greece in 1965, has since expanded to include 223 new and novel variants from all over the globe (Rahman et al., 2018; Steward et al., 2000). Other clinically relevant ARGs of putative environmental heritage include the *qnr* gene family which confers mild resistance to fluoroquinolones. These genes, often found in *Enterobacteraciae* affecting humans, are thought to have originated in several aquatic species of bacteria like *Shewanella algae* and *Vibrio splendidus.* (Cantón, 2009; Lupo et al., 2012).

Evidence exists indicating prevalent ARGs and high potential for mobility in environments such as water runoff from animal feedlots; air, soils and groundwater surrounding wastewater and solid waste treatment plants; air surrounding poultry farms and markets; and other sites where concentrated fecal wastes exist (Chapin et al., 2005; de J. Sosal et al., 2010; Echeverria-Palencia et al., 2017; Gao et al., 2018, 2016; Gibbs et al., 2006; Hu et al., 2018; Li et al., 2018, 2016; Liu et al., 2012; McEachran et al., 2015; Neher et al., 2020; Pal et al., 2016; Rizzo et al., 2013; Sancheza et al., 2016; Stange and Tiehm, 2020; Zhang et al., 2017; Zhu et al., 2013). In a few high-income cities across the globe, a variety of ARGs have been detected and quantified in aerosols. Xie *et al.* (Xie et al., 2019) and Wang *et al.* (Wang et al., 2019) detected ARGs encoding resistance to β-lactams, tetracyclines, and fluoroquinolones in ambient urban air from Chinese cities. Echeverria-Palencia *et al.* (Echeverria-Palencia et al., 2017) detected aerosolized bla_{SHV}, a β-lactam resistance-encoding ARG, in densities ranging from 0.2 to 600 gene copies (gc)/m^3 in four cities in California. Less well characterized are sources and densities of specific ARGs of concern in cities in low- and middle- income countries (LMICs), however. These are settings where conditions favor AR emergence and transfer, and where uncontained, concentrated fecal waste may be present in densely populated areas with a high burden of enteric disease and poor antibiotic stewardship (Graham et al., 2018; Nadimpalli et al., 2020; Witte, 2000; Zhang et al., 2017). Even where wastewater treatment is successful in removing a majority of fecal bacteria in these settings, AR-bacteria can persist post-treatment (Kumar et al., 2020). Where sanitation systems are completely absent or operating ineffectively, ARG diversity and AR protein concentrations are even more likely to be widely disseminated in the environment (Pehrsson et al., 2016), presenting possible exposure risks to nearby populations.

We hypothesized that where urban wastewater flows are uncontained and open to the atmosphere, ARGs would be detectable and quantifiable in aerosols nearby. The absolute densities of specific ARGs of concern – necessary for developing mechanistic models for fate, transport, and exposure – remain a critical unknown in these settings. We further hypothesized that aerosolized ARGs would decrease with distance from potential sources. The city of La Paz, Bolivia, where wastewater is conveyed in open, engineered channels shared with surface water, provided an opportunity to test these hypotheses.

METHODS

Sample sites and collection.

We collected samples during both the rainy (March) and dry (May-July) seasons in La Paz, Bolivia (March/2018, May-June/2018, March/2019, June-July/2019) where a network of rivers receives untreated sewage discharge, industry effluent, and stormwater runoff. Most of the waterway flows in a series of engineered channels (Alarcon Calderon, 1996; Poma et al., 2016) consisting of highly impacted surface waters, which we refer to here as open wastewater canals (OWCs). The main waterway, the Choqueyapu River, begins as a small stream at Pamapalarama, flowing south through central La Paz, home to 900,000 people (1900 persons/km^2) (INE, 2018; Ohno, A; Marui, A; Castro, ES; Reyes, AA; Elio-Calvo, D; Kasitani, H; Ishii, Y; Yamaguchi, 1997). As it flows through the city, the Choqueyapu is joined by tangential tributaries including the Orkojahuira, Irpavi, and Achumani rivers (Figure 1).

