A clinically important, plasmid-borne antibiotic resistance gene (β-lactamase TEM-116) present in desert soils

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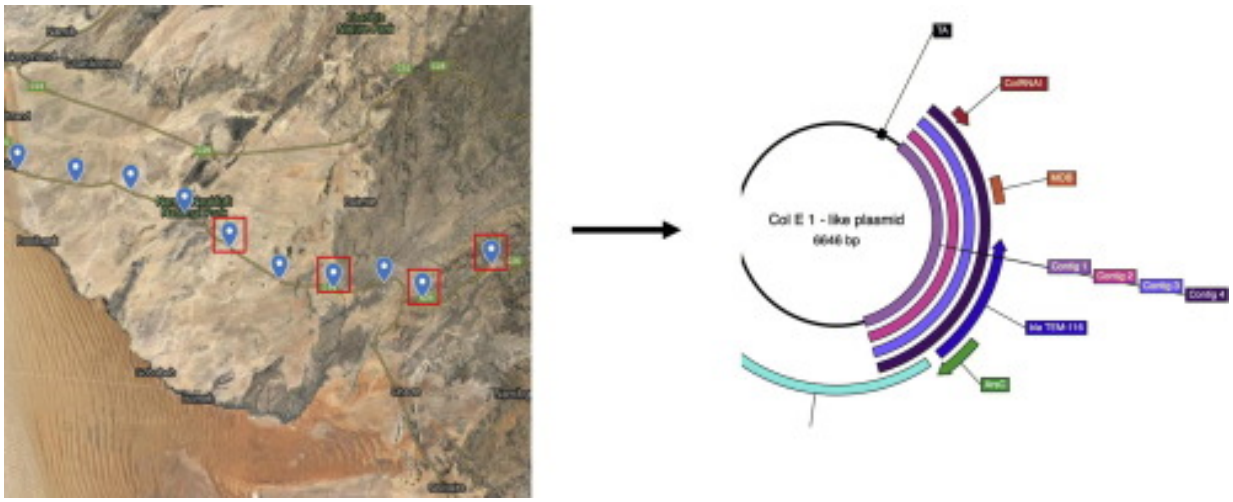
Highlights

• Shotgun metagenomics was used to investigate antibiotic resistance genes (ARGs) in desert soils.
• A clinically relevant plasmid-borne ARG was detected.
• The plasmid also carries a metal resistance gene and a P7 entero-bacteriophage.
• These findings have important implications for the One Health initiative.

Abstract

The exhaustive use of antibiotics in humans, animal farming and other agricultural practices has resulted in the frequent appearance of antibiotic resistant bacteria in human-impacted habitats. However, antibiotic resistance in natural (less-impacted) habitats is less understood. Using shotgun metagenomics we analysed soils from relatively low anthropogenic impact sites across the Namib Desert. We report the presence of a clinically significant extended spectrum β-lactamase (TEM-116), on a ColE1-like plasmid also carrying a metal resistance gene (arsC). The co-occurrence of resistance to antimicrobial drugs and metals encoded on a single mobile genetic element increases the probability of dissemination of these resistance determinants and the potential selection of multiple resistance mechanisms. In addition, the presence of a P7 entero-bacteriophage on the same plasmid, may represent a new vehicle for the propagation of TEM-116 in these soil communities. These findings highlight the role of the environment in the One Health initiative.

Graphical abstract



Keywords: Metagenomics; Low anthropogenic impact; Namib desert; Mobile genetic element

1. Introduction

The One Health approach refers to the multidisciplinary collaboration on a local, national and global scale to ensure optimal health for people, animals and the environment. It recognises that antimicrobial resistance (AMR) has clear links to all components of this triad (Robinson et al., 2016). Most initiatives in understanding and combatting AMR have focused solely on human and animal sectors, while the extent of antibiotic resistance in the environment is poorly understood (Essack, 2018; Finley et al., 2013). However, recent studies have reported that many of the resistance genes found in clinical pathogens originate from soil-borne bacteria (Finley et al., 2013; Martínez et al., 2015) and that soils act as a reservoir of resistance genes (Larsson et al., 2018). The potential mobilization (e.g., through plasmids and other mobile elements) of resistance genes within the soil reservoir and the extent to which soils provide transmission routes for the propagation of resistance determinants to humans and animals is therefore a matter of immediate concern (Bengtsson-Palme et al., 2017; Bengtsson-Palme and Larsson, 2016).

