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Genomic and Functional Characterization of CTX-M-15-Producing *Klebsiella pneumoniae* ST307 Isolated from Imported Leopard Tortoises in Germany

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Abstract: The *Klebsiella pneumoniae* ST307 clone, identified in the mid-1990s, has emerged as a global antimicrobial-resistant (AMR) high-risk clone, significantly contributing to the global health challenge also posed by other AMR *K. pneumoniae* lineages. The acquisition of a $bla_{\text{CTX-M-15}}$-carrying plasmid has facilitated its widespread dissemination. At Europe's major transport hub for the movement of live animals, Frankfurt Airport, a shipment of 20 live leopard tortoises was sampled during German border control in 2014. Phylogenetic analysis (MLST) identified a *K. pneumoniae* ST307 strain, prompting further investigation. Our analysis revealed the presence of a ~193 kb plasmid carrying a broad range of AMR genes, including $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1B}}$, $bla_{\text{OXA-1}}$, *aac(3)-IIa*, *aac(6′)-Ib-cr*, *aph(3″)-Ib*, *aph(6)-Id*, and *qnrB1*. Additionally, mutations in the quinolone resistance-determining region in *gyrA* (S83I) and *parC* (S80I) were detected. Phenotypic testing demonstrated resistance of the isolate to the most common antimicrobials used in both human and veterinary medicine; exceptions included carbapenems and newer β-lactamase inhibitor combinations. Because the role of imported exotic animals in the dissemination of AMR genes is largely deficient, the present study fills yet missing mosaic pieces in the complete picture of extended-spectrum β-lactamase (ESBL)-producing *Enterobacterales*.

Keywords: *Klebsiella pneumoniae*; ST307; CTX-M-15; plasmid; tortoise; antimicrobial resistance; virulence

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1. Introduction

Klebsiella (*K.*) *pneumoniae* is a Gram-negative opportunistic pathogen of the *Enterobacterales* order that is frequently associated with antimicrobial-resistant (AMR) nosocomial infections worldwide. *K. pneumoniae* sequence type ST307 is an emerging AMR high-risk clone that was described in Europe in 2008 for the first time [1,2]. Since then, it has spread globally among humans and animals, causing severe hospital- and community-acquired infections such as pneumonia, urinary tract and bloodstream infections. Moreover, *K. pneumoniae* ST307 has become a threat to public health as it frequently produces extended-spectrum β-lactamases (ESBL) and carbapenemases, both of which are β-lactamases inactivating their corresponding antibiotic by cleaving the β-lactam ring. Therefore, both enzyme groups contribute to an AMR phenotype by hydrolyzing penicillins and cephalosporins, and in the case of carbapenemases, carbapenem antibiotics [2–5]. Among ESBLs, cefotaximase-Munich (CTX-M)-type ESBLs have become dominant in clinical *Enterobacterales* isolates [6]. CTX-M-15 ESBL-producing *K. pneumoniae* was first isolated from human urine and pulmonary specimens collected from hospitalized patients in India in 1999 [7]. Today, CTX-M-15 frequently emerges in *K. pneumoniae* with a multidrug-resistant (MDR) phenotype and is often associated with the presence of multiple ESBL genes, thereby accounting for limitations in antimicrobial treatment [2,6,8]. CTX-M-15-producing *K. pneumoniae* ST307 has often been isolated from humans worldwide and is increasingly found in various animal species as

well [5,9–16]. In Europe, it was first isolated from an African spurred tortoise (*Centrochelys sulcata*) in the UK in 2019, followed by several reports on the isolation in other species, such as cats, dogs, horses, cattle, and birds [17–24]. Because the importation of live exotic companion animals is still at a high level, fundamental research on the potential risk posed by the dissemination of antimicrobial-resistant bacteria seems to be justified [25]. Therefore, it is imperative to screen for antimicrobial resistance and AMR genes of bacteria present in these imported animals. In the context of another research project analyzing the prevalence and AMR genes of *Enterobacterales* (unpublished data) and *Acinetobacter* spp. in imported pet reptiles, a *K. pneumoniae* ST307 strain was isolated and subsequently further analyzed [26,27]. As a result, we report here the isolation of an MDR CTX-M-15-producing *K. pneumoniae* ST307 from leopard tortoises (*Stigmochelys pardalis*) imported to Germany from Ecuador.