During the first three sampling events, we identified 13 sites along the Choqueyapu River meeting the following criteria: (1) proximity to OWCs, (2) accessible at ground level, and (3) unintrusive to residents or passers-by. We identified two control sites >1 km from known concentrated wastewaters or other contaminated sources: (1) Chacaltaya, a weather station and environmental observatory located at 5380 m in elevation and far from human habitation and (2) Pampalarama, a pristine site near the Choqueyapu headwaters. In a final sampling event (June-July 2019), we chose an expanded range of sites to further interrogate the spatial relationship of targets in relation to the OWCs, with particular attention to distances of 200 m or less from OWCs. We based selected sampling locations on the Advanced Spaceborne Thermal Emission and Reflection Radiometer (ASTER) Global Digital Elevation Model (GDEM) version 2 data for the study area (NASA/METI/AIST/Japan Space Systems and Team, 2009) and a hydrographic model (ESRI Inc., 2019) in ArcGIS Version 10.7.1 to generate the major streamlines according to the topography for the La Paz metro area. Based on the four main OWCs, we randomly selected sites ≤ 200 m from the mid-point of OWCs and at least 150 m from the nearest other site. Given the diurnal cycles of atmospheric stability and the resulting impact on bioaerosol dissemination (Jacob, 1999; Jones and Harrison, 2004), we assessed the potential temporal associations of ARG densities in the air through sampling both in the morning and afternoon at each site.

Sample collection, culture, extraction, and analysis.

We used the Six-Stage Viable Andersen Cascade Impactor (ACI) with selective media in six partitioned chambers at a flow rate of approximately 28.5 L/min to assess viability of *E. coli* at a subset of sites (Andersen, 1958) (ACI, Thermo Scientific™, USA), as an indicator of aerosolized fecal material. We used AquaTest Medium (Sisco Research Laboratories PVT. LTD., India) to select for *E. coli* (Bain et al., 2015; Brown et al., 2020; Genter et al., 2019; Magro et al., 2014). We incubated plates overnight at 37°C. The limit of detection for the culture analysis was determined by dividing 1 colony-forming unit (CFU, the minimum count per plate) by the volume of air sampled.

To collect larger volumes for molecular analysis, we sampled for approximately 4 hours per sampling event using the ACD-200 BobCat Dry Filter Air Sampler (InnovaPrep, Drexel, MO, USA) with 52 mm electret filters and a flow rate of 150 L/min, to yield a total sample volume of 36 m^3 of air per sample. We used a single-use wet foam carbon compressed elution kit (InnovaPrep, Drexel, MO, USA) to flush the filter following the manufacturer's instructions, yielding approximately 6 mL of liquid. In a subset of locations, we collected 150 mL grab samples of OWC water for further molecular analysis.

We treated filter eluant with a guanidine thiocyanate-based lysis buffer (UNEX; Microbiologics, St. Cloud, MN, USA) in a 1:1 volumetric ratio, stored these in SK38 bead tubes (Bertin Corp, MD, USA) and cryovials and transported them to our laboratory in Atlanta. We used 900 μL of sample eluant and UNEX mixture for extraction. After DNA extraction per the bacterial/viral UNEX protocol (Hill et al., 2015), we stored extracted nucleic acids in 50–75 μL of 10 mM Tris-1 mM EDTA (pH 8) in a −80°C freezer until further analysis. We vortexed OWC samples and then followed the same extraction and storage procedures as for eluted air samples.

In total, we collected 27 ACI samples near OWCs (13 wet season, 14 dry season) for *E. coli* enumeration by culture, 71 high-volume samples for molecular analysis near OWCs (13 wet season, 58 dry season), and 4 grab samples of OWC water. The distance to nearest OWCs was a mean of 93 m (range: 1 – 778 m). We further collected 4 ACI (3 wet season, 1 dry season) and 4 high-volume (2 wet season, 2 dry season) control samples in the unimpacted settings far from human habitation. Sampling locations are shown in relation to OWCs in Figure 1.

ARG detection and quantitative analysis.

