Global evolutionary epidemiology and resistome dynamics of *Citrobacter species, Enterobacter hormaechei, Klebsiella variicola, and Proteeae clones*

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Tweet: Global *E. hormaechei, C. freundii, P. mirabilis, P. stuartii* & *P. rettgeri* strains contain multiple resistance mechanism to important and reserved antibiotics in globally circulating clones. These portend the dawn of pandrug resistance and a return to the pre-antibiotic era.

Running head: Global phylogenomics of resistant Enterobacterales

Author summary

Citrobacter spp., *Enterobacter hormaechei subsp., Klebsiella variicola* and *Proteae* tribe members are rarely isolated Enterobacterales increasingly implicated in nosocomial infections. Herein, we show that these species contain multiple genes encoding resistance to important antibiotics and are widely and globally distributed, being isolated from human, animal, plant, and environmental sources in 67 countries. Certain clones and clades of these species were internationally disseminated, serving as reservoirs and mediums for the global dissemination of antibiotic resistance genes. As they can easily transmit these genes to more pathogenic species, additional molecular surveillance studies should be undertaken to identify and contain these antibiotic-resistant species.

Abstract

Background. The global epidemiology and resistomes dynamics of multidrug-resistant *Citrobacter* spp., *Enterobacter hormaechei, Klebsiella variicola, Morganella morganii, Proteus mirabilis* and *Providencia* spp. have not been described, despite their importance as emerging opportunistic clinical pathogens.

Methods. The genomes of the above-mentioned organisms were curated from PATRIC and NCBI and used for evolutionary epidemiology, phylogeography and resistome analyses. The phylogeny trees were drawn using RAxML and edited with Figtree. The resistomes were curated from GenBank and the phylogeography was manually mapped.

Results and conclusion. Mcr-9 and other mcr variants were common in *E. hormaechei subsp.* and substantial in *C. freundii* whilst KPC, OXA-48, NDM, IMP, VIM, TEM, OXA and SHV were abundant in global *E. hormaechei subsp., Citrobacter freundii, P. mirabilis, P. stuartii* and *P. rettgeri* clones/clades; these resistance genes were mainly borne on IncF, IncX, IncN, IncH, Inc, IncM, A/C, and Col plasmids. Species-specific ampCs were highly conserved in respective species whilst fluoroquinolones, aminoglycosides, macrolides, fosfomycin, chloramphenicol, tetracycline, sulphamethoxazole and trimethoprim resistance mechanisms were abundantly enriched in almost all clades of most of the species, making them extensively and pandrug resistant; *K. variicola, C. amalonaticus* and *C, koseri* had relatively few resistance genes. Resistome similarities as well as local and international dissemination of strains evolving from common ancestors were observed, suggesting the anthroponotic, zoonotic, and food-/water-borne infectiousness of these pathogens. There is a global risk of pandrug resistant strains escalating local and international outbreaks of antibiotic-insensitive infections, initiating the dawn of a post-antibiotic era.

Keywords: Resistome; epidemiology; *Citrobacter; Klebsiella variicola; Morganella; Proteus; Providencia; Enterobacter hormaechei*

Introduction

Antibiotic resistance is mainly disseminated via horizontal and vertical transmission through mobile genetic elements such as plasmids, insertion sequences, integrons and transposons and through clonal and multiclonal expansion of same species [1–6]. Conjugative plasmids have been implicated in the transmission of several resistance determinants within and across species, resulting in the presence of same or very similar resistomes in same and different species and clones [1,4,7–9]. Thus, the emergence of plasmid-borne resistance genes is always a cause for concern as they help breach the species barrier and shuttle resistance genes (ARGs) from commensals and non-pathogenic bacteria to pathogenic ones or vice versa [3,4,10,11]. Such has been the case with the emergence and rapid spread of extended-spectrum β-lactamases (ESBLs) viz., TEM, SHV, OXA and CTX-M, carbapenemases such as NDM, IMP, VIM, KPC and GES, the mobile colistin resistance gene *mcr-1* (to *mcr-10*) and recently, the mobile tigecycline resistance gene, *tet(X)* [12–17]. Thus, such conjugative plasmids influence the genomic plasticity of several related and unrelated species and genera of bacteria [8,11,18–21].

Coupled with plasmid-borne dissemination of ARGs is the selection and expansion of specific drug-resistant clones [11,21], which quickly spread under antibiotic pressure to overpopulate their environments, facilitating their survival and subsequent spread to other environments [14,22,23]. In cases where such clones harbour resistance plasmids, their expansion almost always lead to the concomitant replication and intra-clonal as well as inter-clonal spread of such plasmids [4,5,7,8,24,25]. Thus, as such clones are disseminated through contact, food, water, farms, hospitals, and the environment, they carry with them these resistance plasmids to colonize new hosts and environments [6,26–28]. It is thus not surprising to have same

clones hosting the same plasmids, contain the same resistomes [4–6,21,28]. This explains the presence of multi-drug resistance (MDR) in particular international clones such as *Klebsiella pneumoniae* ST258 and *E. coli* ST131 [21,25,29,30].

Hence, tracing the phylogeography of clones and their associated resistance genes is highly critical in epidemiology and public health as it provides necessary data to contain the further spread of ARGs [26,28,31]. In this work, the global evolutionary epidemiology and resistome dynamics of clinically important but relatively less isolated Enterobacterales pathogens are described [8]. It is notable that most of the recently emerged or novel resistance genes in bacteria have occurred in Enterobacterales more than in any other family of bacteria, making Enterobacterales particularly important medically [28,32,33]. These include ESBL-, carbapenemase-, *mcr*- and *tet(X)*-producing producing Enterobacterales, which have been classified by the World Health Organization (WHO) as high and critical priority pathogens due to their implication in high mortalities and morbidities [32,33].

Although *Citrobacter* spp., *Enterobacter hormaechei, Klebsiella variicola, Morganella morganii, Proteus* spp. and *Providencia* spp. are not mostly reported as *Escherichia coli, Klebsiella pneumoniae,* and *Salmonella enterica,* they have been associated with multiple resistance and clinical fatalities [1,3,7,14,25,28]. *Proteus, Providencia,* and *Morganella* species are known as the tribe Proteeae and are referred to as such in this article [34,35]. Clinical and non-clinical (veterinary and environmental) data from around the world suggest that *Citrobacter* spp., *Enterobacter hormaechei, Klebsiella variicola* and Proteeae tribe are relatively less isolated albeit implicated in occasional outbreaks or local epidemics [1,24,35–41]. Moreover, the use of colistin to treat MDR infections in humans and animals are increasingly selecting for the tribe Proteeae as they are intrinsically resistant to colistin [1,7,35,42].

Due to the transferability of resistance plasmids between members of the Enterobacterales, the global resistome epidemiology of these six genera is important as they could be eventually transferred to commonly isolated Enterobacterales species, and vice versa [11,14,21,25,43,44].

Results

Included genomes

Out of about ~3000 genomes, a final total of 2,377 genomes from *C. freundii* (n=569 genomes), *C. koseri* (n=82 genomes), *C. amalonaticus* (n=35 genomes), *E. steigerwaltii* and *E oharae* (n=121 genomes), *E. xiangfangensis* (n= 90 genomes), *E. hormaechei* (n=563 genomes), *K. variicola* (n=574 genomes), *M. morganii* (n=59 genomes), *P. mirabilis* (n= 156 genomes), and *Providencia* spp. (n=128 genomes) were obtained from PATRIC and NCBI databases as at January 2020 and used for downstream analyses (Tables S1-S3). Included in *C. freundii, C. koseri, E. xiangfangesis, K. variicola* and *Providencia* spp. genomes were genomes of other Gram-negative bacterial species or genera that were initially classified within these respective species but later reclassified by NCBI's ANI (average nucleotide identity) analysis. The genomes of these reclassified species were however maintained and included in the phylogenomics and resistome analyses to both confirm their species with the phylogenetic tree analyses and their resistomes (Tables S1-S3).

The genomes were mainly isolated from human specimens, followed by animal (including food animals), plants (including food crops) and environmental specimens. The human and animal specimens used included urine, blood, stool, catheter tip, and swabs whilst the environmental specimens used included soils, hospital environments, water, wastewater, sinks etc. (Tables S1-S3). In all, these genomes were obtained from 67 countries globally, with the USA having the most genomes for all species: *C. freundii*/spp. (USA=233,

China=34, France=29, Spain=22; other countries = 243); *C. amalonaticus* (USA=17, other countries =18); *C. koseri* (USA=55, other countries = 27); *E. steigerwaltii/oharae* (USA=40, Japan=15; other countries = 63); *E. hormaechei* (USA=142, China=78, Japan=72, France=21, UK=12; other countries = 231); *E. xiangfangensis* (USA=50, China=14, India=12; other countries = 13); *K. variicola* (USA=289, Germany=50, China=24, Bangladesh=23; other countries = 186); *M. morganii* (USA=16; other countries = 49); *P. mirabilis* (USA=38, France=27, China=17; other countries = 74); *Providencia* spp. (USA=61; other countries = 66) (Tables S2).

C. freundii, the only species among the species included in this analysis to have an MLST scheme, had 84 different clones or sequence types (STs). ST100 (n=51), ST22 (n=51), ST62 (n=18), ST11 (n=14), ST299 (n=11), ST8 (n=10), ST114 (n=8), and ST98 (n=8) were the commonest clones. Although *E. hormaechei, E. xiangfangensis, E. oharae/steigerwaltii,* and *K. variicola* have no MLST schemes, their metadata had them (Tables S2),

Species epidemiology

Citrobacter species

Amongst the *C. freundii* genomes were other *Citrobacter* spp. such as *C. werkmanii, C. youngae, C. brakii, C. portucalensis* etc. *C. werkmanii* were isolated from humans, sprouts, and sinks whilst *C. brakii* were obtained from humans, vegetables, and hospital environments. C. *werkmanii* and *C. brakii* strains on the same clade (bootstrap of 100%, suggesting very close evolutionary distance) were found in USA, Germany, and India (Fig. 1-5). Notably, *Citrobacter sp.* wls707 and wls717 (of ST32) from Poland are recently evolved (branching length and bootstrap of 100%) strains from *C. werkmanii* strains (Fig. S1A)

C. koseri were mainly found from humans and from a mouse (clade B2) (Fig. 1-4). *C. portucalensis* of the same clade (but different clones) were found in Nigeria (vegetables) and

