Phylogeographical Landscape of *Citrobacter portucalensis* Carrying Clinically Relevant Resistomes

Fábio P. Sellera,[a,b,c] Miriam R. Fernandes,[d] Bruna Fuga,[b,d,e] Herrison Fontana,[b,d] Felipe Vásquez-Ponce,[b,e] Daphne W. Goldberg,[f] Daniel F. Monte,[b,g] Larissa Rodrigues,[b,e] Adriana R. Cardenas-Arias,[b,e] Ralf Lopes,[e] Brenda Cardoso,[b,e] Daniela G. C. Costa,[h] Fernanda Esposito,[b,d] Nilton Lincopan[b,d,e]

[a]Department of Internal Medicine, School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, Brazil
[b]One Health Brazilian Resistance Project (OneBR), São Paulo, Brazil
[c]School of Veterinary Medicine, Metropolitan University of Santos, Santos, Brazil
[d]Department of Clinical Analysis, School of Pharmacy, University of São Paulo, São Paulo, Brazil
[e]Department of Microbiology, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brazil
[f]Aquatic Biodiversity Monitoring Program, Federal University of Rio Grande, Rio Grande do Sul, Brazil
[g]Department of Food and Experimental Nutrition, Faculty of Pharmaceutical Sciences, Food Research Center, University of São Paulo, São Paulo, Brazil
[h]Pró-Tamar Foundation, Ubatuba, São Paulo, Brazil

Fábio P. Sellera and Miriam R. Fernandes contributed equally to this article. Author order was determined by the corresponding author after negotiation.

ABSTRACT During a surveillance study conducted to assess the occurrence and genomic landscape of critical priority pathogens circulating at the human-animal-environment interface in Brazil, as part of the Grand Challenges Explorations-New Approaches to Characterize the Global Burden of Antimicrobial Resistance program, two multidrug-resistant (MDR) *Citrobacter portucalensis* carrying $bla_{CTX-M-15}$ extended-spectrum β-lactamase (ESBL) genes, isolated from green sea turtles, were characterized. Genomic and phylogeographical analysis of *C. portucalensis* genomes available in public databases revealed the intercontinental dissemination of clades carrying different arrays of clinically relevant genes conferring resistance to carbapenems, broad-spectrum cephalosporins, cephamycins, aminoglycosides and fluoroquinolones, disinfectants, and heavy metals. Our observations suggest that *C. portucalensis* could be emerging as critical priority bacteria of both public and One Health importance worldwide.

IMPORTANCE The global spread of antibiotic-resistant priority pathogens beyond the hospital setting is a critical issue within a One Health context that integrates the human-animal-environment interfaces. On the other hand, next-generation sequencing technologies along with user-friendly and high-quality bioinformatics tools have improved the identification of bacterial species, and bacterial resistance surveillance. The novel *Citrobacter portucalensis* species was proposed in 2017 after taxonomic reclassification and definition of the strain A60[T] isolated in 2008. Here, we presented genomic data showing the occurrence of multidrug-resistant *C. portucalensis* isolates carrying $bla_{CTX-M-15}$ ESBL genes in South America. Additionally, we observed the intercontinental dissemination of clades harboring a broad resistome to clinically relevant antibiotics. Therefore, these findings highlight that *C. portucalensis* is a global MDR bacteria that carries intrinsic bla_{CMY}- and *qnrB*-type genes and has become a critical priority pathogen due to the acquisition of clinically relevant resistance determinants, such as ESBL and carbapenemase-encoding genes.

KEYWORDS Enterobacterales, emerging pathogens, multidrug-resistant, international clade, critical priority, One Health, genomic surveillance

embers of the genus *Citrobacter* are part of the normal intestinal flora of humans and animals and have been isolated from a variety of environmental sources, including soil, water, and food; being considered opportunistic pathogens for humans (1, 2). The clinical significance of *Citrobacter* is due to its ability to easily acquire multiple resistance genes to antibiotics used in therapy. In this regard, some species of the genus carry intrinsic AmpC-type β-lactamase genes (i.e., *bla*$_{CMY}$-type), which can be related to mobile genetic elements (1, 3). Additionally, ESBLs and carbapenemases have been identified in *Citrobacter* spp., expanding the resistance to broad-spectrum cephalosporins and carbapenems. Currently, this genus comprises 15 species (https://lpsn.dsmz.de/genus/citrobacter) with the most recently described *Citrobacter* species being *C. portucalensis*, *C. europaeus*, and *C. cronae* (1, 4, 5). Although the novel *C. portucalensis* species was proposed in 2017, the strain A60^T was isolated in 2008 from a water well sample collected in Cantanhede city, Centre region of Portugal (1, 2). Consequently, by using genome data of *C. portucalensis* A60^T, the average nucleotide identity (ANI) results have led to change originally genomes submitted as *C. freundii* and *Citrobacter* spp., to *C. portucalensis* since 2018.