We conducted absolute quantification of ARGs via droplet digital PCR (ddPCR™, Bio-Rad, Hercules, CA, USA). ARG targets spanned three major antibiotic groups commonly used in low-income settings and whose ARGs have been detected previously in environmental samples: tetracyclines (*tetA*) (Guarddon et al., 2011), fluoroquinolones (*qnrB*) (Cavé et al., 2016), and β-lactams (bla_{TEM}) (Lachmayr et al., 2009). Resistance to tetracycline in clinical isolates has been reported in multiple studies in Bolivia, including in two urban cities of Bolivia where 93% of *E. coli* isolates from children's stool were not susceptible to the antibiotic and another study in La Paz, Bolivia where 28% of ETEC isolates from children's stool were not susceptible (Bartoloni et al., 2006; Rodas et al., 2011). Furthermore, tetracycline resistant *Enterobacteriaceae* were detected in ambient air in the same area as ours at 28% prevalence (Salazar et al., 2020) and 50% prevalence (Medina et al., 2020). Fluoroquinolone resistance has been reported in one study in Bolivia where overall, *qnr* genes were detected in 63% of commensal enterobacteria isolated from healthy children's stools and specifically, *qnrB* was detected in 60% of isolates (Pallecchi et al., 2009). Additionally, another study in Bolivia reported fluoroquinolones as a common antibiotic used to treat upper respiratory infections (Cordoba et al., 2017). Class A β-lactamases such as TEM varieties are known to be present in South American countries due to the widespread distribution of β-lactam antibiotics (Villegas et al., 2008). Though the other three assays are specific to the individual gene, the bla_{TEM} assay used in our analysis

incorporates 135 variants within the TEM family of β-lactam resistance including resistance to penicillins, cephalosporins, carbapenems and other antibiotics that have a β-lactic ring in their structure. The TEM β-lactamases can be both ESBL (extended spectrum β-lactamases) and inhibitor resistant and are the most clinically significant. Therefore this assay accounts for a wide range of resistance mechanisms and target drugs that are commonly used in this sampling locations (Lachmayr et al., 2009).

An additional target, integron class 1 (*intI1*), was included to assess potential genetic mobility and has also been previously detected in environmental media. Through enabling gene cassette movement and their physical association to MGEs, integrons aid in the spread of antibiotic resistance in gram negative bacteria and when present in the environment or in bacteria, they indicate that resistance has either been acquired or may be acquired in the future (Barraud et al., 2010; Gillings et al., 2015; Mazel, 2006). One study by Leverstein-Van Hall et al. found that the detection and presence of integrons in *Enterobacteriaceae* is strongly associated with resistance to multiple antibiotics and in the case of some antibiotics, predictive of their resistance. (Leverstein-Van Hall et al., 2003).

We experimentally determined LODs for each assay using a probit analysis outlined by Stokdyk et al. (Bivins et al., 2020; Stokdyk et al., 2016). Reaction mixes, conditions, ARG target sequences, and experimentally determined limits of detection (LOD) for each target are described in Table 1.

Spatial analysis.

We conducted a spatial analysis to estimate the relationship between lateral distance from OWCs and ARGs in aerosols, using data from the fourth sampling event (2019). First, we calculated the two-dimensional distance from each sampling location to the nearest OWC segment and then, to account for the city's variable topography and elevation, we adjusted distances by applying the elevations we extracted from the ASTER GDEM (NASA/METI/AIST/Japan Space Systems and Team, 2009) to the horizontal distances of the sampling points in ArcGIS, calculating the adjusted distance through using Pythagorean theorem. We performed a linear regression analysis in RStudio version 1.1.383 to assess the significance of the relationship between lateral distance from OWCs and ARGs density based on 95% confidence (alpha=0.05), subsequently disaggregating the data based on time of sampling (morning or afternoon) and conducting a multiple linear regression analysis to assess the potential diurnal impacts.

RESULTS AND DISCUSSION

E. coli, ARG and MI detection in aerosol samples.

We analyzed high-volume samples for ARG and MI targets (mean volume 48 m^3, range 9–216 m^3). We set all densities below the experimentally derived LOD to zero. Our LODs can be interpreted as the density at which we are 95% confident that the density detected is accurate. Among samples located <1 km from OWCs (n=71), we detected the MI, *intI1*, in 47% of samples (n=33) and we detected ARGs *qnrB*, *tetA*, and bla_{TEM} in 11%, 17%,

and 68% of samples respectively (n=8,12, and 48; Table 3). We then plotted the density distribution and the corresponding standard error for each target (Figure 2).