The persistent exposure of bacterial strains to a multitude of β-lactam antibiotics has led to the production and mutation of β-lactamases (Bush, 2018; Pitout and Laupland, 2008), known as extended-spectrum β-lactamases (ESBLs). ESBLs are generally plasmid-borne and have the ability to induce host cell resistance to newer β-lactam antibiotics, specifically to third and fourth generation cephalosporins and monobactams, but not cephamycins and carbapenems (Ur Rahman et al., 2018). ESBLs were primarily found in *Klebsiella pneumonia* and *Escherichia coli* in the 1980's, but have since been reported in several other species of bacteria, including *Pseudomonas*, *Shigella* and *Salmonella* (Mesa et al., 2006; Tissera and Lee, 2013). More recently, with the application of metagenomics screening methods, ESBLs have been reported to be ubiquitous in different natural environments, such as ancient soils (Allen et al., 2009; Perron et al., 2015), deep terrestrial sub-surfaces (Brown and Balkwill, 2009), isolated caves (Bhullar et al., 2012) and in cold deserts (Segawa et al., 2013; Wei et al., 2015). However, little is known about the presence of ESBLs in hot deserts, which represent 20% of the global land area (Ayal, 2007), are major and consistent sources of long-

distance airborne particulates (Belov et al., 2018) and therefore of particular significance as reservoirs of these genes.

TEM-116 is an ESBL characterized by two amino acid substitutions at residues 84 and 184, (Usha et al., 2008) and thought to have evolved directly from the first-discovered plasmid mediated β-lactamase, TEM-1 (Usha et al., 2008). It has been demonstrated that TEM-116 has given rise to over 50 TEM variants (Jacoby and Bush, 2016; Zeil et al., 2016), and has been postulated that TEM-116 acts as a second-generation progenitor of TEM variants. TEM-116 is widespread in clinical (Jeong et al., 2004; Naiemi et al., 2005; Usha et al., 2008; Vignoli et al., 2005) and non-clinical environments (Balsalobre et al., 2010; Forcella et al., 2010; Maravić et al., 2016; Mondal et al., 2019), including agricultural soils (Anand et al., 2016; Demaneche et al., 2008). The reports describing TEM-116 in soils, suggest that this ESBL is native to soil resistomes and is chromosomally mediated (Jacoby and Bush, 2016). In this study we provide evidence, for the first time, of the clinically relevant plasmid-borne TEM-116 in desert soils.

2. Materials and methods

2.1. Study site and sampling

The central Namib is a hyper-arid desert located on the south west coast of Africa. The Namib Desert has scarce and unpredictable rainfall patterns with rainfall increasing gradually from the coast, inland, producing a variable west-east rainfall gradient (Eckardt et al., 2013). Previously, anthropogenic influences across the desert have been minimal and were mostly limited to scientific expeditions. However, nature based tourism has increased drastically in the last decade (Dowling and Grunert, 2018), possibly increasing the anthropogenic pressure in the Namib desert. Surface soils (0 to 5 cm) were collected across this west-east transect on the 10th April 2018 (23°11′76.1″S 15°16′69.2″E). The transect spans three xeric zones (fog, arid and rainfall), samples were collected from the arid (n = 3) and rainfall (n = 3) zones. Four aliquots of 50 g of soil were taken at each site at 100 m spacing, using sterile methods and stored in sterile 50 ml polypropylene Falcon tubes (Grenier, Bio-One). Soils were stored at −80 °C for molecular analysis.

2.2. Sample preparation and DNA sequencing

Metagenomic DNA was extracted from the soil samples (n = 6), using the DNeasy Powersoil Kit (Qiagen) as per the manufacturers' instructions. Samples were submitted for sequencing at a commercial supplier (MR DNA Lab, Shallowater, TX, USA). Sequencing was performed on a HiSeq 2500 Ultra-High-Throughput Sequencing system (Illumina) using paired-ends (2 × 250 bp) for 500 cycles, as per the manufacturers' instructions.