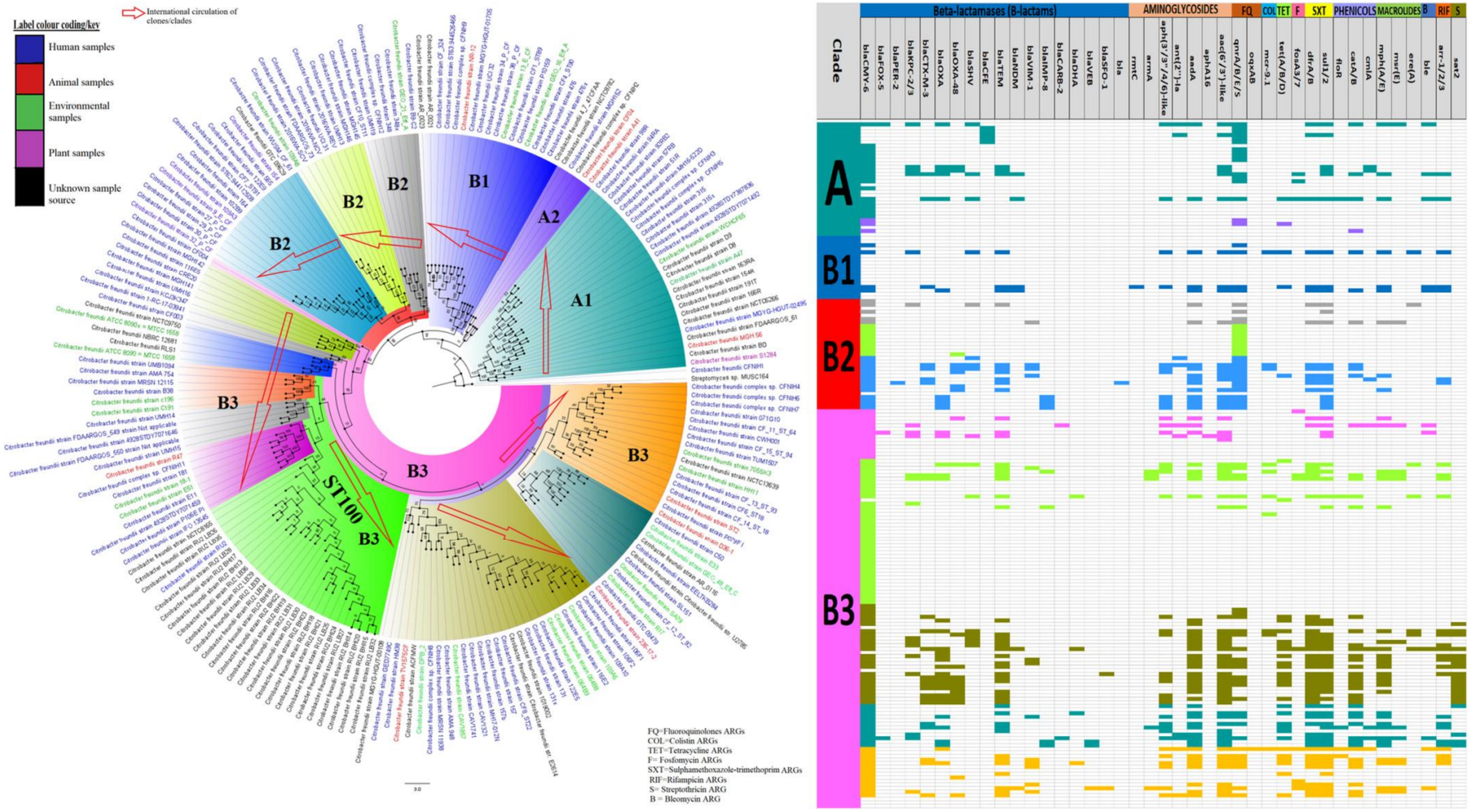


Fig. 1. Evolutionary epidemiology and resistome of global *Citrobacter freundii* isolates. *C. freundii* clustered into four main clades (A, B1, B2 and B3), highlighted with distinct colours. Clade B3 had the most resistome abundance and diversity. Strains from humans (blue labels), animals (red labels), plants (purple/mauve labels) and the environment (green labels) were found in the same clade/cluster. *Bla*$_{CMY}$ was conserved in these genomes. Branches with bootstrap support values of ≥50 were defined as belonging to the same clade. The branch lengths also show the evolutionary distance between the isolates. Blue and red arrows show the direction of evolution as well as local and international dissemination of strains of the same clone/clade through different hosts.

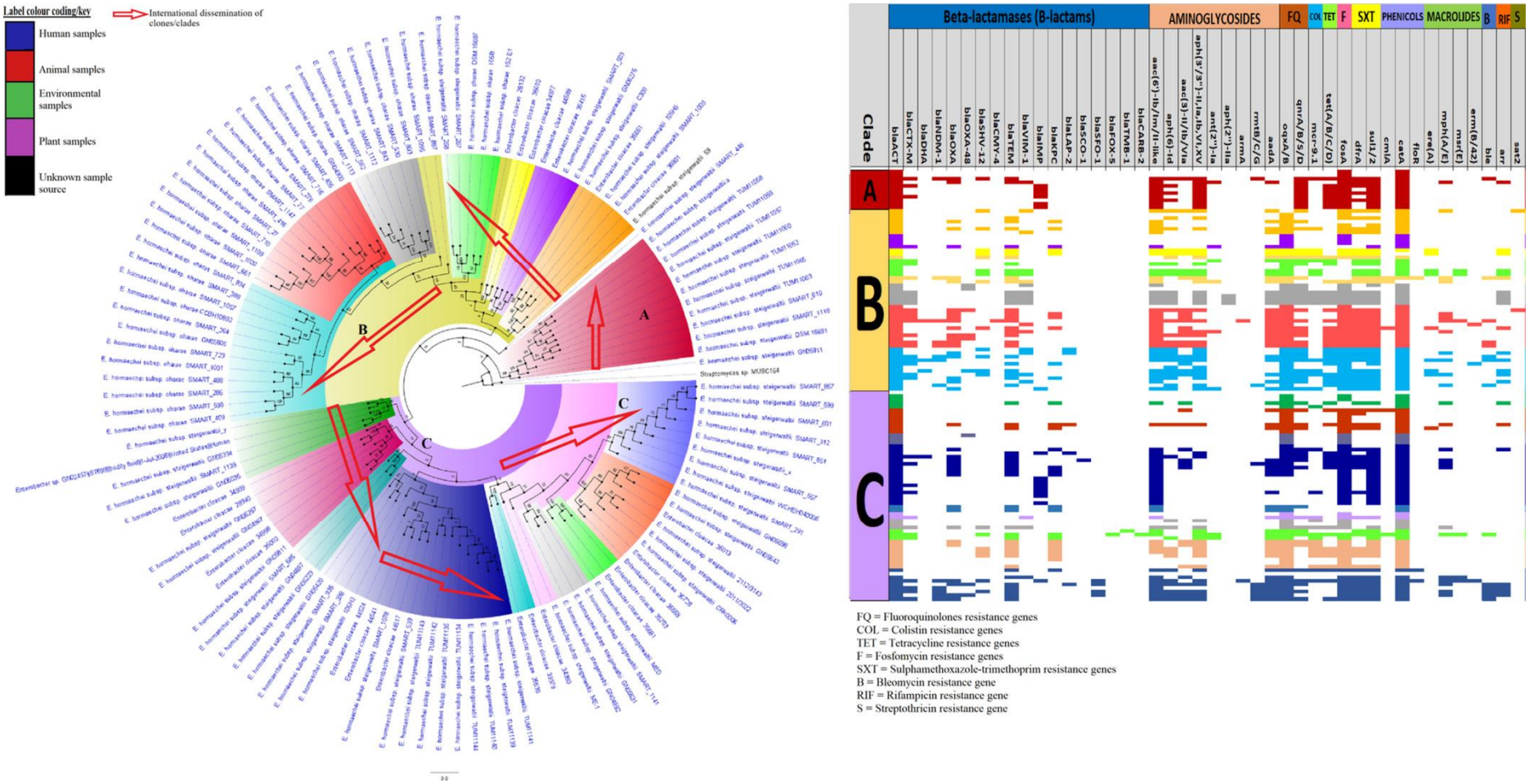


Fig. 2. Evolutionary epidemiology and resistome of global *Enterobacter steigerwaltii/oharae* isolates. There were three main clades on this tree, with *E. steigerwaltii* being found on all three branches/clades of the tree whilst *E. oharae* isolates clustered mainly on clade B of the tree. Within clade/branch A and C were also *E. cloacae* isolates. All the three main clades A, B and C (with distinct highlights) contained strains distributed globally from humans (blue labels). Clades B and C contained diverse and rich resistome repertoire. bla_{ACT}, *cat*A, and *fos*A were conserved in these genomes. Branches with bootstrap support values of ≥50 were defined as belonging to the same clade. The branch lengths also show the evolutionary distance between the isolates. Blue and red arrows show the direction of evolution as well as local and international dissemination of strains of the same clone/clade through different hosts.

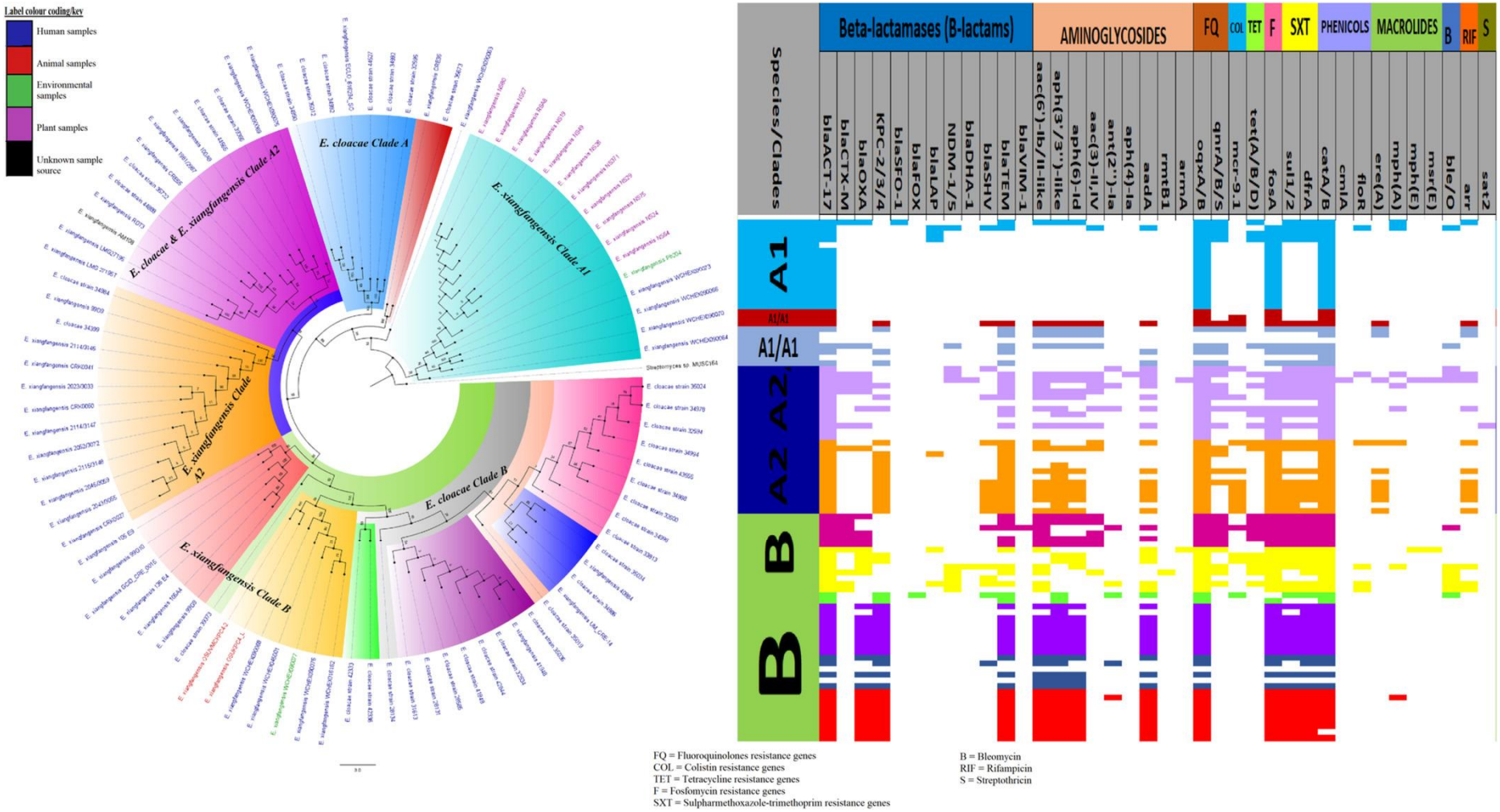


Fig 3. volutionary epidemiology and resistome of global *Enterobacter xiangfangensis* isolates. The *E. xiangfangensis* contained *E. cloacae* genomes and they clustered into clades A1, A2 and B, with clades A2 and B having rich and diverse resistome repertoire; these clades were distributed globally from humans (blue labels) and animals (red labels). *bla*$_{ACT}$ was conserved in these genomes. Branches with bootstrap support values of ≥50 were defined as belonging to the same clade. The branch lengths also show the evolutionary distance between the isolates. Blue and red arrows show the direction of evolution as well as local and international dissemination of strains of the same clone/clade through different hosts.