To confirm the presence of aerosolized fecal material, we measured culturable *E. coli* in aerosol samples (Cronholm, 1980; Dueker, 2012; Farling et al., 2019; Rocha-Melogno et al., 2020; Salazar et al., 2020). We detected *E. coli* in 52% of samples (n = 14) with an average density of 11 CFU/m^3 air across all positive samples (Figure 2). We detected culturable *E. coli* in aerosols with aerodynamic particle sizes of 0.6–7 μm, in 27 % of culturable *E. coli* under 2.1 μm, the size cutoff for fine aerosol particles. These data indicate that fecal indicator bacteria may linger in the air on a scale of hours, with a settling velocity in air of 0.5 m/hour for a typical particle with 2 μm diameter (Flagan and Seinfeld, 1988), indicating high transport potential in air near OWCs. While ARGs can be free-floating or contained within viable or non-viable cells, we make no inference about the relationship between *E. coli* and ARGs in this context, instead using *E. coli* only as a marker for aerosolized fecal waste that may be attributed to many sources in a contaminated urban setting such as La Paz. We do note that *E. coli* cultured from aerosols near the Choqueyapu River has indicated the presence of a range of resistant phenotypes in members of the coliform group (Medina et al., 2020; Salazar et al., 2020).

We detected all targets above the LOD among OWC-adjacent sites (Table 3). We detected bla_{TEM} at one control site. Other studies in high-and-middle-income countries have reported comparable ARG absolute densities in contaminated settings where fecal wastes may be enriched, primarily indoors (Gao et al., 2018; Ling et al., 2013). Studies reporting relative abundances of ARGs in outdoor ambient air, such as Li et al. (Li et al., 2018), show that a wide range of ARG and MI subtypes may persist in the environment and shed light on the potential threat of urban aerosol transmission of these contaminants. However, where relative abundance reveals target diversity and presence within the microbial communities of a particular sample, absolute quantification in environmental media allows for characterization in a broader context that can be directly applied to population and environmental exposures through fate and transport modeling and risk assessments. Our results further confirm the range of ARGs present in outdoor ambient air at detectable levels and additionally through absolute quantification allow for public health exposure applications. This suggests widespread distribution of ARGs and associated MIs in ambient air, with uncontained urban wastewater flows now implicated as a potential source in cities with poor sanitation. Once airborne, ARGs and MIs in microorganisms may be transported through the air, inhaled or deposited on surfaces and fomites and subsequently ingested (de Man et al., 2014). Free floating ARGs and MIs may be picked up by other microbes in the environment through horizontal gene transfer. Both mechanisms may contribute to the dissemination of AR in the environment and potentially pose a public health threat to the affected populations.

ARG and MI detection in OWCs.

To confirm the presence of targets in OWCs, we collected samples during the third sampling event (n = 4). We detected *intI1* and bla_{TEM} in all OWC samples and at the highest averages (1.4×10^8 and 6.4×10^7 gc/100mL respectively). We detected *qnrB* and *tetA* in

3/4 OWC samples at averages of 1.7×10^7 and 1.4×10^7 gc/100 mL respectively, indicating AR contaminated surface waters that are likely contributing to the dissemination and proliferation of AR in the surrounding environment. Comparably, ESBL-producing bacteria, such as *bla*$_{TEM}$, have been detected in the Choqueyapu River previously (Guzman-Otazo et al., 2019; Poma et al., 2016). Although there are limited data on antibiotic usage in Bolivia, previous studies have reported that in some areas in Bolivia and more widely in South America, the most commonly used antibiotics include penicillin, ampicillin, and amoxicillin (Bartoloni et al., 1998; Cordoba et al., 2017), all members of the β-lactam family. High usage of these drugs combined with poor wastewater treatment may lead to the release and spread of resistant organisms. A study in Cochabamba, Bolivia, a city south-east of La Paz, detected the presence of β-lactam resistance encoding ARG variants (also covered by the *bla*$_{TEM}$ ARG target we used in our study) in rivers that flow throughout the city (Saba Villarroel et al., 2017). Our results support these studies that β-lactam resistance encoding ARGs, in addition to tetracycline and fluoroquinolone resistance encoding ARGs are highly prevalent in these urban settings.

Spatial analysis.