Antibiotic-resistant priority pathogens have globally disseminated beyond the hospital environment, threatening wildlife and ecosystems within a One Health context that integrates the human-animal-environment interface (6–8). During a nationwide surveillance study (One Health Brazilian Resistance Program, OneBR), conducted in Brazil to assess the occurrence and genomic features of World Health Organization (WHO) priority pathogens circulating at the human-animal-environment interface (6), two ceftriaxone-resistant Gram-negative bacilli isolated from cloacal swab samples collected from 2 green sea turtles affected by fibropapillomatosis (a debilitating neoplastic disease with immunosuppressive effects), were sequenced. Both green sea turtles were admitted to a rescue and rehabilitation center in Southeastern Brazil (23°27'08.0"S, 45°04'13.6"W). Isolates were recovered from MacConkey agar plates supplemented with ceftriaxone (2 μg/mL) and incubated at 37℃ overnight. Bacterial identification and antimicrobial susceptibility testing were performed by Vitek 2 system (bioMérieux, USA), disc diffusion, Etest, and/or agar dilution methods (9). Colistin MIC was determined by broth microdilution method, according to EUCAST (http://www.eucast.org). Extended-spectrum β-lactamase (ESBL) production was screened by the double-disk synergy test (DDST) (10).

For whole-genome sequencing (WGS), genomic DNAs were extracted using the PureLink Quick Gel Extraction kit (Life Technologies, Carlsbad, CA). DNA concentrations were evaluated using a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA). Genomic libraries were constructed using a Nextera XT DNA library preparation kit (Illumina Inc., Cambridge, UK) and genomic DNA was sequenced by the Illumina NextSeq 500 platform, using paired-end reads (150 bp). Short-read sequence data were *de novo* assembled using CLC Genomics Workbench v.10. Unassembled reads and contigs less than 200-bp long were removed from the genome. The sequences were annotated using PGAP v.3.2 (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/), and ANI was based on the NCBI report (https://www.ncbi.nlm.nih.gov/) and by using DFAST (https://dfast.ddbj.nig.ac.jp/). Resistome and plasmidome were evaluated using ResFinder 4.1 and PlasmidFinder 2.1 databases (http://www.genomicepidemiology.org/). Disinfectants (quaternary ammonium compounds [QACs]), pesticides, and heavy