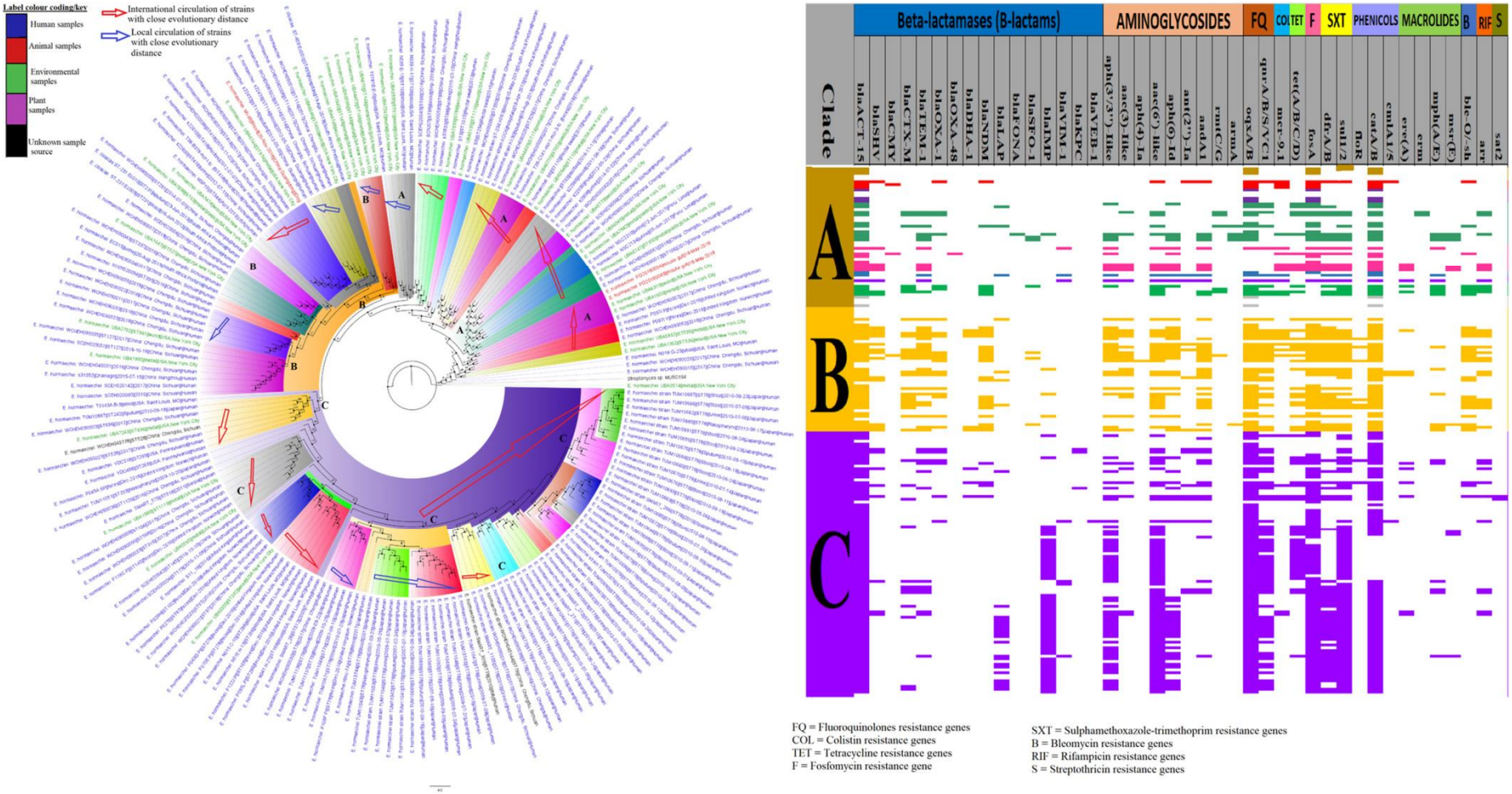


Fig 4. Evolutionary epidemiology and resistome of global *Enterobacter hormaechei* isolates. The *E. hormaechei* isolates clustered into three main clades A, B and C (with distinct highlights) that contained strains distributed globally from humans (blue labels), and animals (red labels), plants (purple/mauve labels) and the environment (green labels). Clades B and C contained diverse and rich resistome repertoire. *bla*$_{ACT}$ was conserved in these genomes. Branches with bootstrap support values of ≥50 were defined as belonging to the same clade. The branch lengths also show the evolutionary distance between the isolates. Blue and red arrows show the direction of evolution as well as local and international dissemination of strains of the same clone/clade through different hosts.

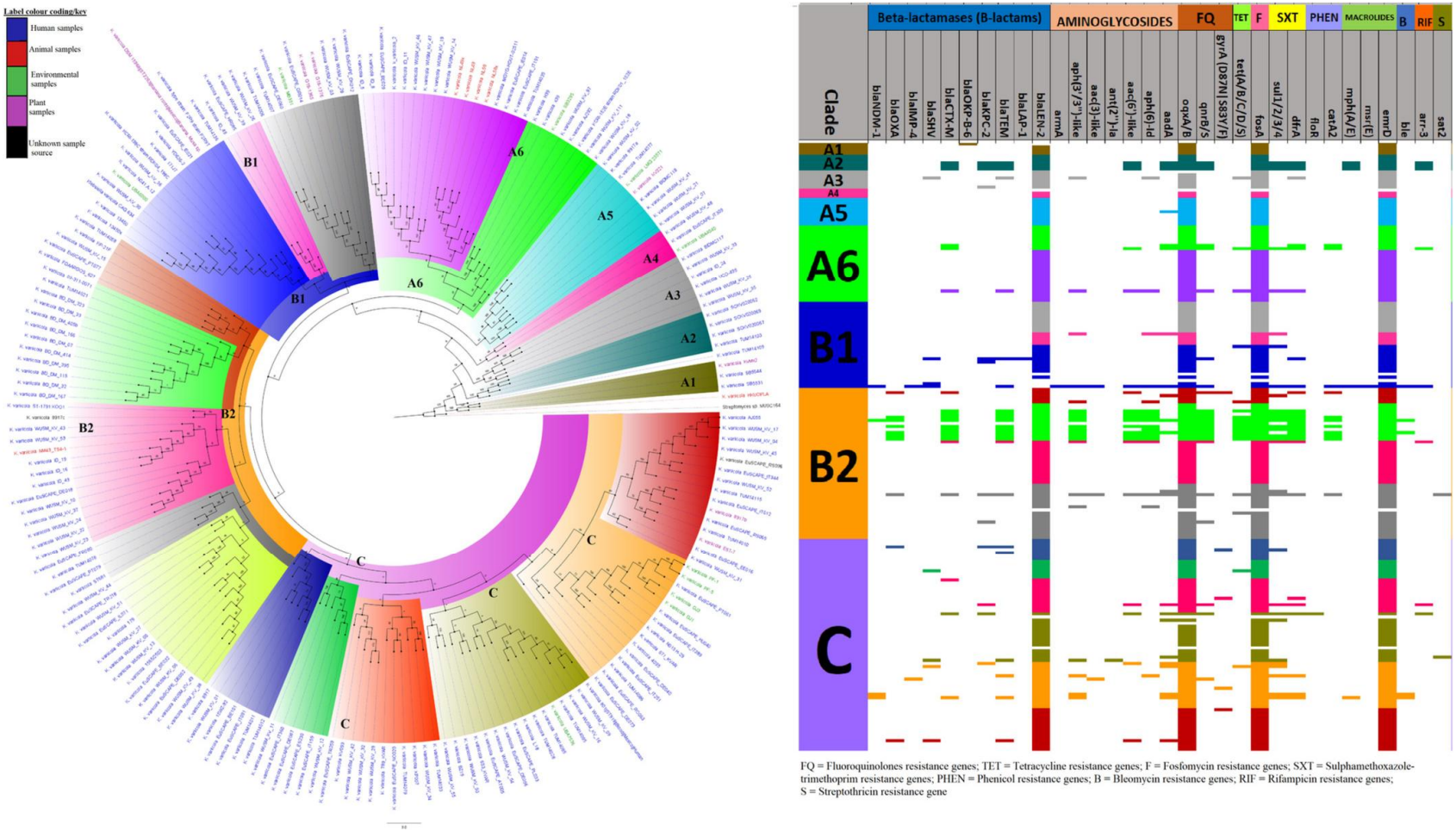


Fig 5. Evolutionary epidemiology and resistome of global *Klebsiella variicola* isolates. The *K. variicola* strains clustered into nine clades viz., A1, A2, A3, A4, A5, A6, B1, B2 and C, which were highlighted with distinct colours and were isolated from countries around the globe. The clades contained strains distributed globally from humans (blue labels), animals (red labels), plants (purple/mauve labels) and the environment (green labels). Besides a few strains in clade B2, the other strains contained very few resistance genes. *bla*$_{LEN}$ was conserved in these genomes. Branches with bootstrap support values of ≥50 were defined as belonging to the same clade. The branch lengths also show the evolutionary distance between the isolates. Blue and red arrows show the direction of evolution as well as local and international dissemination of strains of the same clone/clade through different hosts.

Brazil (turtle) as well as from effluents (UK), humans (China), chives, carrots and salad (Germany); these clustered in *C. freundii* clade B1. *C. freundii* strains of different STs and countries clustered together into clades, showing the wide distribution of strains of close evolutionary distance (same clone or close genetic make-up); they were isolated from humans, plants, animals, and the environment (Fig. 1-5).

C. werkmannii, C. brakii and *C. youngae* clustered within *C. freundii* clade A whilst *C. portucalensis* and *C. youngae*, were clustered in clade B; *C. koseri* clustered in clade B3 of *C. freundii* (Fig. 1-2, &5). *C. freundii* clades B2 and B3 had abundant (and diverse) resistome than clades A and B1, although *bla*$_{\text{CMY}}$, *qnr*A/B/D/E/S/VC1, *sul1/2, aac*(6'/3')-like ARGs were common in all the clades; *mph*(A/E), *catAB, dfrAB, aadA* and *bla*$_{\text{TEM}}$ were common in clades B2 and B3. Important ARGs such as *mcr*, *bla*$_{\text{KPC}}$, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{VIM}}$, and *bla*$_{\text{OXA-48}}$ were relatively rare and mainly found in clades B2 and B3 than in A and B1. Comparatively, other Enterobacterales species (*E. coli, K. pneumoniae,* and *S. marcescens*) had strains with richer (between 50-70 ARGs) resistomes diversity than *C. freundii* clades A and B1, including chromosomal mutations and MDR efflux pumps (Table S4). However, Clades B2 and B3 had comparable resistome diversity and abundance to the above-mentioned Enterobacterales species, in which CMY was well-nigh absent except in *E. coli* (Table S4; Fig. 1-2, &5).

A local outbreak of *C. braakii* was observed in the UK (in humans in 2018), with closely related strains (bootstrap of ≥87%) being isolated from biosolids (Canada, between 2006-2010), carrots (Germany in 2005), chicken (China) and beef (Canada) (Fig. S1A). Further, *C. brakii* strains of the same clone (bootstrap of 100%) were disseminating through leafy vegetables in the USA (2018) (Fig. S1D). *C. amalonaticus* was also found in humans, animals, plants, and the environment (Fig. S1B): *bla*$_{\text{CMY}}$ was not common in this species, but *bla*$_{\text{SED}}$ and *Oqx*A/B were almost conserved in clades B1 and C, with clade B2 (all from

France) having the richest resistome repertoire (22 ARGs) that included *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{NDM}$ and *mcr*-9.1 (Fig. S1B). *C. koseri* had 18 unique ARGs, with *bla*$_{MAL-1/2}$ (clade B2), *bla*$_{CKO}$ (clade B3) and *fos*A7 (clade B2) being the commonest ARGs. Comparatively, the other Enterobacterales species (e.g., *E. coli, K. pneumoniae, Enterobacter* spp., *Serratia* spp., and *Providencia* spp.*)* had richer resistome diversity (47 ARGs) than *C. koseri* (18 ARGs) (Fig. S1C).