We sought to assess the relationship between ARGs and one MI near OWCs. From the randomly generated points (n=50) for the final sampling event, we only sampled at 25 sites due to time restraints and in some cases, lack of accessibility. One site we sampled at was observed to be much further (~450 m) than within the intended buffer because the actual river location varied from the model near this point. We excluded the 2 samples taken at this site from further analysis and the sample taken at the control site. Hence, we assessed 46 samples from 23 sites in our spatial model, all located within 150 m from OWCs. We performed a linear regression calculation for target densities as distance from OWCs increased and examined the relationship between lateral distance and OWCs for all targets detected (Figure 3).

Despite observed mean higher densities of targets at locations close to OWCs, specifically for *bla*$_{TEM}$, overall regression results showed no significance between distance from OWCs and ARG density based on 95% confidence both when separated by time of day and when not separated. However, when not separated by time of day we observed a tendency of *bla*$_{TEM}$ density in aerosols to decrease (p=0.082) as distance from OWCs increases within the 150 m. The apparent decrease may indicate that proximity to OWCs is important when assessing human risk through exposure, though we cannot rule out that this observation is attributable to chance.

Our interpretation of findings is constrained by the study limitations. Our data are observational and limited in time and space, where phenomena such as aerosolization of waste and aerosol transport may be highly variable according to local conditions. We identified a limited number of ARGs to assess *a priori*, and these may or may not be the most relevant targets for the study area or for exposure relevance more generally. Additionally, we acknowledge that though identification of low gene target densities via ddPCR is improved when compared to qPCR (Cavé et al., 2016), false positives are possible (Cao et al., 2015) even though we have applied conservative estimates of LODs derived

experimentally. Though the detection of *E. coli* in air near OWCs indicates the presence of fecal waste in aerosols, we cannot unambiguously attribute *E. coli* to these OWCs. Additionally, though ARGs are present in air near OWCs where the same ARGs are also present, we cannot unambiguously identify the OWCs as the source.

CONCLUSION

Bioaerosols near urban wastewater flows may be an important factor in the environmental transmission of AMR in cities with poor sanitation. We found that as distance from fecal waste sources such as OWCs increases, bla_{TEM} density decreases, indicating that proximity to these OWCs is important to consider in the context of dissemination of AR. While the exposure and health risks are unknown, uncontained, concentrated fecal wastes in densely populated cities may present a range of health risks related to exposure to sanitation-related aerosols. Further exploration into the spatial relationship between fecal sources and ARG and MI presence in the environment – including in cities of LMICs – should be pursued, along with quantitative risk models to assess the potential for exposures in this poorly characterized pathway.

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ABBREVIATIONS

AR	antibiotic resistance
ARG	antibiotic resistance gene; mobile genetic element (MGE)
MI	mobile integron
LMICs	low- and middle-income countries
OWC	open waste cana
ASTER	Advanced Spaceborne Thermal Emission and Reflection Radiometer
GDEM	Global Digital Elevation Model
ACI	Andersen Cascade Impactor
FIB	fecal indicator bacteria
CFU	colony-forming unit
ddPCR	droplet digital PCR
ESBL	extended spectrum β-lactamases
LOD	limit of detection