metal resistance genes were predicted using ABRicate (https://github.com/tseemann/abricate) and comparing the contigs against the NCBI database (https://www.ncbi.nlm.nih.gov). Genes were predicted using a coverage and identity threshold $>$ 80%. Sequence types were predicted using the *Citrobacter* spp. Multilocus sequence type (MLST) scheme (https://pubmlst.org/organisms/citrobacter-spp). Phylogenomic analysis was performed using publicly available genomic data (https://www.ncbi.nlm.nih.gov) from 69 *C. portucalensis* strains isolated from different sources and countries (Table S1). In this regard, CSI Phylogeny v.1.4 (https://cge.cbs.dtu.dk/) was used for SNP-based phylogenetic tree inference using the *C. portucalensis* FDAARGOS_617 complete chromosome sequence
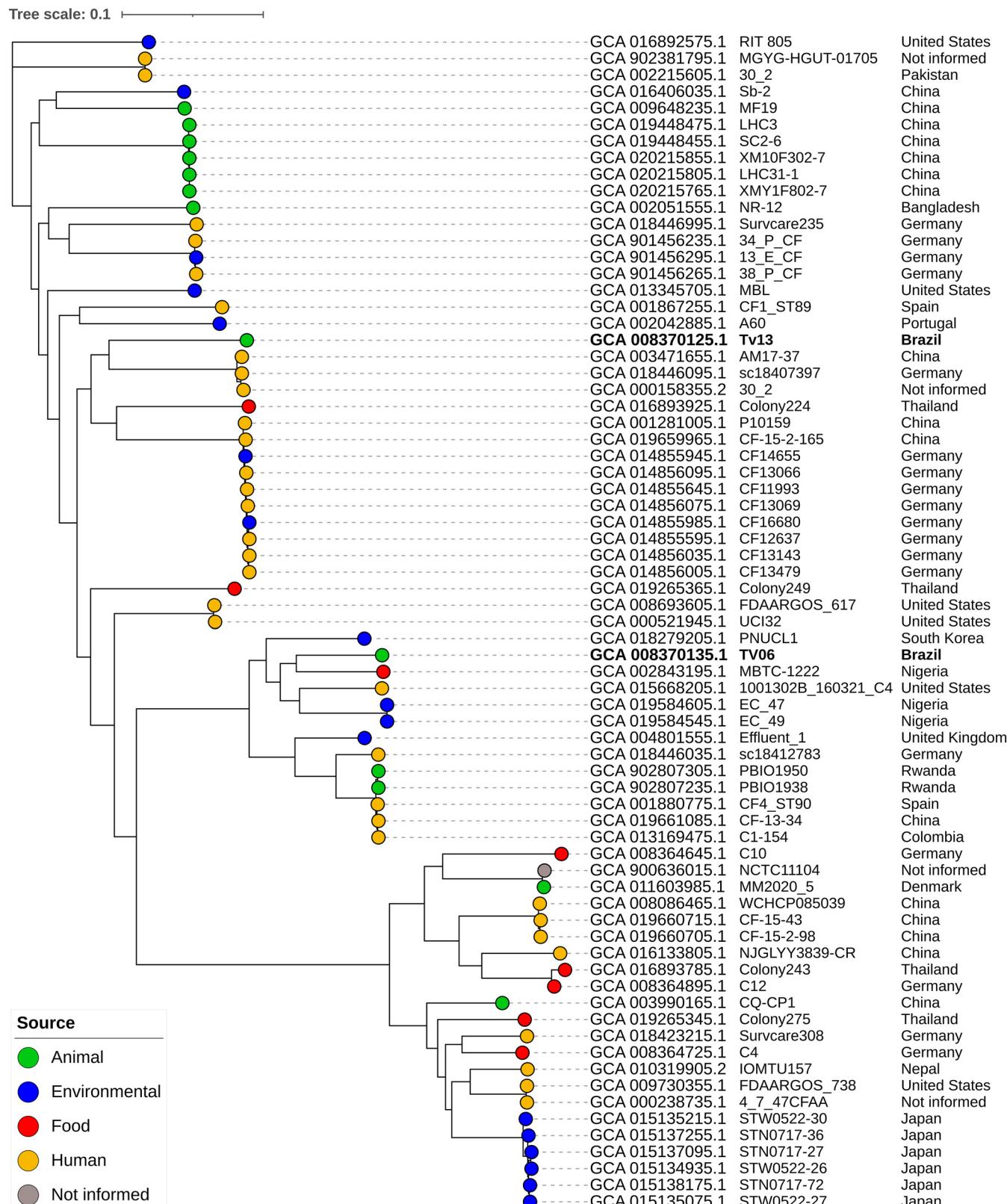
FIG 1 SNP-based phylogenetic analysis of CTX-M-15-positive *Citrobacter portucalensis* strains TV06 (GenBank accession number: VTZD01000001.1) and TV13 (GenBank accession number: VTZC01000001.1) identified in this study, and 69 previously sequenced, assembled, and annotated *C. portucalensis* human and nonhuman *C. portucalensis* strains identified in European, Asian, North American, South American, and African countries (Table S1). Phylogenetic relationships were inferred using CSI Phylogeny 1.4 (https://cge.cbs.dtu.dk/). The *C. portucalensis* FDAARGOS_617 complete chromosome sequence (GenBank accession

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(GenBank accession number: CP044098.1) as reference. Tree topology visualization and annotation were performed with iTol v.6 (https://itol.embl.de/).

Initially, cephalosporin-resistant isolates TV06 and TV13 were identified as *Citrobacter* spp. by Vitek 2 system, being further investigated by WGS. In this regard, genomes of TV06 and TV13 strains were 5,357,700-bp and 5,414,211-bp in size with 126 and 113 of average coverage and 51.98% and 51.54% GC content, respectively; and both presented an average read length of 150-bp. Additionally, genome sequences of TV06 (GenBank accession number: VTZD01000001.1) and TV13 (GenBank accession number: VTZC01000001.1) strains shared 98.37% and 98.53% average nucleotide identity with the genome of *C. portucalensis* strain A60^T (GenBank accession number: MVFY01000001.1), respectively. Thus, these sequences were assigned to the *C. portucalensis* species by the NCBI taxonomy staff.