C. freundii strains of clade B2 (bootstrap of 100%) were isolated in Seattle (2016), Boston (2015/6), Michigan (2013/4), Germany (2012/3), France (2017), and Spain (2014). Intercountry dissemination of *C. freundii* clade B3 (bootstrap of 100%) between Australia (2012/3) and UK (2016) as well as between Japan (2001), Tanzania (2009), Spain (2014), China (2014), and France (2017) were observed; clade B1 strains also had international dissemination (Fig. S1D). As well, a local circulation (and possible outbreak) of *C. freundii* ST100 in clade B3 in Texas (USA, 2011-13) was also observed (Fig. S1D). Notably, *C. freundii* ST22 in clade B3 (bootstrap of ≥83%) were circulating between France (2017) (Fig. S1A & 5) and *C. freundii* strains of the same genotype (bootstrap of 100%) were circulating in the USA (Texas, 2015-18) (Fig. S1D).

A C. amalonaticus clonal expansion (clade C, bootstrap of 100%) was observed in the USA (2014/5) and Switzerland (2019) whilst a carbapenemase and an MDR *C. amalonaticus* of clade B2 dissemination was observed in France. A cross-country dissemination of *C. amalonaticus* clade B1 in humans in USA, 2013-15, and in seafood in Malaysia, 2014, was also identified whilst a local dissemination of clade A strains (bootstrap of 100%) in dairy and beef in Ontario (2014) and leafy vegetables in Montreal, Canada, was also observed (Fig. S1D).

Enterobacter species

The resistomes of *E. steigerwaltii* and *E. oharae* were extraordinarily rich and diverse (44 ARGs), although clade A of *E. steigerwaltii* had lesser resistome abundance and diversity compared to clades B and C (Fig. 2). *E. oharae* only clustered in clade B of *E. steigerwaltii* and had most *bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{VIM}}$ ARGs than *E. steigerwaltii* clade C. *bla*$_{\text{ACT}}$ was present in almost all *Enterobacter* spp. strains whilst other AmpCs such as *bla*$_{\text{LAP}}$, *bla*$_{\text{SCO-1}}$, *bla*$_{\text{SFO}}$, *bla*$_{\text{TMB}}$, *bla*$_{\text{DHA}}$, and *bla*$_{\text{CARB}}$ were virtually absent. *E. cloacae* strains clustered closely with *E. steigerwaltii* clades B and C. overall, *E. steigerwaltii* and *E. oharae* strains were richly endowed with clinically important ARGs including carbapenemases, ESBLs, *aac(6')-like, aac(3')-like, aph(3'/3")-like, aadA, dfrA, catA, fosA, oqxAB, qnrA/B/S/D, sul1/2,* and *tet*(A/B/D). *Mcr-9* genes were particularly abundant (total count) in clade B than in clades A and C of *E. steigerwaltii/cloacae* (Fig. 2).

E. cloacae strains clustered with all *E. xiangfangensis* strains except clade A1, from which it was evolutionarily distant (Fig. 3). ARGs such as *bla*$_{\text{ACT}}$, *cat*A/B, *Oqx*A/B and *fos*A were universally present in almost all the *E. xiangfangensis* and *E. cloacae* strains. Furthermore, *E. xiangfangensis* clade A1 had the least ARGs diversity and abundance whilst *E. cloacae* A1 and the remaining *E. xiangfangensis* (clades A2 and B) were richly endowed with multiple ARGs such as *bla*$_{\text{KPC}}$, *mcr-9*, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{TEM}}$, and genes mediating resistance to fluoroquinolones (*aac*(6'/3')-like, *qnr*A/B/S etc.) and aminoglycosides (*armA*, *rmtC/G*, *aad*A, *aph*(4)-, *aph*(3'/3")- and *aph*-(6')-like) as well as *tet* (A/B/D), *dfrA*, and *arr* (particularly in clades A2 and B). Thus, the resistomes diversity and abundance of *E. xiangfangensis* (38 unique ARGs, 1168 total ARGs, 30.74 relative richness) and *E. cloacae* were comparable to that of *E. oharae/steigerwaltii* (44 unique ARGs, 1588 total ARGs, 36.09 relative richness) (Table S4; Fig. 2-7).

An *E. hormaechei* outbreak or persistence of clones ST116 and ST128 (under clades A, B and C) was observed in Germany in 2017, consisting of closely related strains (with ≥50% bootstrap) whilst clade A strains (≥50% bootstrap) circulated in the USA (2013, 2015, and 2019) and in Australia (2013) (Fig. 4-S2A-B). Local and international (between Norwich, UK, and Missouri, USA) transmission of *E. hormaechei* clade C strains in 2016 were observed. A similar observation was made in Japan in 2007, 2009, and 2007, in which strains of the same clone (ST78) were isolated. A potential outbreak of ST78 was also observed in New York and Boston (2012) and in Boston in 2007 and 2009. Transmission of the same clone (ST78) was also identified in China, Italy, Greece, Spain, Portugal (2016), Lebanon (2018), and Taiwan as well as in Nigeria (2015) and between France and Spain (2016/7), making ST78 an international clone with bla_{IMP}, aminoglycosides, fluoroquinolones, tetracyclines, sulphonamide-trimethoprim, phenicols, and fosfomycin ARGs.

E. hormaechei ST190 strains (clade B, ≥50% bootstrap) were isolated from USA, Germany, and Pakistan whilst ST510 strains in Colombia (2014) and ST113 strains in USA (2011-12), Australia (2007 and 2013), and Lebanon (in 2017) were found circulating among humans; these suggest local outbreak in Lebanon and international dissemination of ST113 (Fig. S2A). In addition, *E. hormaechei* ST102 (clade C) transmission and possible outbreak (100% bootstrap) was observed in Germany (2018) whilst a clonal (of mainly ST114) and polyclonal expansion of strains in clade C (between 2013-19), particularly in Australia, China, France, Lebanon, Germany, Malaysia, Poland, Morocco, South Africa, USA, etc. was identified, making ST114 an international clone (Fig. S2A). Furthermore, local circulation of ST105 occurred in Croatia in 2011-2013.

E. steigerwaltii and *oharae* were mainly isolated from humans whilst *E. xiangfangensis* and *E. hormaechei* were from humans, plants (*E. xiangfangensis* from rice in India), animals and the environment. Strains of closely related (i.e., close evolutionary distance) *E. hormaechei* in

clade B were from the US, South Africa, Colombia, China, Germany, Australia and Lebanon from humans, animals, and plants (Fig. 4-10). Other closely related *E. hormaechei* strains from humans, animals, plants, and the environment were found in different countries within short time periods, showing a persistent dissemination of closely related strains across countries through different hosts. These observations were made in *E. hormaechei* clades B and C, and represented local and international outbreaks spanning UK, USA, China, Serbia, Japan etc. (Fig. 4-10). The presence of these closely related strains in humans, animals, and the environment buttress the need for One Health surveillance studies to comprehensively map out the epidemiology of pathogens of MDR bacteria.

Moreover, *bla*$_{\text{ACT}}$, *fos*A, *Oqx*A/B and *cat*A/B were almost conserved in almost all the clades of *E. hormaechei.* As observed with the other *E. hormaechei* subsp. (*oharae, steigerwaltii* and *xiangfangensis*), *E. hormaechei* clades B and C were richly endowed with ARGs than some clade A strains (except clade A in Fig. 4). Specifically, clades B and C strains were relatively enriched with *bla*$_{\text{TEM-1}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{CTX-M}}$, *aph(3'/3")*-like, *aac(3')*-like, *aac(6')*-like, *dfrA/B, mcr-9, arr, qnrA/B/S, sul-1/2,* and *tet*(A/B/C/D). Further, clade B was mostly rich in *bla*$_{\text{KPC}}$ and *bla*$_{\text{NDM}}$ whilst clade C was rich in *bla*$_{\text{NDM}}$ and *ble.* A substantial number of clade A strains also had *mcr-9* and *bla*$_{\text{TEM}}$ (Fig. 4-10).

E. oharae was evolutionarily closer to *E. xiangfangensis,* clustering on the same branches, with a relatively few *E. oharae* strains clustering with *E. steigerwaltii.* However, *E. steigerwaltii* was mostly distant from *E. oharae* and *E. xiangfangensis,* with a few *E. xiangfangensis* strains (from rice in India) clustering within *E. steigerwaltii* clades A and C (Fig. S2B). Figure S2B summarises the ARGs in *E. hormaechei* and its subspecies: *bla*$_{\text{ACT}}$, *fosA, OqxAB, qnrA/B/D/S,* and *catA/B* were conserved whilst *bla*$_{\text{OXA}}$, ble, *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{KPC}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{VIM}}$, *mcr, tet, mph(A), sul-1/2, dfrA,* and fluoroquinolone and aminoglycoside ARGs were richly abundant, particularly in clusters IV, V and VII.

E. xiangfangensis ST66 was isolated in France and Egypt between 2016-2017 whilst *E. oharae* ST114, which was evolutionarily closer to *E. oharae* ST511 (Argentina) and ST418 (India), had an international circulation (cluster V). Notably, *E. xiangfangensis* clade C strains were tightly clustered together, with little branching lengths that suggests fewer genomic differences (evolution) between the strains. Moreover, an *E. steigerwaltii* dissemination and possible outbreak was observed in Japan in 2010 (Fig. S2B).

K. variicola

K. variicola strains were isolated mainly from humans, with some being from animals, plants, and the environment. On the individual branches/clades were closely related strains with very close evolutionary distance i.e., of the same clone/clade, (≥50% bootstraps) but disseminated across countries, suggesting international dissemination of that clade and showing little evolution during the spread from host to host, e.g., clades A3, A4, A5, A6, B1, B2 and C (Fig. 5-S3A-B). As well, local outbreaks of closely related strains in the USA, Bangladesh, Canada, and Germany were observed. A reassessment of the *K. variicola* tree with genomes of *K. pneumoniae* and *K. quasipneumoniae* largely confirmed the initial clustering of the *K. variicola* strains, with only a few rearrangements of some strains within different clades (Fig. 5-S3A-B).