gc gene copies

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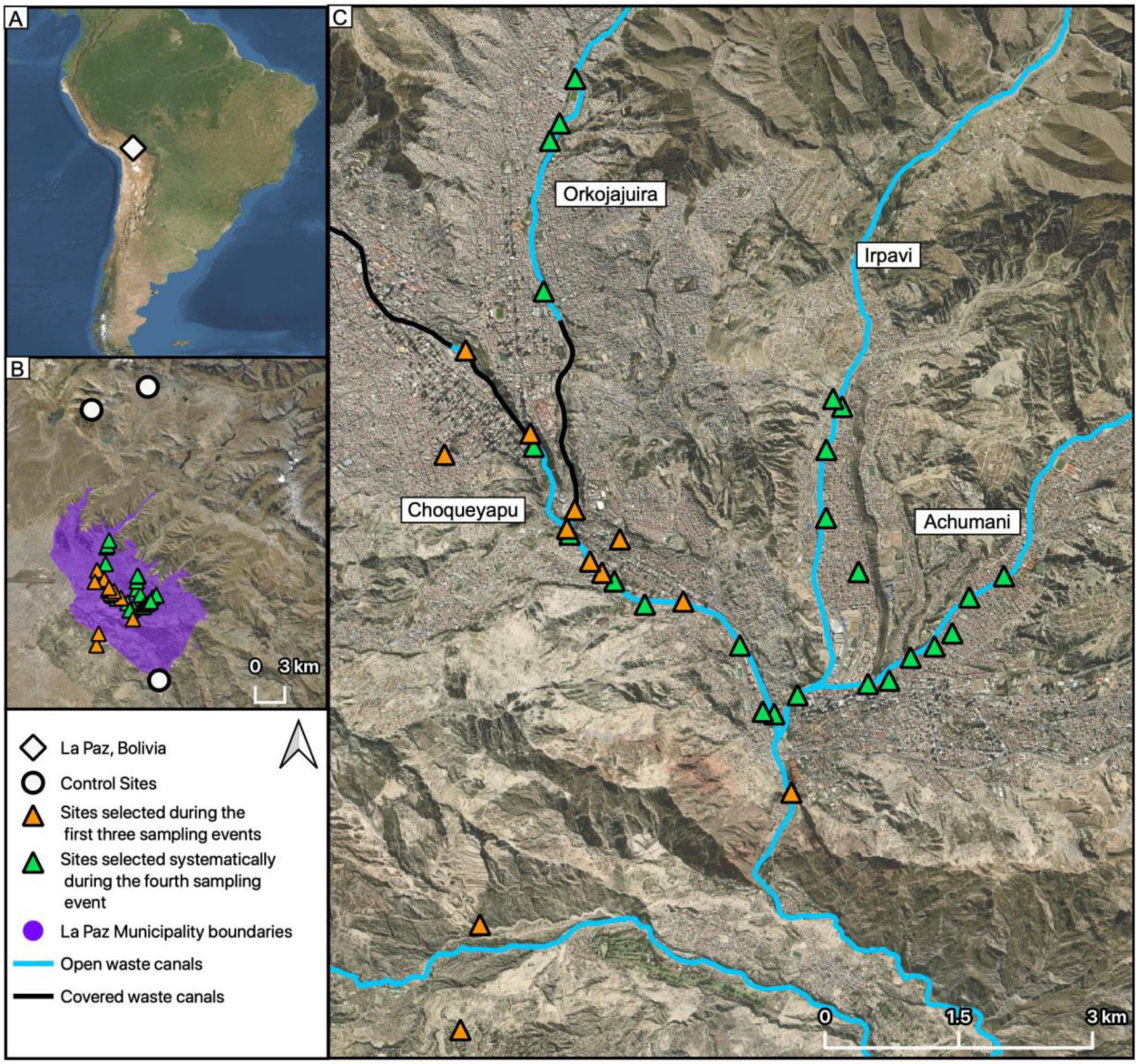

Figure 1.

(A) Location of La Paz, Bolivia. (B) La Paz Municipality boundaries with all sampling sites and control sites. (C) Sampling locations in relation to covered and uncovered OWCs.

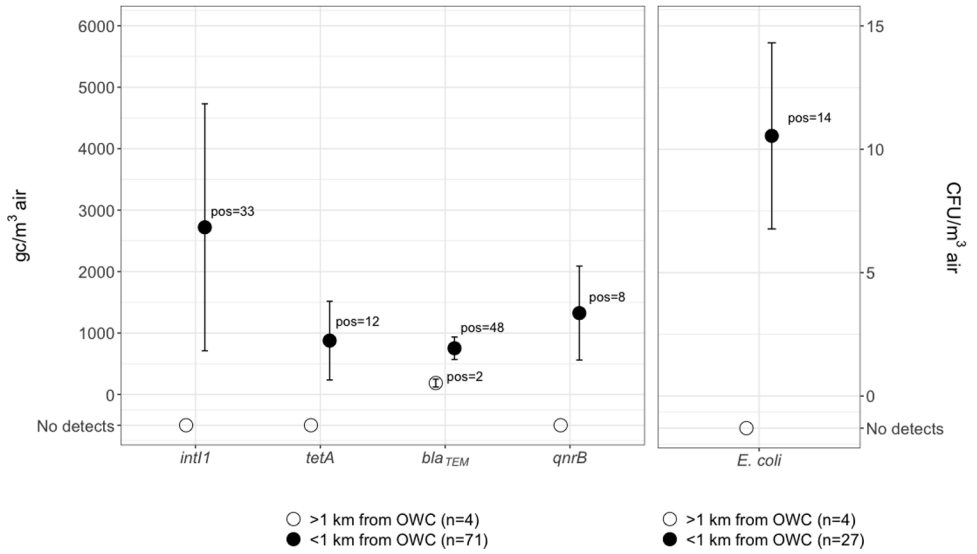

Figure 2.