2.3. Metagenomic assembly and acquired ARG identification

The number of reads per sample ranged from 9,868,998 to 11,726,910 (10,632,908 on average), with a sequence depth of 5GB. Raw reads were quality filtered using FastQC (Andrews, 2010), and trimmed using PrinSeq (Schmieder and Edwards, 2011). Reads were assembled using SPAdes v3.12.0 (Bankevich et al., 2012), with default settings and the “meta” parameter specified. The quality of each assembled metagenome (n = 6) was assessed using QUAST v5.0.2 (Mikheenko et al., 2018). The contigs were aligned using EPSript 3.0 (Robert and Gouet, 2014) to identify regions of similarity. To identify the acquired antibiotic

resistance genes, contigs were compared against the ResFinder (Zankari et al., 2012) database by means of BLASTn with an E-value threshold of 1×10^{-6} and the following parameters: minimum identity 87% and minimum match length 25 bp. Similar cut-off values were used in a study carried out in Antarctic soil (Van Goethem et al., 2018) and in a more recent study analysing antibiotic resistance in sewage impacted environments (Karkman et al., 2019). In this study, these values were used to identify genes that do not stretch over the entire contig.

2.4. Mobile genetic elements and taxonomic identification

To identify mobile genetic elements associated with ARGS, contigs were compared to the Mobile Genetic Elements Database (Pärnänen et al., 2018) by means of BLASTn with an E-value threshold of 1×10^{-6} and the following parameters: minimum identity 87% and minimum match length 25 bp. The plasmid detected was confirmed with the PlasmidFinder database (Carattoli et al., 2014) using the same parameters. To identify taxonomic markers associated with ARGs, gene prediction was performed using Prodigal v2.6.3 (Hyatt et al., 2010). The genes predicted by Prodigal were compared against the ProGenomes database (Mende et al., 2017) using blastp in DIAMOND v0.7.9.58 (Buchfink et al., 2014) at an E-value cut off of 1×10^{-5}. Only contigs larger than 1000 bp were annotated.

2.5. Phage identification and metal resistance gene annotation

To identify bacteriophage sequences, contigs were compared to the MetaPhinder database, which contains whole bacteriophage genome sequences. BLASTn was run with an E-value threshold of 1×10^{-6}. Metal resistance genes were detected by running BLASTp with these contigs against the BacMet database (Pal et al., 2014) using DIAMOND v0.7.9.58 at an E-value cut off of 1×10^{-6} and the following parameters: minimum identity 87% and minimum match length 25 bp. The raw sequence data and contigs are available on NCBI (PRJNA592367).

3. Results and discussion

We used de novo assembled shotgun metagenomic sequences (contigs) from six surface soil sample sites in the Namib Desert. Analysis using ResFinder (Zankari et al., 2012) revealed the presence of an acquired ESBL, TEM-116, at four of the sample sites (Table S1). The length of TEM-116 reported here was 861 bp and showed 100% similarity to the reference TEM-116 gene (accession no: AY425988.1) (Table S2). TEM-116 has been globally reported in a number of gram negative organisms, it was first identified in clinical strains of *K. pneumonia* and *E. coli* in Korea (Jeong et al., 2004). Thereafter, on conjugate plasmids in *E. coli* isolates in Uruguay (Vignoli et al., 2005) and later, in *Acinetobacter baumanii* of both clinical and non-clinical origin (Maravić et al., 2016; Naiemi et al., 2005), indicating the widespread dissemination of this ARG. TEM-116 has an extended spectrum of activity against several third and fourth generation cephalosporins (Song et al., 2005; Usha et al., 2008), for this reason it is considered an ESBL.

A comparison of our contigs (Fig. S1) against the Mobile Genetic Elements (Pärnänen et al., 2018) and PlasmidFinder (Carattoli et al., 2014) databases revealed the presence of a ColRNAI replicon (with a length of 131 bp and 90% similarity to the reference replicon) (Table S2). Mapping of the contigs to the Col E1 plasmid pIGMS32 (accession no: DQ298019) indicates that TEM-116 reported here is carried on a plasmid similar to pIGMS32 (Fig. 1). pIGMS32 was initially identified as a narrow host range plasmid.

However, it was later reported (Smorawinska et al., 2012) that this plasmid can contain different mobilization (MOB) modules which enables its dissemination among evolutionarily distinct bacterial species (that is, this plasmid has a wider host range than previously thought). Since then, pIGMS32 has been found in clinically relevant Gram-negative organisms, such as a *K. pneumoniae* strain implicated in a carbapenem-resistant infection outbreak (Espedido et al., 2013), and in *Serratia marcescens*, in a clonal multi-drug resistant outbreak (Moradigaravand et al., 2016). Consequently, it has been suggested that pIGMS32 is a carrier of resistance mechanisms (e.g., efflux pumps) of clinical significance (Ares-Arroyo et al., 2018), and of genes conferring resistance to several antibiotics including β-lactams (Papagiannitsis et al., 2015).