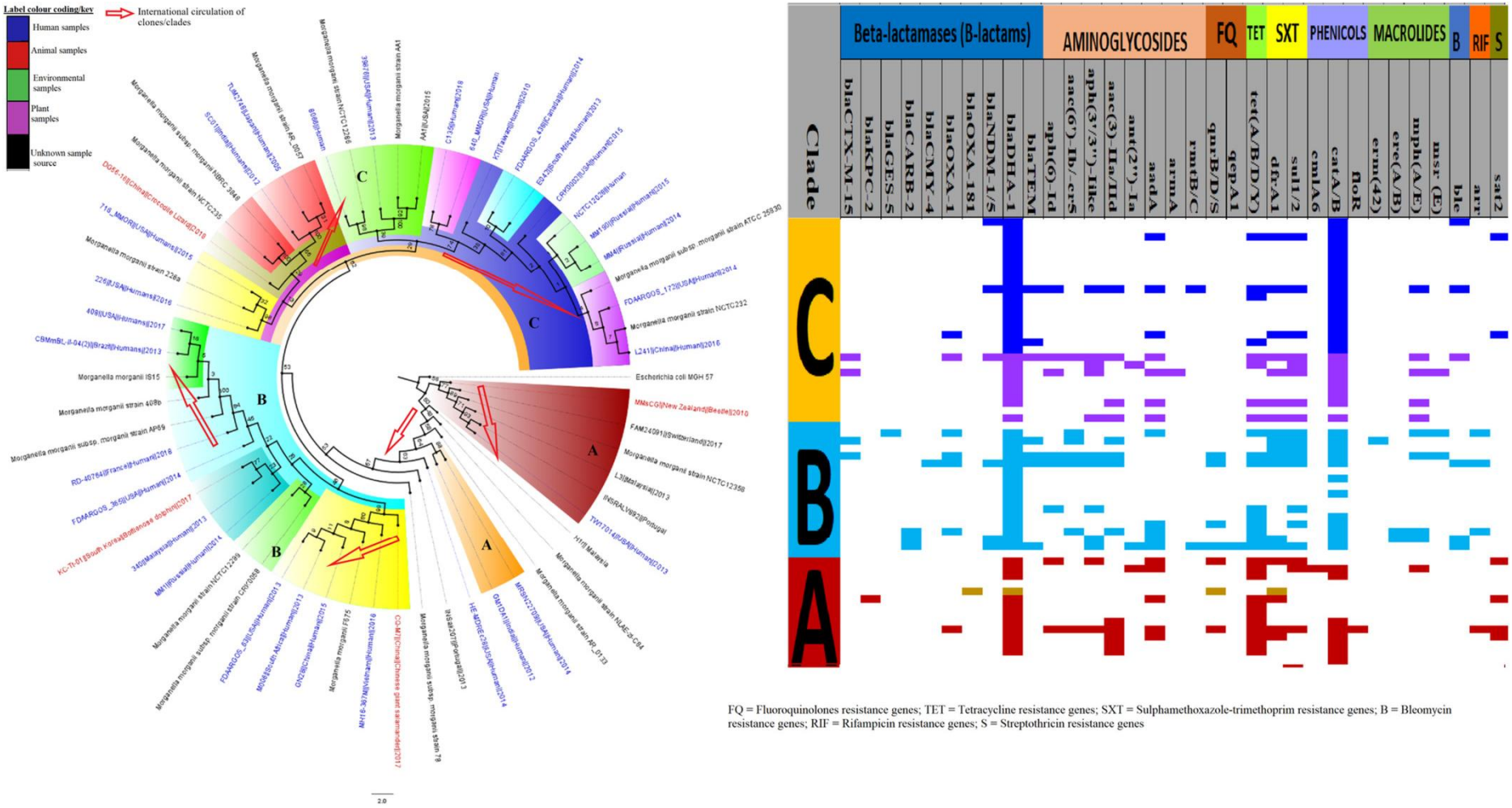
Notably, *K. variicola* had fewer resistome diversity and abundance (36 ARGs) than *Citrobacter* spp. (58 ARGs), *Enterobacter* spp. (38-48 ARGs), and *K. pneumoniae* spp. (68 ARGs). Conserved within the *K. variicola* genomes were *emrD*, *fosA*, *OqxAB* and $bla_{\text{LEN-2}}$, whilst the other ARGs were sparse. Whilst bla_{SHV} was conserved in *K. pneumoniae*, it was virtually absent in *K. variicola;* further, bla_{LEN} was present in the latter but absent in the former. *K. variicola* clade B2 strains (on branch VIII), specifically those from Bangladesh, had richer and more diverse resistomes (36 ARGs) than the other *K. variicola* clades/clusters.

Whilst *mcr* and *bla*$_{NDM}$ were virtually absent in these genomes, *bla*$_{KPC}$ occurred in substantial abundance. These suggest that *K. variicola* is least likely to be MDR and/or harbour ESBLs, carbapenemases, *mcr* and other clinically important ARGs compared to other Enterobacterales species (Fig. 5-S3A-B).

K. variicola clusters VI and VII (clades A and C) comprised strains found in USA (2017), suggesting a possible local polyclonal (ST641 and ST318) outbreak, whilst a similar circulation was observed in Germany (2014) and in Belgium. Similarly, local transmission of ST205 was found in dairy in Canada (2012) and large isolations of ST771 (clade B, cluster III) was reported in Bangladesh (2016-17), suggesting a possible outbreak or endemicity of this clone there. Local disseminations of other clones within clade B (cluster VIII) such as ST1142 in China (2016-17), and ST2609, ST1556 and ST695 in USA (2017) were also identified (Fig. S3A-B).

M. morganii

There were three *M. morganii* clusters/clades viz., A, B and C, which were mainly from humans with a few in clades A and C being from animals (Fig. 6). Within each clade are closely related strains with very close evolutionary distance (≥ 50% bootstrap) that were distributed across several countries; indeed, strains of the same clone were found in different countries, suggesting international dissemination of the same clones. The branching order of the trees within each clade shows the gradual evolution of the strains as they moved from host to host. *bla*$_{DHA}$ and *catA/B* genes were almost conserved in almost all the *M. morganii* genomes. Other highly abundant ARGs in the *M. morganii* strains were *sul-1/-2*, *tet*(A/B/D/Y), *bla*$_{OXA-1}$, *ble, dfrA1, aadA, aac(3)*-like and *aph(3'/3")*-like. Notably, clade B had more ARG diversity and abundance than clade C and A; clade A had the least diversity and abundance of ARGs (Fig. 6).



FQ = Fluoroquinolones resistance genes; TET = Tetracycline resistance genes; SXT = Sulphamethoxazole-trimethoprim resistance genes; B = Bleomycin resistance genes; RIF = Rifampicin resistance genes; S = Streptothricin resistance genes

Fig 6. Evolutionary epidemiology and resistome of global *Morganella morganii* isolates. The *M. morganii* strains clustered into three clades, A (red highlight), B (light blue highlight) and C (yellow/gold highlight), containing isolates obtained globally from humans (blue labels) and animals (red labels). Branches with bootstrap support values of ≥50 were defined as belonging to the same clade. The branch lengths also show the evolutionary distance between the isolates. Blue and red arrows show the direction of evolution as well as local and international dissemination of strains of the same clone/clade through different hosts.

P. mirabilis

P. mirabilis clustered into three major clades and included isolates from humans, animals, and the environment. Within the three clades were sub-clades consisting of closely related strains (≥ 50% bootstrap) from the same as well as different countries, showing local and international outbreaks involving human and animal hosts, and in some cases environmental mediators (Fig. 7). Specifically, local isolations of same strains were seen in the USA (clades A3, B2 and B3), France (clade C2) and Japan (C3) whilst international dissemination of clades A2, A3, A4, B, C2 and C3 were observed. Clade C2 had the richest abundance of resistomes (~46 ARGs), with all the members having a uniform/conserved diversity of the same ARGs, except for *bla*$_{\text{CTX-M}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{TEM}}$, and *Inu*(F/G). Clade C3 had the 2nd most abundant but more diverse ARGs than C2. Notably, clade B and its subclades had lesser ARGs than clades A and C. *CatA* and *tet*(A/B/D/Y) ARGs were virtually conserved in all the clades whilst *aad*A, *aac(3')*-like, *aph(3'/3")*-like, *aph(6')*-like, *dfrA7*, *sat*2, and *sul-1/2/3* were substantially prevalent in all the clades. In particular, *bla*$_{\text{CARB2}}$ and *flor*R were highly conserved in clade C2; *flor*R was however less abundant than *bla*$_{\text{CARB}}$. Notably, *mcr* was almost absent except in a few strains in B3. As well, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{CMY}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{OXA-1}}$, *bla*$_{\text{TEM}}$ and *ble-O/Sh* were mainly found in clades A and C3, with traces in B and C1. Thus, ESBLs, carbapenemases and *mcr* genes were relatively less common in *P. mirabilis* strains, compared to other Enterobacterales (Fig. 7 & S1-S6).

Providencia species

There were five *Providencia* spp. viz., *stuartii, rustigianii, alcalifaciens, heimbachae,* and *rettgeri,* with *P. rettgeri* being most isolated and branching into two clades, A and B (Fig. S4). The other species had single clades. *Providencia* spp. were isolated from animals, humans, and the environment. Highly similar strains of the same species were found across

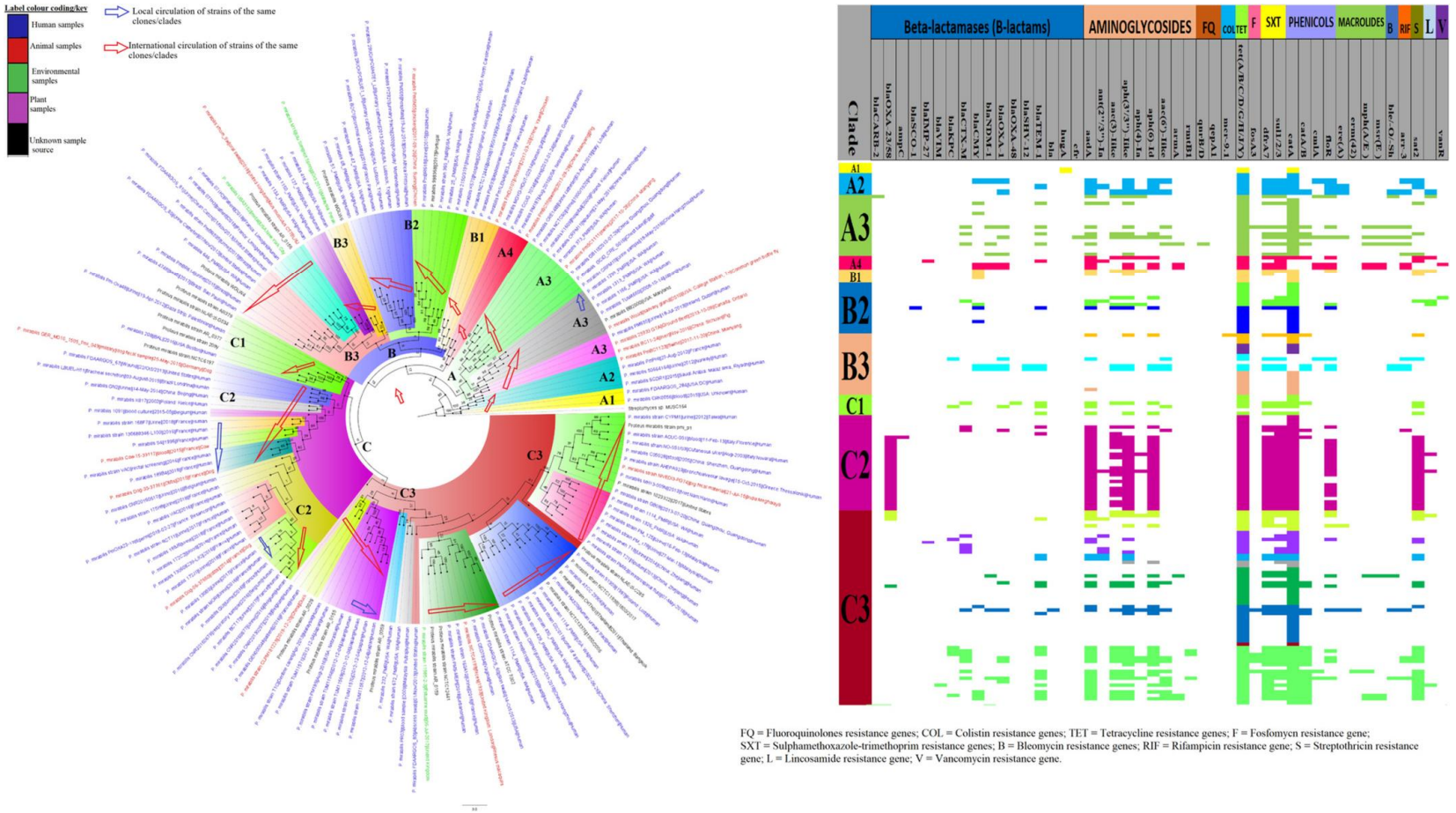


Fig 7. Evolutionary epidemiology and resistome of global *Providencia* spp. isolates. The *Providencia* spp. clustered into 12 branches, with *P. stuartii*, *P. rustigianii*, *P. alcalifaciens*, *P. heimbachei* and *P. rettgeri* clustering into branch VIII to XII respectively. *P. rettgeri* clustered into clades A and B, consisting of globally distributed isolates. Included in this tree are *K. pneumoniae* and *Escherichia coli* species that were originally classified as *Providencia sp*. but later reclassified into their actual species using ANI; their clustering away from the *Providencia sp*. confirms the ANI results that they were initially misclassified. *P. stuartii* and *P. rettgeri* contained richer and more abundant resistomes than the other *Providencia* strains and contained strains distributed globally from humans (blue labels), animals (red labels), plants (purple/mauve labels) and the environment (green labels). Branches with bootstrap support values of ≥50 were defined as belonging to the same clade. The branch lengths also show the evolutionary distance between the isolates. Blue and red arrows show the direction of evolution as well as local and international dissemination of strains of the same clone/clade through different hosts.

countries (*P. stuartii, P. alcalifaciens, P. heimbache* and *P. rettgeri*) and within countries (*P. rettgeri* clade A). The distinction between the various species of Providencia was depicted by the clustering patterns on the tree as strains of the same species clustered together; *Providencia* spp. was closest evolutionarily to *S. marcescens* whilst *E. coli* was closest to *Citrobacter* spp., and *E. hormaechei.* As well, *K. pneumoniae, K. aerogenes,* and *K. michiganensis* clustered together with relatively short evolutionary distance (Fig. S4 & S1-S6).