Left: average ARG and MI densities with mean standard error bars for the distribution in gene copies per cubic meter of air, where targets were detected at levels equal to or above the LOD. Right: mean culturable *E. coli* per cubic meter of air with mean standard error bars for the distribution in coliforming units per cubic meter of air.

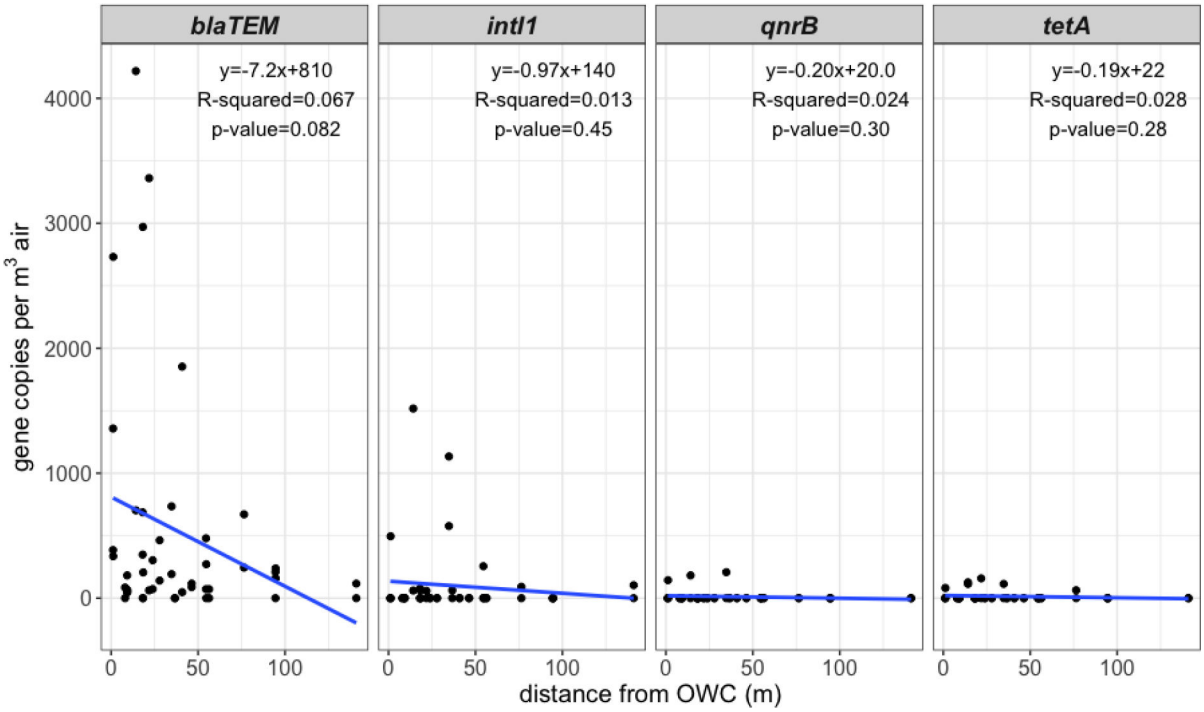

Figure 3.

ARG density in gene copies per m^3 of air in relation to the distance from OWCs. The blue line indicates the linear regression calculation for each data set and the data is not separated by time of day. Each plot includes the corresponding regression line equation, R-squared value, and p-value.

Table 1.

Forward and reverse primers and probes for each assay are listed. Reaction mixes were set to a total volume of 20 μL, containing a primer concentration of 900 nM, probe concentration of 250 nM and 1X Supermix for Bio-Rad's QX200TM Droplet Digital PCR system (Bio-Rad, Hercules, CA, USA). We used the ddPCRTM Supermix for Probes for all targets except blaTEM, for which we used ddPCRTM Supermix for Residual DNA Quantification due to the known presence of residual sequence in less purified commercial supermixes (Chiang et al., 2005). On each ddPCR™ plate for all assays, we included 2 positive control wells using a gBlock™ (IDT, Coralville, Iowa, US) containing all of the target ARG sequences in its length, diluted to approximately 10^3 gc/uL of reaction mixture. For reproducibility, the positive control sequence is also included in a supplementary file. Additionally, we included at least 2 no template controls using molecular water to control for contamination via human or other error and to assess the rate of false positives. For 2 replicates of each sample extract, we quantified gene copies of each target in the ddPCR reaction mixture (2 μL extract, 21 μL of ddPCR reagents) and averaged the results together.