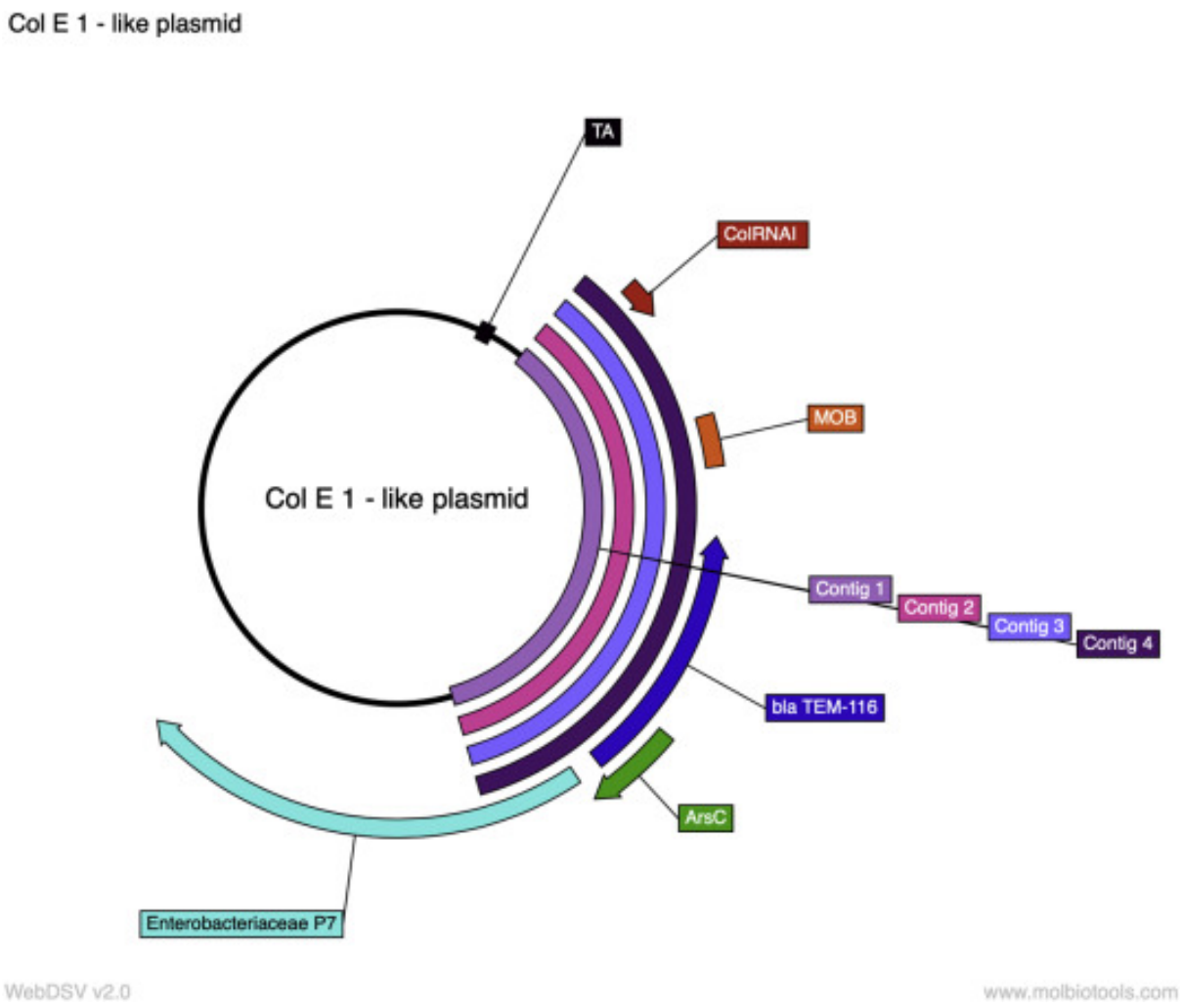


Fig. 1. Graphical representation of the part of the contigs (~90% of the total length) that contain the overlapping sequences for the ARG TEM-116, metal resistance gene ArsC and Bacteriophage P7 mapped to the ColE1-like plasmid profile (accession no: DQ298019). The ColE1 plasmid contains putative genetic modules (MOB: mobilization system, ColRNAI: origin of replication and TA: toxin-antitoxin stabilization system) which, are depicted in the figure. The figure showing the plasmid profile was determined using WebDSV2.0 and can be found at (www.molbiotools.com).