Both strains exhibited a multidrug-resistant profile (i.e., resistance to at least one agent in three or more antimicrobial categories) (11), against amoxicillin-clavulanic acid, aztreonam, ceftriaxone (MIC > 32 μg/mL), cefotaxime, ceftazidime (MIC $\geq$ 64 μg/mL), cefepime (MIC $\geq$ 32 μg/mL), cefoxitin (MIC $\geq$ 64 μg/mL), gentamicin (MIC $\geq$ 64 μg/mL), nalidixic acid, ciprofloxacin (MIC $\geq$ 4 μg/mL), and trimethoprim-sulfamethoxazole ($\geq$320, 16/304). On the other hand, both strains showed an ESBL phenotype, remaining susceptible to carbapenems (ertapenem, MIC $\leq$ 0.12 μg/mL; imipenem, MIC $\leq$ 0.25 μg/mL; and meropenem, MIC $\leq$ 0.25 μg/mL), fosfomycin (MIC $\leq$ 4 μg/mL), and colistin (MIC = 0.5 μg/mL).

Results from WGS analysis are summarized in Table S1. The resistome of both *C. portucalensis* strains was composed of genes conferring resistance to β-lactams ($bla_{CTX-M-15}$, $bla_{CMY-129}$-like, bla_{TEM-1B}, bla_{OXA-1}), aminoglycosides, quinolones, sulfonamides, and trimethoprim. *C. portucalensis* TV06 harbored additional resistance genes to phenicols, fosfomycin, and tetracyclines. On the other hand, genes conferring resistance to heavy metals (arsenic, mercury, silver, and tellurium), disinfectants (quaternary ammonium compounds [QACs]), and pesticides (glyphosate) were detected in both genomes (Table S1). Plasmid analysis revealed the presence of IncHI2 in *C. portucalensis* TV06, and IncHI1, IncFII, and IncFIB plasmids in the strain TV13. In this regard, *in silico* analysis mapping the contigs against paired-end short reads along to BLASTn analysis revealed that $bla_{CTX-M-15}$ genes were carried by IncHI and IncFII plasmids in *C. portucalensis* TV06 and TV13, respectively (Table S1). Unfortunately, because we used short-read sequencing technology, it was not possible to assemble the complete plasmids.

Although, MLST analysis revealed that the TV13 strain belonged to ST63, it was not possible to predict the ST of the TV06 strain. SNP-based phylogenetic analysis grouped CTX-M-15-positive TV06 and TV13 strains in two distinct clades, both related to *C. portucalensis* strains isolated from human and nonhuman sources in distinct geographic regions (Fig. 1). The matrix of SNP-based phylogeny analysis is quoted in Table S2. Specifically, the TV06 strain clustered (14549 to 15041 SNP differences) with *C. portucalensis* strains MBTC-1222 (isolated from food in Nigeria; assembly accession number: GCA_002843195.2), 1001302B_160321_C4 (isolated from a human in the USA; assembly accession number: GCA_015668205.1), and EC_47 and EC_49 (isolated from environmental sources in Nigeria; Assembly accession numbers: GCA_019584605.1 and GCA_019584545.1) (Fig. 1, Table S2). On the other hand, TV13 was clustered (21254 to 21829 SNP differences) with the human strains AM17-37 and sc18407397, identified in China and Germany, respectively (assembly accession numbers: GCA_003471655.1 and GCA_018446095.1); the food strain Colony224 from Thailand (GCA_016893925.1); human strains P10159 and CF-15-2-165 from China (Assembly accession numbers: GCA_001281005.1 and GCA_019659965.1); and CF14655, CF13066, CF11993, CF13069, CF16680, CF12637, CF13143 and CF13479 (Assembly accession numbers: GCA_014855945.1, GCA_014856095.1, GCA_014855645.1,