2. Materials and Methods

2.1. Sample Collection and Bacterial Species Identification

The acquisition and cultivation of samples were performed as previously described [26]. In brief, a pooled fecal sample was collected in 2014 at Frankfurt Airport, Germany, from a shipment of 20 farm-bred leopard tortoises from Ecuador, as declared in the relevant travel documents, in cooperation with authorized veterinarians from the Border Control Post of Frankfurt Airport. The sample was stored in a sterile, fecal sample tube at 4–7 °C and transported to the Institute of Hygiene and Infectious Diseases of Animals in less than 48 h. For cultivation, the sample was streaked onto 5% sheep blood agar (Merck, Darmstadt, Germany), water-blue-metachrome-yellow-lactose-agar (Gassner agar; Oxoid, Wesel, Germany), and MacConkey agar (Oxoid) containing 1 mg/L cefotaxime (Sigma-Aldrich/Merck, Darmstadt, Germany) directly after arrival of the sample in the laboratory. The plates were incubated at 37 °C for 24 h in ambient air. Species identification was accomplished by MALDI-TOF MS (Bruker Daltonics, Bremen, Germany; DB 9045). All obtained isolates were stored in Brain Heart Infusion (BHI) broth (Oxoid) containing 30% glycerol at −70 °C until further use.

2.2. Genomic Analysis

The *K. pneumoniae* isolate IHIT34097 was subjected to whole-genome sequencing using the Nextera XT Library Preparation Kit (Illumina GmbH, Munich, Germany), which was sequenced on a MiSeq sequencer (Illumina Inc., San Diego, CA, USA) in 250 bp paired-end mode with a minimum coverage of 100-fold. Genomes were de novo assembled using SPAdes v.3.15.1. and annotated using RAST v.2.0 [28,29]. To verify the plasmid sequence, the DNA was additionally sequenced on an Oxford Nanopore GridION GI1-5 (Oxford Nanopore Technologies, Oxford, UK) using FLO-MIN114 (R10.4.1) flow cells. Sample preparation was accomplished according to the manufacturer's protocol without any optional steps or size selection using the Rapid Barcoding Kit SQK-RBK114-24, running on ONT's MinKNOW control software Version 4.5.0.

The Bacterial Isolate Genome Sequence Database (BIGSdb) Version 1.42.3 was used for the detection of virulence genes, sero(geno)typing, and multilocus sequence typing (MLST) [30]. Capsule biosynthesis (K) and lipopolysaccharide antigen (O) loci were typed using Kaptive [31]. Additionally, BacWGSTdb was utilized to determine isolates with close synteny based on a core genome multilocus sequence typing (cgMLST)-based comparison [32]. All isolates with a maximum difference of 50 alleles were extracted from the National Center for Biotechnology Information (NCBI) database, and cgMLST allelic distances were used for the calculation of a minimum spanning tree as implemented in Ridom Seqsphere+ v9.0 [33,34]. Additionally, isolate BL714 (SRA ERS9590854), a CTX-M-15-producing *K. pneumoniae* ST307 strain that was isolated in 2019 from a tortoise in the UK, was included in the minimum spanning tree (Supplementary Table S1) [24]. For the determination of plasmids and AMR genes, PlasmidFinder 2.1 and ResFinder 4.4.2 were employed [35,36]. Based on the results of PlasmidFinder, highly related plasmids were searched with the NCBI microbial nucleotide basic local alignment search tool (BLAST). They were

aligned using Geneious 8.1.9 (Biomatters, Auckland, New Zealand) in order to display the complete plasmid of IHIT34097. Alignment was verified using bridging polymerase chain reactions (PCR) (Supplementary Table S2). Closely related plasmids according to single nucleotide polymorphism (SNP)-based analysis of BacWGSTdb containing a $bla_{\text{CTX-M-15}}$ gene were extracted from the NCBI database, and BLAST Ring Image Generator (BRIG) was used to display circular comparisons [32,33]. Plasmid pIHIT34097 was used as a reference with an upper identity threshold of 90% and a lower identity threshold of 70%, and nucleotide sequence search was performed with BLASTn (Supplementary Table S1).

2.3. Phenotypic Analysis

Antimicrobial susceptibility testing was performed by broth microdilution using the Merlin Micronaut-S software Version 6.00 with the companion animal layout E1-319-100 (Merlin Diagnostika GmbH, Bornheim-Hersel, Germany), which includes antimicrobial substances typically used in veterinary medicine. For antimicrobial substances utilized in human medicine, the VITEK®2 compact 15 with the VITEK®2 system (v9.03.3, bioMérieux, Nürtingen, Germany) was employed (AST-N430, AST-XN24, bioMérieux).

For the detection of a hypermucoviscous phenotype, a string test was performed as previously described by Fang et al. (2004) [37]. Therefore, a fresh single colony cultivated on a 5% sheep blood agar plate (Merck, Darmstadt, Germany) was touched with a sterile inoculation loop. The test was considered positive if a viscous filament of >5 mm was formed.