Gene transfer and acquisition in nature occurs mostly between taxonomically homogenous groups (Courvalin, 1994; Kelly et al., 2009). However, the taxonomic annotation of all TEM-116 identified in the Namib Desert indicated that TEM-116 was found in *Rhodococcus ruber* strain Chol-4. *Rhodococcus* strains are considered good candidates for processes such as

bioremediation and biocatalysis since they display wide metabolic versatility with an increased tolerance to varying stress conditions (Guevara et al., 2019). The Chol-4 strain in particular is a model organism for studying biodegradation of steroids to produce pharmaceutically active steroid drugs (Guevara et al., 2019). As far as we know, *R. ruber* has not been previously associated with antibiotic resistance to β-lactams. The presence of this ESBL in *Rhodococcus ruber*, a Gram-positive bacterium, indicates an "inter-Gram" gene transfer event. The first report of inter-Gram transfer was in 1987, where a plasmid present in *E. coli* was successfully transferred to several Gram-positive bacterial strains (Trieu-Cuot et al., 1987). Initially, it was thought that this transfer could only occur via conjugation (Kelly et al., 2009), but most recently it has been demonstrated that it can also occur via transformation (Jiang et al., 2017). The presence of TEM-116 in a gram-positive organism implies that inter-Gram transfer in soil bacteria may be more common than previously thought (Popa et al., 2011). Soils are highly diverse and can act as hot spots for gene acquisition from phylogenetically distant groups (Popa et al., 2011), and therefore, facilitate a direct connection of a large part of the bacterial gene pool (Klümper et al., 2015).

Antibiotic resistance genes have been reported as wide-spread in both hot and cold deserts (Belov et al., 2018; Fierer et al., 2012; Van Goethem et al., 2018), and the ubiquity of TEM-116 in soil environments cannot be disputed (Jacoby and Bush, 2016). However, to the best of our knowledge, resistance genes of clinical relevance on conjugative plasmids have not been reported in desert soils. Hence, the presence of TEM-116 on a conjugative plasmid in this desert soil is unexpected since such soils are thought to be subject to low anthropogenic impact. However, the Namib Desert harbors highly transient populations of wildlife (Hedman et al., 2014), such as Springbok (*Antidorcas marsupialis*), Oryx (*Oryx gazella*), and several native and migratory species of birds (Stein et al., 2008). Consequently, these animals and birds in such natural environments are potentially at risk for the acquisition of antibiotic resistant bacteria (Allen et al., 2010; Literak et al., 2010). Although the routes of transmission are difficult to establish, direct contact with domestic livestock, via sharing drinking water and food sources, may be highly significant in transmission flows (Radhouani et al., 2014; Van den Honert et al., 2018). The spread of environmental ARGs mediated specifically by migratory birds, has been well recognized (Wellington et al., 2013; Wu et al., 2018). We note that, following sporadic rain events in the Namib Desert and the resulting growth and seeding of the dominant grass species *Stipagrostis ciliate* (Dean and Williams, 2004), very large numbers (millions) of Gray-backed Sparrow-larks (*Eremopterix verticalis*) invade the region. As ground-feeders, these populations have the capacity to support regional transport of soil microorganisms (and ARGs) via fecal deposits. High proportions of resistance determinants have been detected in feces of migratory birds across countries (Foti et al., 2011; Guenther et al., 2012; Radhouani et al., 2012), including pristine areas (Hernández and González-Acuña, 2016; Rabbia et al., 2016).

There is strong evidence that levels of human and animal fecal pollution correlate positively with the presence of antibiotic resistant organisms (Karkman et al., 2019; Sapkota et al., 2007). However, these studies are largely limited to aquatic environments, which are highly susceptible to anthropogenic contamination (Karkman et al., 2019). Detecting markers for fecal indicator bacteria (FIB) using metagenomics is problematic because of the low abundance of these marker bacteria in microbial communities (Bengtsson-Palme et al., 2017; Karkman et al., 2019). An alternative approach is the use of bacteriophages as indicators of fecal pollution (Ballesté et al., 2019; McMinn et al., 2017; Muniesa et al., 2018). Using MetaPhinder (Jurtz et al., 2016) we detected enterobacteria S-13 phages in these soil bacterial

communities and a partial construct of an enterobacteria P7 phage encoded in the plasmid (Fig. 1).