The other Enterobacterales species had more ARGs than *Providencia* spp. Within *Providencia* spp., *P. stuartii* and *P. rettgeri* were most endowed with richer and more diverse resistomes whilst *P. alcalifaciens* and *P. rustigianii* were almost bereft of ARGs. Common ARGs within *P. stuartii,* were *aac(2')-Ia, aph(3')*-like, *bla*$_{\text{OXA}}$, *bla*$_{\text{TEM}}$, *aadA1, dfrA/B, sul1, fosA, ampC, tet* and *catA/B* whilst *P. rettgeri* had *aac(6')*-like, *aph(3'/3")*-like, *bla*$_{\text{SRT}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{NDM}}$, *aadA, dfrA/B, OqxAB, sul1, qnrA/B/D/E/S, ampC, catA/B, tet, arr, mph*(A/E), *cmlA, msr*(E), and *Inu*(F/G). Hence, *P. rettgeri* has the most abundant and diverse ARGs (~70) than all other *Providencia* spp. (Fig. S4).

Phylogeography

North America (particularly USA) and Europe (particularly Western and Southern Europe) had the highest concentration of the various species, followed by South East Asia, South America (particularly Brazil and Colombia) and South Africa. There were sparse reports on these species from Australasia, the Middle East and Africa (except South Africa) and the Caribbean (Fig. 8).

C. freundii clade A was distributed mainly in North America, Europe, and South-East Asia whilst clade B was found worldwide on almost all continents. *C. amalonaticus* clade A was found in North America and South Korea; clade B was found in Malawi, USA, Malaysia, and

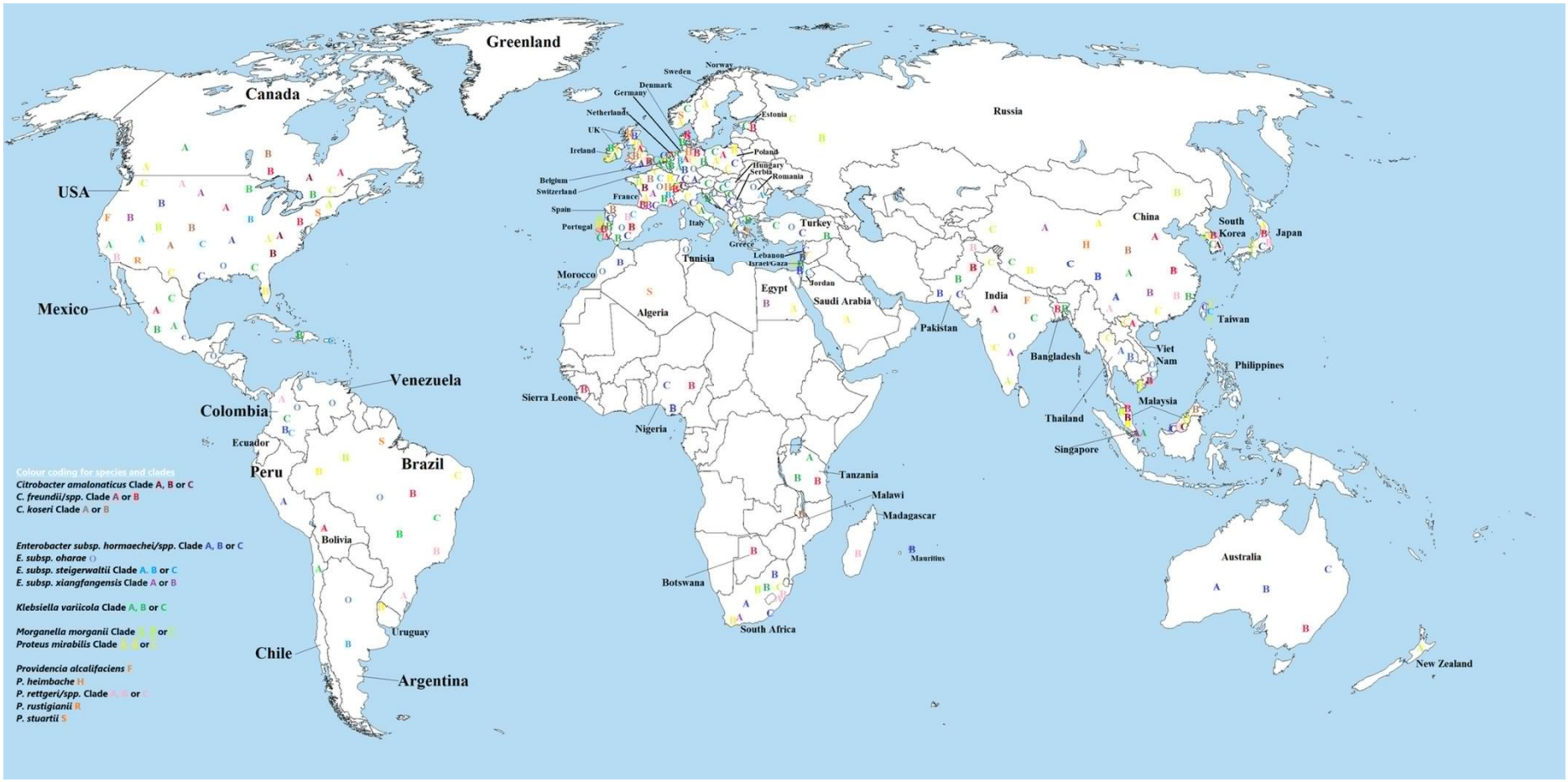


Fig 8. Global geographical distribution of *Citrobacter freundii*, *Citrobacter amalonaticus*, *Citrobacter koseri*, *Enterobacter hormaechei subsp. hormaeche*i, *xiangfangensis*, *steigerwaltii* and *oharae*, *Klbesiella variicola*, *Morganella morganii*, *Proteus mirabilis*, and *Providencia* spp. Most of these genomes were obtained from USA, Europe, South-East Asia and South America in a descending order of frequency. *C. freundii*, Enterobacter spp., *K. variicola*, and *P. mirabilis* had more diverse distribution across the globe. Each species is designated with a different colour code. Branches with bootstrap support values of ≥50 were defined as belonging to the same clade. The branch lengths also show the evolutionary distance between the isolates. Blue and red arrows show the direction of evolution as well as local and international dissemination of strains of the same clone/clade through different hosts.

France whilst clade C was only found in Malaysia, USA and Switzerland. *C. koseri* clade A (USA) and clade B (USA, Spain, UK, France, Canada, China and Malaysia) were relatively less reported with clade B being more widely distributed globally (Fig. 8).

E. oharae strains and *E. hormaechei* clades A, B and C were globally disseminated, with clade C being most widely distributed, followed by clades B and A. *E. steigerwaltii* clade C was more globally distributed than clades A (USA, Japan and Europe) and B (Argentina, USA, France and Germany). Although *E. xiangfangensis* was globally disseminated, the respective clades A (China, India, USA, S. Africa, and France) and B (France, USA, Egypt, and China) were reported from very few countries (Fig. 8).

K. variicola strains, particularly clades B and C, were of wide geographical distribution; clade A was found in relatively fewer countries globally. *M. morganii* strains were found in North America, Europe (including Russia), and South-East Asia, with clades B and C being found in South Africa. *P. mirabilis* clades A, B and C were found globally, with clade C being reported in most countries. *P. rettgeri* strains had the widest global distribution and reports from most countries among the *Providencia* spp., followed by *P. stuartii*. *P. rettgeri* clade A (China, Brazil, South Africa, Colombia, and USA) was found in fewer countries than clade B. *P. stuartii* strains were of global distribution whilst *P. rustigianii* (UK and USA), *P. alcalifaciens* (USA and India) and *P. heimbachae* (France, China, and Germany) were not (Fig. 8).

Count and distribution of ARGs per species

Providencia spp. had the highest resistome diversity (70 ARGs against 15 antibiotic classes), followed by *C. freundii* (58 ARGs against 14 antibiotic classes), *E. hormaechei* (48 ARGs against 12 antibiotic classes), *P. mirabilis* (46 ARGs against 14 antibiotic classes), *E. steigerwaltii/oharae* (44 ARGs against 12 antibiotic classes), *E. xiangfangensis* (38 ARGs

against 12 antibiotics), *K. variicola* (36 ARGs against 12 antibiotic classes), *M. morganii* (33 ARGs against 10 antibiotics), *C. amalonaticus* (22 ARGs against 11 antibiotics), and *C. koseri* (18-20 ARGs against 13 antibiotics classes) (Table S4). In all the species, β-lactamase genes were more diverse and abundant, followed by aminoglycoside, macrolide, phenicol, fluoroquinolone, tetracycline, and sulphamethoxazole-trimethoprim (SXT) ARGs; fluoroquinolone ARGs were however more diverse than macrolides and phenicols in *Citrobacter freundii/koseria* and *K. variicola* (Table S2 & Fig. S5-S10). *E. hormaechei* had the highest ARGs abundance and relative resistome richness, followed by *K. variicola, C. freundii, E. steigerwaltii/oharae,* and *E. xiangfangensis.* Chi-square analyses found the ARGs diversity, abundance, and relative richness of the species to be statistically significant; p-value < 0.0001 (Table S4).

Mcr ARGs

Mcr-9.1 ARGs were the commonest *mcr* variants identified, with very few *mcr-1* and *mcr-3* being found in *E. hormaechei* and *C. freundii* (*mcr-1, -3* and *-10*) and a single *mcr-4.3* gene being found in *P. rettgeri.* Notably, the highest prevalence of *mcr-9* was in *E. hormaechei* (n=67 *mcr-9* genes), *E. steigerwaltii/oharae* (n=32 *mcr-9* genes) and *E. xiangfangensis/cloacae* (n=19 *mcr-9* genes), followed by *C. freundii* (n=19), *C. amalonaticus* (n=5) and other *Citrobacter* spp., some of which had very few or no *mcr* genes (Fig. S5-S10).

Carbapenemases

One of the most prevalent carbapenemase among the species was KPC, with KPC-2 (n=123), KPC-3 (n=97), KPC-4 (n=14), and KPC-6 (n=1) being common. KPC-2 was higher in all the species except in *E. xiangfangensis* for which KPC-3 was more abundant (n=35) than KPC-2 (n=9). KPC was most prevalent in *E. hormaechei/oharae/steigerwaltii/xiangfangensis* and *C. freundii* strains whilst *C. amalonaticus, C. koseri,* and *Providencia* spp. *had no* KPC ARGs.

After Ambler class A KPC, Ambler class D OXA-48-like serine carbapenemases (n=65) were also very prominent in all species except *C. amalonaticus, E. xiangfangensis* and *K. variicola*, with other 0XA-48 variants such as OXA-181 (n=1, *M. morganii*) and OXA-396 (n=1, *P. rettgeri*) being relatively scarce. *C. freundii* (n=43), *E. hormaechei* (n=10), *E. oharae/steigerwaltii* (n=9) and *C. koseri* (n=3) had OXA-48 genes whilst OXA-58 (n=2) and OXA-23 (n=23) were only found in *P. mirabilis*. Other class A serine carbapenemases i.e., GES-5 (n=1, *M. morganii*) and IMI-2 (n=1, *E. hormaechei*), were also rare (Fig. S5-S10).