FIG 1 Legend (Continued)

number: CP044098.1) was used as the reference genome. The phylogenetic tree was visualized by using iTOL v.6 (https://itol.embl.de/). For each *C. portucalensis* isolate the assembly accession number, strain name, and the country is quoted. The scale bar refers to branch lengths and indicates the number of substitutions per site based on 3303375 nucleotide positions (67.04% of the reference genome covered by all isolates) found in all analyzed genomes, using 10 bp as the minimum distance between SNPs. The minimum and maximum SNP differences among all *C. portucalensis* isolates were 0 and 40262, respectively.
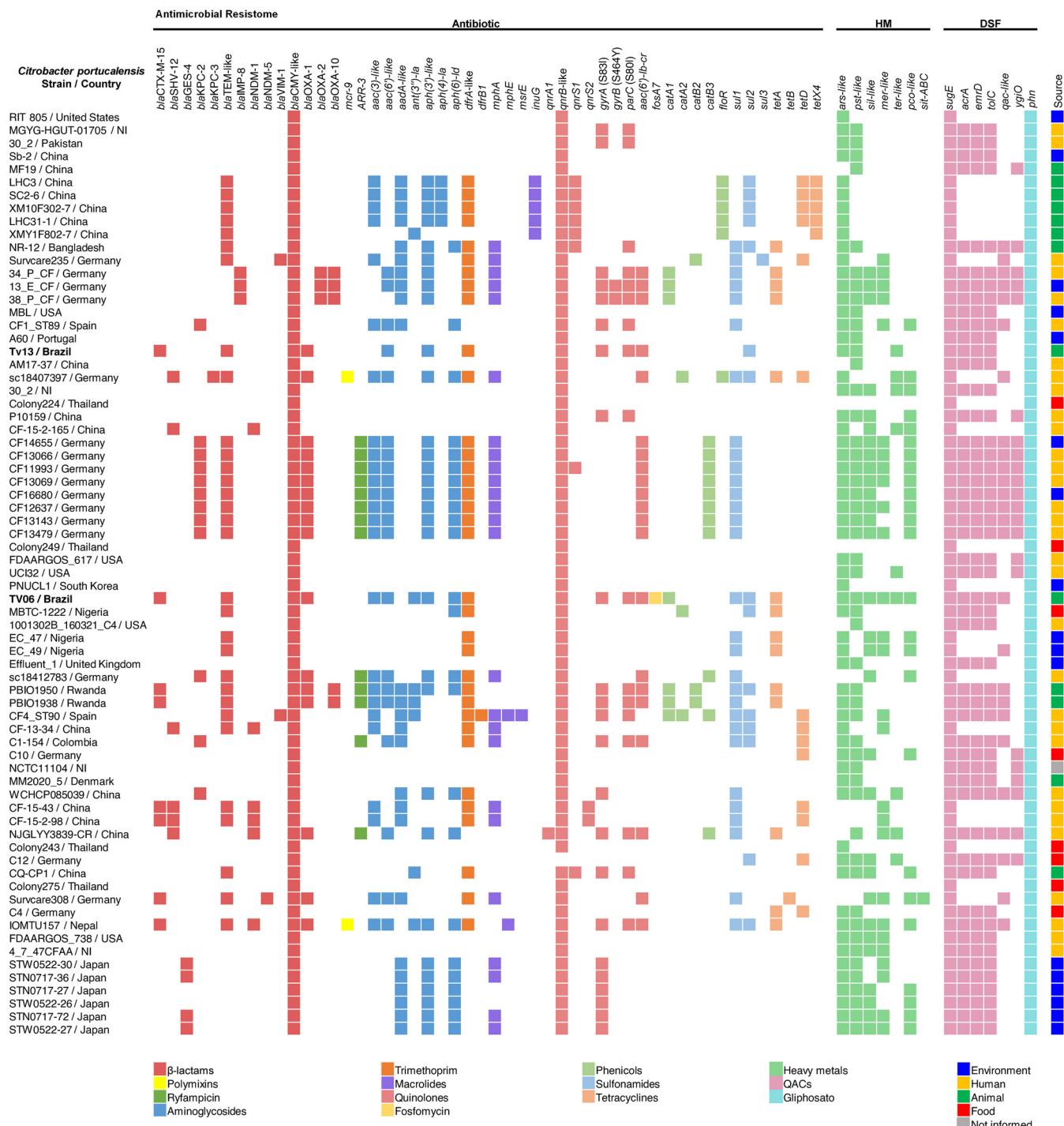
FIG 2 Heatmap showing the antimicrobial resistome of *Citrobacter portucalensis* strains isolated from human and nonhuman sources worldwide (Table S1). HM, heavy metal resistome. DSF, disinfectant resistome. NI, not informed.

GCA_014856075.1, GCA_014855985.1, GCA_014855595.1, GCA_014856035.1, and GCA_014856005.1) strains isolated from human and environmental sources in Germany.