The role of bacteriophages in the dissemination of ARGs in aquatic environments has recently been highlighted (Balcazar, 2014; Colavecchio et al., 2017; Ross and Topp, 2015). For example, the phage-mediated mobilization of quinolone resistance genes (qnrA) in urban wastewater (Colomer-Lluch et al., 2014) and their capacity to facilitate inter-species transfer of β-lactamase genes in hospital effluents (Marti et al., 2014) have both recently been reported. However, the extent to which bacteriophages facilitate the dissemination of ARGs in soil environments is currently unknown. S-13 bacteriophages are somatic coliforms which have been used as indicators of fecal contamination in domestic and municipal sewage as well as in primary and activated sludge (Syngouna and Chrysikopoulos, 2014). However, these phages have the ability to multiply in several species of Enterobacteriaceae, which means that their presence in these soil communities could be unrelated to fecal contamination (Leclerc et al., 2000). The P7 bacteriophage has the ability to replicate in its host as independent plasmid-like elements and reportedly harbors β-lactamases conferring resistance to various β-lactam antibiotics (Billard-Pomares et al., 2014). The presence of P7 on this Col E1-like plasmid may represent a new vehicle for the dissemination of TEM-116 in these communities. Although we cannot explicitly correlate the presence of wildlife to the impact of ARGs in the Namib Desert, our data does imply a link between fecal inputs and the acquired TEM-116 gene, probably mediated by birds.

Antibiotic resistance frequently co-occurs with resistance to heavy metals (Li et al., 2017). This is a result of the colocalization or comigration of genes conferring multiple resistance mechanisms (e.g., target inactivation, target protection, efflux pumps) (Li et al., 2017). Using the BacMet database (Pal et al., 2014), which contains metal resistance genes (MRGs), we demonstrated the presence of arsC genes (coding for arsenate reductase) in the Namib Desert soil metagenome contigs. The arsenate reductase reported here was 113 amino acid residues long (85% of the length of the reference arsC) with a 94% similarity to the reference protein (accession no: BAA24824.1) (Table S2). Interestingly, the arsC gene was encoded within the plasmid. Co-resistance to arsenic and β-lactams has been reported to frequently occur on the same mobile element (Pal et al., 2017). The co-selection potential of arsC and TEM-116 may have functional significance in this particular plasmid due to the presence of a toxin-antitoxin (TA) system (Fig. 1), which stabilizes the plasmid in the host by killing off any of the daughter cells that do not inherit the plasmid (Pal et al., 2015). Therefore, plasmids with ARGs and MRGs, together with a TA system would likely be more persistent in the environment even in the absence of antibiotic selective pressure (Di Cesare et al., 2016). Additionally, if resistance genes for both antibiotics and metals are physically located on the same plasmid, horizontal transfer of the entire gene cluster to other bacteria is likely (Knapp et al., 2011; Pal et al., 2015).

In summary, this work supports the evidence that clinically significant ARGs are widespread even in environments without obvious anthropogenic exposure to antibiotics. It is likely that in bacterial communities, the mobilization of existing resistance determinants happens continuously, although very few of these determinants are selected for and remain in the community. Consequently, resistance genes such TEM-116, which are already circulating between the clinic and the environment, may easily re-emerge in the clinic during antibiotic therapy further reducing treatment options. The likely exchange of resistance genes between soil dwelling bacteria and clinical pathogens highlights the crucial role of the environment in the emergence and re-emergence of these resistance determinants. The value of the One

Health approach is undisputed, considering the dynamics of ARGs as they move from clinical environments to natural environments.

CRediT authorship contribution statement

Yashini Naidoo Conceptualization, Investigation, Writing - original draft. **Angel Valverde:** Writing - review & editing, Supervision, Funding acquisition. **Errol D. Cason:** Software, Writing - review & editing. **Rian E. Pierneef:** Software, Validation, Writing - review & editing. **Don A. Cowan:** Supervision, Funding acquisition, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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