NDM was the commonest class B carbapenemase (n=159), followed by IMP (n=97) and VIM (n=83). NDM-1 (n=137) was the most prevalent variant and was found in *E. hormaechei* (n=68), *C. freundii* (n=15), *E. steigerwaltii* (n=13), *Providencia* spp. (n=12), *P. mirabilis* (n=10), *E. xiangfangensis* (n=6), *C. amalonaticus* (n=5), *M. morganii* (n=5), and *K. variicola* (n=3), with NDM-5 (n=16; 12 in *E. hormaechei,* 3 in *E. xiangfangensis,* and 1 in *P. mirabilis*), NDM-7 (n=3 in *E. hormaechei*) and NDM-9 (n=3 in *K. variicola*) being less prevalent. IMP-1 (n=71; 67 in *E. hormaechei* and 4 in *C. freundii*), IMP-8 (n=14; 13 in *C. freundii,* 1 in *E. steigerwaltii*), IMP-4 (n=9; 5 in *C. freundii,* 4 in *E. hormaechei*), IMP-27 (n=2 in *P. mirabilis*) and IMP-13 (n=1 in *E. oharae*) were the identified variants. VIM-1 (n=65; 26 in *C. freundii,* 14 in *E. steigerwaltii*, 21 in *E. hormaechei,* 1 in *E. xiangfangensis*, 2 in *Providencia* spp., 1 in *P. mirabilis*), VIM-4 (n=12; 5 in in *E. steigerwaltii,* 5 in *E. hormaechei,* 2 in *C. freundii*), VIM-2 (n=2 in *Providencia* spp.), VIM-31 (n=2; *E. steigerwaltii* and *E. hormaechei*), VIM-5 (n=1 in *E. hormaechei*), and VIM-67 (n=1 in *E. hormaechei*) were identified in the strains (Fig. S5-S10).

ESBLs and ampCs

TEM was the commonest ESBL and TEM-1 (a narrow-spectrum Ambler class A β-lactamase) was the most common variant to be identified in all species; particularly, TEM-1

was most abundant in *Enterobacter* spp. and *C. freundii*. OXA (particularly OXA-1, -9, and -10), CTX-M (particularly CTX-M-15) and SHV (particularly SHV-12) were also common in almost all species in a descending order of prevalence, but were very abundant in *Enterobacter* spp., specifically *E. hormaechei; C. koseri, C. amalonaticus, P. mirabilis,* and *M. morganii* had relatively low abundance of SHV, OXA and CTX-M genes. Other ESBLs genes such as bla_{SCO}, bla_{LAP}, bla_{VEB}, bla_{TMB}, bla_{SFO}, bla_{SMB}, bla_{CARB}, *bla* etc. were also rare in the various species (Fig. S5-S10). LEN and CKO/MAL class A β-lactamases were also found to be common in *K. variicola* and *C. koseri* respectively.

AmpC ARGs were basically strain-specific, with ACT, CMY, DHA, and SED being conserved in *Enterobacter* spp., *C. fruendii, M. morganii,* and *C. amalonaticus* (except clade A) respectively; FOX was rare (Fig. S4-S9).

Aminoglycoside ARGs

ARGs mediating resistance to aminoglycosides such as *aadA, aph(2")*-like, *aph(3')*-like, *aph(4')*-like, *aac(6')*-like, *aac(3)*-like, *aph(6)*-like, and *ant(2'')*-like were abundantly prevalent in almost all the clades of *C. freundii, Enterobacter* spp., and *P. mirabilis* and sparsely abundant in the other species; *aadA, aph(4')*-like, *aac(6')*-like and *aac(3')*-like ARGs were most common. 16S rRNA Methyltransferases such as *rmtB1* (*E. steigerwaltii* and *P. mirabilis,*), *rmtC/G* (*E. hormaechei, C. freundii* and *M. morganii*), and *armA* (*C. freundii, E. xiangfangensis, P. mirabilis, P. stuartii, M. morganii* and *P. rettgeri*) were rare (Fig. S5-S10).

Fluoroquinolone ARGs

Aac(6')-like, *OqxAB, QnrA/B/D/S* and *qepA* ARGs were identified, albeit *qepA* (*M. morganii*) was rare and *Oqx*A was less prevalent than *Oqx*B in all but one species. Notably,

OqxAB were virtually absent in *C. fruendii* clades. whilst chromosomal mutations in *gyr*AB and *par*CE were only observed in *K. variicola* (Fig. S5-S10).

Other ARGs

Chloramphenicol ARGs, *cmlA, catA* and *catB* were found in all the species, albeit *cmlA* was relatively rare in all the species whilst *catA/B* were conserved in *Enterobacter* spp. and *M. morganii; catA* was more prevalent than *catB. catA/B* were also abundant in *Citrobacter* spp., *P. mirabilis* and *Providencia* spp. (Fig. S5-S10). *Sulphamethoxazole-trimethoprim* ARGs, *sul-1/2/3* and *dfrA,* were enriched in *Providencia* spp., *P. mirabilis, M. morganii, Enterobacter* spp., *C. koseri* (*dfrA was virtually absent*), *C. amalonaticus* clade B2, and *C. freundii. Sul1* was more prevalent than *Sul2,* with *Sul3* being relatively rare whilst the *dfrA* variants were remarkably diverse. Indeed, both *sul1, sul2* and/or *sul3* as well as several *dfrA* variants were present concurrently in some single strains (Fig. S5-S10; Tables S2).

Several tetracycline ARGs such as *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(G), *tet(J), tet*(S), *tet*(Y), and *tet*(41), were present in all the species. Notably, *tet*(A), *tet*(B), and *tet*(D), were highly enriched in the various genomes with a descending order of frequency; *tet*(J) was most prevalent in *P. mirabilis* (Fig. S5-S10). *fosA* variants were present in all the species except *M. morganii but* were most enriched and conserved in *Enterobacter* spp. *K. variicola, P. stuartii* and *P. rettgeri.* As well, *ere*(A), *emr*(D), *erm*(B), *msr*(E), *mph*(A) and *mph*(E) macrolide ARGs were common in the various species, with *mph*(A) being richly abundant*; mph*(E) and *msr*(E) were enriched in *C. freundii, Providencia* spp. and *P. mirabilis, emr*(D) was abundant in *K. variicola* whilst *ere*(A) was abundantly enriched in *E. hormaechei* and *E. xiangfangensis.* Rifamycin ARG, *arr,* was identified in the various species, represented by *arr-3 arr-2* and *arr-1* in all the species (Fig. S5-S10).

ARGs genetic environment and mobility

The ARGs, particularly the carbapenemases, ESBLs, *mcr-1* and *mcr-10, aac(3)-IIa* *aac(6')-Ib-cr5, oqxB, qnr, sul, tet(A),* and *catA/B* were based on plasmids belonging to different replicon types. Common plasmid replicons included IncFIB(pECLA), IncFII(pECLA), IncHI2, IncHI2A, IncN, IncM, A/C, IncQ1, IncR, and IncX3. Most of the genomes contained multiple plasmids with different replicons, increasing the probability of horizontal ARGs transfer between clones and species (Table S4).

Discussion

Among Gram-negative bacterial species, *Pseudomonas aeruginosa, Acinetobacter baumannii, Vibrio cholerae,* and Enterobacterales such as *K. pneumoniae, E. coli, S. enterica,* and *Shigella* spp. are commonly isolated and implicated in nosocomial (anthroponotic), zoonotic and water/food-borne infections with MDR, extensively and pandrug resistant (XDR and PDR respectively) phenomes [4,26,40,45–47]. However, other less isolated Enterobacterales species such as *Citrobacter* spp., *Enterobacter hormaechei subsp., K. variicola, P. mirabilis, M. morganii and Providencia* spp., are increasingly being implicated in MDR, XDR and PDR infections globally as opportunistic pathogens [1,7,8,28,34,41,48]. We show herein, that *C. freundii, Enterobacter hormaechei subsp. hormaechei, xiangfangensis, oharae* and *steigerwaltii,* and Proteeae strains harbour multiple resistance mechanisms that can make them antibiotic-resistant pathogens. More concerning is the global distribution and multiple (human, animal, plants and environmental) specimen sources of these strains, which suggest that they can cause anthroponotic, zoonotic and food- and water-borne infections [8,40,49].

Hence, these opportunistic pathogens demand more attention than they have been given hitherto as the rich resistome repertoire (Table S4) identified in their genomes makes them reservoirs of ARGs [11,14,21,50]. Moreover, being intestinal denizens and commensals, they can

easily share these ARGs with facultative and obligate pathogens of humans and animals [22,51–53]. Further, their presence on plants and the environment further suggests that they can share their ARGs with food-borne and water-borne pathogens [51–53]. Notably, the *E. xiangfangensis* strains found in rice from India had very few ARGs, albeit a few had multiple ARGs (Fig. 3).

Of greater concern is the rich resistome repertoire and abundance of globally distributed *E. hormaechei subsp.* strains (Table S4). Specifically, *E. hormaechei subsp.* contained clinically important ARGs such as *mcr-9,* carbapenemases, and ESBLs, alongside fluoroquinolones (*oqxAB*), aminoglycoside, tetracycline, macrolide, fosfomycin, chloramphenicol, rifamycin and sulphomethoxazole-trimethoprim resistance mechanisms (Fig. 2-10); *oqxAB* is known to be conserved in *Enterobacter* spp. [54]. This resistome repertoire was also seen in *K. variicola, C. freundii, P. rettgeri P. mirabilis, P. stuartii,* and *M. morganii,* to a relatively lesser degree in a descending order (Table S4). This is a worrying observation as colistin, carbapenems and tigecycline are last resort antibiotics used to treat fatal bacterial infections [55,56]. In most cases, these antibiotics are used in combination with fosfomycin, fluoroquinolones and aminoglycosides to treat carbapenem-resistant Enterobacterales (CRE) infections [55,56]. Evidently, the presence of all these resistance mechanisms to these antibiotics, could make these species PDR and automatically qualify them as critical priority pathogens per the WHO criteria [32,33].

As well, members of the tribe Proteeae viz., *M. morganii*, *P. mirabilis*, and *Providencia* spp., are known to have intrinsic resistance to colistin, tigecycline, aminopenicillins, amikacin, tobramycin, lincosamides, macrolides, fosfomycin and first- and second-generation cephalosporins [1,7,35,57]. Thus, the presence of additional resistance determinants in this tribe is especially worrying. Already, there are increasing reports on the isolation of Proteeae species in recurrent urinary tract infections (UTIs) infections, which is facilitated by the increasing use of colistin to treat MDR infections; their broad intrinsic resistance mechanisms enable

them to flourish during antibiotic chemotherapy [1,7,8,35,58,59]. These observations evince the growing threat of antimicrobial resistance globally and its associated after-effects, supporting the need for efficient antibiotic stewardship to safeguard current antibiotic arsenals as well as discover novel ones [3,14].

As shown in the phylogenomic and phylogeographic analyses, local and international transmission, or outbreaks of strains within the same clone, clade, and subclade i.e., of very close evolutionary distance, were observed. Notably, these closely related strains were isolated from humans, animals, plants, and the environment and they harboured important resistance determinants as described above. The phylogenomics showed the gradual evolution of a single strain during dissemination from host to host and depict the fact that antibiotic resistance respects no boundaries. Notably, a large part of *E. hormaechei* clade C consisted of strains from human stools/urine in Japan; these were closely related strains evolving from Spain, Taiwan, China, and Greece (Fig. 4-10). A similar observation was made with strains of close evolutionary distance from humans in Nigeria, France, Spain, Portugal, and Lebanon as well as with strains from humans, animals, and the environment in several countries such as France, Germany, USA, Pakistan, Morocco, Lebanon, and Poland in clade C. *E. hormaechei* strains were also found in desert sands in Morocco, showing their broad and diverse niches (Fig. 4-10).