Gene target	Primers	Probes	Limit of Detection (gc/uL ddPCR reaction mix)	Cycling conditions
tetA	F: CCGCGCTTTGGGTCATT R: TGGTCGCGTCCCAGTGA	FAM-TCGGCGAGGATCG-BHQ1	0.19	95°C for 10 min 45 cycles of 95°C for 30 s and 56°C for 1 min 98°C for 10 min
qnrB	F: CAGATTTYCGCGGCGCAAG R: TTCCCACAGCTCRCAYTTTTC	FAM-CGCACCTGGTTTTGYAG YGCMTATATCAC-BHQ1	0.24	95°C for 10 min 45 cycles of 95°C for 30 s and 56°C for 1 min 98°C for 10 min
bla $_{TEM}$	F: CACTATTCTCAGAATGACTTGGT R: TGCATAATTCTCTTACTGTCATG	FAM-CCAGTCACAGAAAAGCATCTTA CGG-BHQ1	0.12	95°C for 10 min 45 cycles of 95°C for 30 s and 56°C for 1min 98°C for 10 min
intI1	F: GCCTTGATGTTACCCGAGAG R: GATCGGTCGAATGCGTGT	6HEX-ATTCCTGGCCGTGGTTCTGGG TTTT-BHQ1	0.10	95°C for 10 min 45 cycles of 95°C for 30 s and 57°C for 1 min 98°C for 10 min
Positive Control	ACTTGTCGGACAGGTGCCGGCCGCGCTTTGGGTCATTTTCGGCGAGGATCGCTTTCACTGGGAC GCGACCACGATCGGCATTTCGCTTGCCGAAATCCTTCTTGGGCGCCACCGTTGGCCTTCCTGTAA AGGATCTGGGTCCAGCGAGCCTTGCGGCGGAACTTCACGCGATCGGCAATGGCGCTGACTACGT CCGCATGGGCACCCATCCAACGGTTTTCCCACAGCTCACACTTTTCCAACACGACTTTCGAAAAA TTGGCGTAGCTTAGATTGGTATTCGTGATATATGCGCTACAAAACCAGGTGCGCGTGGTGATCAT ATTCATAAAGCTTGCGCCGCGGAAATCTGCGCCTTGTGCGCGGCAGTGGAGCAACTCGGTCGCC GCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGAT GGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATCGGCGAGTTCTTGGGA TGGCAGGCGATATTCATTACTTTGGCTATACTGGCGATGCTCGCACTCCTAAATGCGGGTTTCAG GTGGCACGAAACCCGCCCTCTGGATCAAGTCAAGACGCGCCGATCTGTCTTGCCGATCTTCGCG AGTCCGGCTTTTTGGGTTTACACTGTCGGCTTTAGCGCCGGTATGGGCACCTTCTTCGTCTTCTTC TCGACGGCTCCCCGTGTGCTCATACGCACGACACCGCTCCGTGGATCGGTCGAATGCGTGTGCTG CGCAAAAACCCAGAACCACGGCCAGGAATGCCCGGCGCGCGGATACTTCCGCTCAAGGGCGTCG GGAAGCGCAACGCCGCTGCGGCCCTCGGCCTGGTCCTTCAGCCACCATGCCCGTGCACGCGACAGC TGCTCGCGCAGGCTGGGTGCCAAGCTCTCGGGTAACATCAAGGCCCGATCCTTGGAGCCCTTGC			

Table 3.

Summary of positive detections in all air samples after applying the experimentally determined LOD.

	Detections in samples above experimentally determined LOD, n (%)	
	Samples <1 km from OWCs (n=71)	**Samples >1 km from OWCs (n=4)**
intI1	33 (47%)	0 (0%)
qnrB	8 (11%)	0 (0%)
tetA	12 (17%)	0 (0%)
bla_{TEM}	48 (68%)	2 (50%)