Remarkably, *C. portucalensis* global strains harbor genes encoding resistance to critically important antimicrobials, including carbapenems (bla_{VIM-1}, bla_{NDM-1}, bla_{NDM-5}, bla_{IMP-8}, bla_{GES-4}, bla_{KPC-2} and bla_{KPC-3}), broad-spectrum cephalosporins ($bla_{CTX-M-15}$, bla_{SHV-12}, and bla_{OXA}-like), cephamycins (intrinsic bla_{CMY-2}-, bla_{CMY-4}-, bla_{CMY-13}-, bla_{CMY-25}-, bla_{CMY-26}-, bla_{CMY-34}-, bla_{CMY-35}-, bla_{CMY-39}-, bla_{CMY-46}-, bla_{CMY-49}-, bla_{CMY-53}-, bla_{CMY-63}-, bla_{CMY-71}-, bla_{CMY-86}-,

$bla_{CMY-124}$-, $bla_{CMY-127}$- and $bla_{CMY-129}$-like), quinolones (*qnrA1, qnrB1, qnrB2, qnrB6, qnrB9, qnrB13, qnrB15, qnrB17, qnr18, qnrB19, qnrB23, qnrB29, qnrB30, qnrB32, qnrB54, qnrB57, qnrB58, qnrB76, qnrB77, qnrS1, qnrS2, and/or aac[6']lb-cr*), polymyxins (*mcr-9*), and amino-glycosides (Fig. 2, Table S1).

Strikingly, we identified that both TV06 and TV13 isolates harbored the clinically relevant $bla_{CTX-M-15}$ gene, which confers resistance to expanded spectrum cephalosporins, being on the spotlights due to its rapid spread among clinical and environmental members of Enterobacterales (12, 13). CTX-M-15 producers have gained medical attention as important pathogens responsible for life-threatening infections, being frequently associated with MDR profiles (12, 13). Noteworthy, we have recently identified CTX-M-15-producing *Enterobacter hormaechei* and *C. freundii* coinfecting a free-living green turtle (*C. mydas*) (14), supporting that CTX-M-15 producers also represent a threat for wildlife.

After having your updated name formally recognized, *C. portucalensis* has been further isolated from uziza leaves in Nigeria (15), nasal and urine samples in dogs from Japan (3), urinary tract infection in a cat from Austria (16), poultry dropping samples from Bangladesh (17), soil from USA (18), and human stool and sputum samples in China (19, 20). Moreover, from genome data deposited in the NCBI database, vegetables, humans, animals, and the environment have been registered as sources of isolation of *C. portucalensis* in Bangladesh, China, Colombia, Denmark, Germany, Japan, Nepal, Nigeria, Pakistan, Portugal, Rwanda, South Korea, Spain, Thailand, United Kingdom and USA (Table S1).

In this study, we report the emergence of CTX-M-15-producing *C. portucalensis* in marine wildlife, in South America. In this regard, green turtles (*C. mydas*) are endangered marine animals that have a global distribution, occurring throughout tropical and subtropical waters (https://www.iucnredlist.org/species/4615/11037468). Due to their highly migratory behavior, green turtles have been investigated as bioindicators of polluted ecosystems contaminated with MDR bacteria (21). Therefore, the occurrence of this sort of bacteria in natural environments can have serious ecological implications (22), adding another layer of difficulty to marine wildlife conservation strategies. On the other hand, considering that *C. portucalensis* is closely related to *C. freundii* (an important nosocomial pathogen) (1), continuous surveillance is required to provide more information about the global dissemination of this emergent species and its adaptation to different hosts.

In summary, our findings highlight that *C. portucalensis* is a global MDR pathogen with intrinsic genes encoding AmpC enzymes active on cephamycin (CMY), which has become a critical priority specie due to the acquisition of clinically significant resistance determinants, such as plasmid-mediated bla_{CTX-M}-type ESBL and carbapenemase-encoding genes (i.e., bla_{GES}, bla_{VIM}, bla_{NDM}, bla_{IMP}, and bla_{KPC}). Finally, because marine animals have been overlooked in the epidemiology of critically important pathogens, more research focusing on the transmission pathways of ESBL-producing bacteria in marine environments is required to understand the clinical and epidemiological impacts on their populations.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.
SUPPLEMENTAL FILE 1, XLSX file, 0.03 MB.
SUPPLEMENTAL FILE 2, XLSX file, 0.04 MB.

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