Uniform and non-uniform resistome patterns were seen between strains of the same clade/clone in almost all the species. For instance, the same resistome was seen in *C. amalonaticus* clade B2 (Fig. S1B), *E. cloacae* clade B (Fig. 3) and *P. mirabilis* clade C2 (Fig. 7) whilst differing resistome patterns were observed in the other species and clades. This observation supports two phenomena: firstly, the clonal expansion of strains harbouring the same resistome repertoire on both chromosomes and plasmids and secondly, the horizontal transmission of genetic elements bearing the same or different ARGs across clones and

species (Table S4). During clonal expansion of strains, there is a concomitant replication of resistance plasmids alongside chromosomal replication, leading to daughter cells with the same resistome diversity [11,12,21,60]. As well, horizontal gene transfer of ARGs between bacteria can alter the resistome diversity and composition of daughter clones emanating from the same ancestor [11,12,21,60]. In this case, both vertical and horizontal transmission of ARGs could be ongoing during the evolutionary epidemiology of the various clades and species across the globe. The horizontal transmission of the ARGs is depicted by their association with plasmids with diverse replicons and promiscuity i.e., IncF, IncX, IncH, IncN, IncR and (Table S4).

As shown in a recent study, carbapenemases are particularly associated with IncF, IncX, A/C, IncN, and IncI plasmids. Notably, the various plasmid types were associated with particular carbapenemases globally, showing that transmission of carbapenemases are mostly mediated by conjugative plasmids [25,61]. Hence, the identification of these plasmid types in genomes bearing carbapenemases and other ARGs is not surprising. In particular, the similar genetic environment of the ARGs on similar plasmid types suggests their mobility through these plasmids into different cells of the same species.

The presence of multiple ARGs in a single strain might not necessarily mean they are all being expressed in the bacteria's phenome as antibiotic-susceptible strains have been found to harbour ARGs. For instance, colistin- and fosfomocyin-sensitive Enterobacterales have been reported in strains harbouring the *mcr-9* and *fosA* genes [1,5,7,8]. Nevertheless, the ability of these ARGs to be expressed in the presence of strong promoter or transferred to another host with a stronger promoter for subsequent expression cannot be gainsaid [62]. Indeed, antibiotic abuse could serve as an inducer to trigger the transfer and expression of these vast resistomes in the microbial phenomes [35,63], necessitating the importance for judicious antibiotic use.

It is revealing to note that *K. variicola, M. morganii, C. amalonaticus* and *C. koseri* strains had very fewer ARGs diversity except for *K. variicola* clade B2 and *C. amalonaticus* clade B2, despite the global distribution of *K. variicola* (Table S4). Notably, *C. amalonaticus* clade B2, which were all from France, were remarkably enriched with ARGs including bla_{NDM}, - representing a local outbreak of XDR *C. amalonaticus* strains (Fig. S1B). Thus, even in species with fewer resistome diversity and abundance, there are MDR, XDR and PDR strains causing local outbreaks.

Instructively, the global distribution and concentration of ARGs reflect the global consumption of antibiotics as demonstrated by Van Boeckel et al. (2015), with countries such as China, USA, Brazil, Germany (and Europe), India, Mexico, Thailand, Malaysia and South Africa (Fig. 8) having higher ARGs. This closely reflects the volumes of antimicrobials consumed per country globally [64] as well as the classes of antibiotics used globally in veterinary and clinical infections [15,64,65]. Particularly, penicillins, tetracyclines, sulphonamides, fluoroquinolones and aminoglycosides were commonly used in animal production [15,64,65] whilst β-lactams (specifically penicillins and cephalosporins), fluoroquinolones, SXT, macrolides, tetracyclines, and aminoglycosides were commonly used for humans globally [15,66]. These classes further mirror the types and relative concentrations of ARGs (β-lactamases, aminoglycosides, macrolides, phenicols, fluoroquinolones, tetracyclines, and SXT resistance genes) identified in these species globally (Figure S5-S10), suggesting that the types of antibiotics used in animals and humans are selecting for ARGs in rare Enterobacterales species.

Notably, most of the genomes included in this analysis were from the USA, Europe, and South East Asia. This may be because these regions have higher prevalence and incidence of infections resulting from these pathogens or that these areas have enough financial and technical means to undertake genomic sequencing of these isolates in periodic surveillance

studies. Specifically, genomes of these species were relatively scarce from a large part of Russia, Middle and North-West Asia, Africa, the Caribbean and parts of South America and Canada. Given the alarming resistome diversity and composition realised in this analyses, it is incumbent for all nations to intensify and adopt genome-based epidemiological studies to quickly identify the sources and reservoirs of ARGs to pre-empt outbreaks of MDR, XDR and PDR pathogens.

Conclusion

In conclusion, less described Enterobacterales species viz., *Enterobacter hormaechei subsp. hormaechei, xiangfangensis, steigerwaltii* and *oharae, C. freundii, M. morganii, P. mirabilis, P. stuartii* and *P. rettgeri,* contain globally distributed MDR, XDR and PDR strains that cause local and international outbreaks, transmitting through humans, animals, food, water and other environmental media or sources. Notably, the resistome repertoire of these relatively rare species were equally or more abundant and diverse as those of commonly isolated species. Hence, intensified efforts should be made to increase education on antibiotic stewardship to safeguard the potency of available antibiotics and reduce the selection and dissemination antibiotic-resistant Enterobacterales. Infection prevention and control as well periodic genomic surveillance of communities, hospitals, farms, water bodies and the general environment (One Health) should be undertaken to pre-empt outbreaks of MDR strains and inform infection control measures.

Notwithstanding the revealing details obtained in this study, strains with clinically important ARGs whose genomes are not deposited in NCBI/PATRIC or whose genomes are not sequenced before January 2020 will be missed; hence, the information contained herein are true up to January 2020. Furthermore, not all genomes provided a complete meta-data, which made it difficult to undertake a complete statistical analyses and comparison between species.

The resistome abundance and relative richness were also affected by the total number of genomes per species such that species with more genomes had higher ARG abundance and relative richness ratio; these biases the abundance and relative resistome richness ratio against isolates with fewer genomes. Moreover, species such as *M. morganii* and *C. amalonaticus* had relatively fewer genomes from few countries, which may be due to their relatively lower isolation rates around the globe. Finally, antibiotic-resistant isolates are mainly analysed and sequenced in clinical practise to the exclusion of susceptible ones, which can bias the resistance abundance obtained in this work. Nevertheless, the global phylogeography and resistome epidemiology of these emerging opportunistic pathogens provide an important picture of the ARGs, sources and transmission patterns of these species.

Methods

Included genomes

Genomes (both draft and complete) of *Citrobacter spp*. (including *amalonaticus*, *freundii, koseri, werkmanii, brakii, portucalensis* and *youngae*), *Enterobacter hormaechei subsp. hormaechei, xiangfangensis, steigerwaltii,* and o*harae, Providencia* spp. (including *alcalifaciens, burhodogranariea, heimbachae, rettgeri, rustigianii,* and *stuartii*) and *Proteus mirabilis* deposited at GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and PATRIC (https://www.patricbrc.org/) from the first deposited genomes up to January 2020 were pooled and filtered to remove poor genome sequences. Briefly, genomes with coverage of less than 10X were removed. No distinction was made between genomes sequenced on different sequencing platforms i.e., genomes from all sequencing platforms were included. Other genome characteristics such as L50, N50, contig number and read length were not used to exclude or discriminate between the genomes. The geographical and sample sources of the genomes were also not used as an exclusion criteria. Sequencing adaptors and contigs below

200 nucleotides were removed using Cutadapt (https://cutadapt.readthedocs.io/en/stable/). The metadata associated with the downloaded genomes are found in Table S1.

Phylogenetics and evolutionary epidemiology analyses

Briefly, plasmid sequences, phages, and poor genomes i.e., genomes with sizes below the average genome size of each species viz., 3-4Mb, were removed. Subsequently, the remaining genomes were grouped into the respective species and aligned using MUMmer4 [67]; genomes of isolates that did not share at least 1000 core protein genes with all the included genomes were then removed after building a consensus set of genes among all the aligned genomes; the removal of such genomes were done using the "Protein Family" tab filtering tool in PATRIC. The remaining genomes were aligned (with MUMmer) and run through RAxML in batches of 200 genomes to draw phylogenetic trees using the maximum-likelihood method. A minimum of 1000 genes were used for the alignment (after building a consensus set of core genes from the aligned genomes) and a bootstrap resampling of 1000x was used; a consensus. The trees were drawn as chrono-/phylo-grams (instead of cladograms), with the branch lengths showing the evolutionary distance between the isolates' genomes. The trees were rooted to a reference *Streptomyces* spp. MUSC164 genome and circularised. The trees were annotated using Figtree (http://tree.bio.ed.ac.uk/software/figtree/) to show their sequence type (ST), host (species), country and year of isolation.

A clade is defined as a branch on the phylogenetic tree containing cluster of genomes with very close evolutionary distance and bootstrap support value of ≥50%; the bootstraps values are shown on the branches sub-branches within these clades were designated (with different highlights) as sub-clades. Isolates with a bootstrap value of ≥70% are defined as closely related. Each clade was given a unique colour to distinguish it from other clades. An outbreak in a country was inferred when isolates (genomes) of the same species and clone (ST) were

collected within the same day, month and/or year within a country or across countries. Red and blue arrows were used to show the evolutionary trajectory and dissemination of strains of the same clones or clades, locally (within a country) and internationally (across national boundaries) respectively; the branch length showed the evolutionary distance of each strain from the other.

Geographical distribution of species and clones

The various clades and subclades per species were manually drawn unto a global map to show their phylogeographic distribution using Paint 3D. Different colour codes were used to distinguish between the various species and clades.

Resistome analyses and statistics

A resistance gene is herein defined as a gene that confers resistance to an antibiotic. A resistome is herein defined as all the ARGs found within a given species. Resistome diversity is here defined as the count of unique ARGs within a genome or species whilst resistome abundance refers to the cumulative count (sum) of total ARGs in each genome for a given species. The resistomes of the included genomes were individually obtained from the NCBI Pathogen Detection database (https://www.ncbi.nlm.nih.gov/pathogens/isolates#/search/), which annotates all deposited genomes and curates their ARGs. The resistomes were aligned per strain and colour-coded per clade or species to show their association per species, clone, or clade. These were then associated with the phylogenomic trees to ascertain the resistome dynamics per clone, clade, species, and geographical location.

A count of the various ARGs within each species is summarised in Figures S1-S6

ARG mobility analyses

The genetic location (plasmid or chromosomal) of important ARGs i.e., carbapenemases, colistin, fluoroquinolones, and aminoglycosides resistance genes, was determined by uploading the genomes to PlasmidFinder and pMLST. The contigs with the respective ARGS were analysed with BLASTn to determine their closest nucleotide homology on GenBank (Table S4).

General statistics

A count of genomes, species, clones, countries from which the genomes were obtained, and specimen sources of the genomes are shown in Supplementary Tables S1-S2. The abundance and diversity of ARGs per species are shown in Table S3, with each clone/clade having a unique highlight to mirror that on the phylogeny trees. The counts of all unique ARGs per species were determined to calculate the resistome diversity and the number of antibiotic classes affected whilst the sum of the counts of all ARGs for each species was computed to determine the resistome abundance. The relative resistome abundance (resistome richness) for each species was computed by dividing the resistome abundance by the resistome diversity to get a ratio. Pearson's Chi-squared test was used to determine the statistical significance of the unique ARGs, total ARGs abundance, and relative resistome richness ratio per species; GraphPad Prism 8.4.3 was used to compute the Chi-squared test. A p-value of < 0.05 was defined as statistically significant.

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