Siderophore production was determined qualitatively by using the chrome azurol S (CAS) plate assay. Plates were prepared based on Schwyn and Neilands (1987) and 5 µL of an overnight culture of the isolate in Luria-Bertani (LB) medium [containing NaCl and yeast extract (both Merck), bacto-tryptone (Becton Dickinson GmbH, Heidelberg, Germany) and agar-agar (Carl Roth GmbH + Co. KG, Karlsruhe, Germany)], adapted to an optical density (OD) at 600 nm of 0.1, were deposited on the CAS agar plate and incubated at 37 °C for 24 h [38,39]. Then, the color change in the CAS agar from blue to orange was documented and measured.

To evaluate survival in human blood, a modified version of the serum resistance assay by Taylor and Kroll (1983) was performed [40]. Bacterial cultures were set to 0.5 McFarland standard turbidity in 0.9% NaCl solution (Merck). An amount of 1 mL of the bacterial suspension was centrifuged at 7500× *g* for 5 min at room temperature and resuspended in 1 mL of 1× phosphate-buffered saline (PBS). Next, 100 µL of the suspension was mixed with 100 µL of human serum (Pan Biotech, Aidenbach, Germany) in 1.5 mL tubes. Subsequently, 10 µL of the mixture was serially diluted, and 50 µL of each serial dilution was plated onto LB agar plates (Merck, 0 h count plates). The tubes were incubated at 37 °C for 4 h in ambient air. A second serial dilution was then performed and plated onto LB agar plates (Merck, 4 h count plates). All plates were incubated at 37 °C for 24 h in ambient air. After incubation, colony-forming units (CFU) were counted. Serum-sensitive strain W3110 served as a negative and serum-resistant strain PBIO1289 as a positive control [41]. To validate the bactericidal effect of the serum, serum-sensitive W3110 mixed with heat-inactivated serum was also included as a control. The assay was performed in duplicates with one biological control.

3. Results

3.1. Antimicrobial Resistance Genes and Chromosomal Mutations

The *K. pneumoniae* strain IHIT34097 isolated from a pooled fecal sample of leopard tortoises was identified as ST307, displaying the KL102 capsular type, associated with the *wzi* allele 173 and O2V2 serotype. Antimicrobial resistance genes included $bla_{\text{CTX-M-15}}$ for ESBL production, $bla_{\text{TEM-1B}}$, $bla_{\text{SHV-28}}$ and $bla_{\text{OXA-1}}$ for broad and narrow β-lactam resistance, respectively, and *aac(3)-IIa*, *aac(6′)-Ib-cr*, *aph(3″)-Ib*, and *aph(6)-Id* for aminoglycoside resistance. The genes *qnrB1*, *aac(6′)-Ib-cr*, *oqxA*, and *oqxB*-like conferring quinolone resistance were detected; additionally, mutations in the quinolone resistance determining region (QRDR) were detected in *gyrA* (S83I) and *parC* (S80I). Also, *dfrA14* and *sul2* for folate pathway antagonist resistance, *fosA* for fosfomycin resistance, and *tet*(A) for tetracycline resistance were found.

3.2. Virulence-Associated Genes

Strain IHIT34097 harbored the type 3 fimbrial gene cluster *mrkABCDFHIJ*, type 1 fimbrial gene cluster *fimABCDEFGHIK*, and regulatory genes *rcsAB*, which confer the up-regulation of *cps* gene expression. For siderophore production, the gene cluster for enterobactin (*entABCEF*, *fepABCDG*, *fes*, and *ybdA*) and *iutA* coding for the aerobactin receptor were found. Genes encoding other proteins involved in iron acquisition, such as yersiniabactin, aerobactin synthesis, and salmochelin, were not identified. The isolate was also negative for the genotoxin colibactin encoding chromosomal island gene *pks* and the gene cluster for the bacteriocin microcin E492. Moreover, *peg344* (unspecific metabolite transporter) and *rmpA/rmpA2* (synthesis of capsular polysaccharide), both attributed to a hypervirulent phenotype, were not present.

3.3. Genomic Analysis

The full assembly of strain IHIT34097 resulted in the 5,493,197 bp chromosome and a 193,223 bp IncFIB$_K$:IncFII$_K$ plasmid termed pIHIT34097 (Bioproject ID: PRJNA1095285). Based on a single nucleotide polymorphism (SNP) comparison, the most closely related plasmid was p72_FIBkpn (Acc. No. NZ_CP034282.1), isolated from a human patient of an intensive care unit in a public hospital in South Africa in 2014 [42]. All detected AMR genes could be demonstrated on the plasmid pIHIT34097, except for *bla*$_{SHV-28}$, *fosA*, and *oqxAB*, which were encoded on the chromosome. The genomic comparison showed a high similarity to other global plasmids including a conserved multi-drug resistance region of ~40 kb. This MDR region contains the genes *bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$, *bla*$_{OXA-1}$, *aac(3)-IIa*, *aac(6′)Ib-cr*, *aph(6)-Id*, *aph(3″)-Ib*, *sul2*, *catB*, and partially *qnrB1* and *dfrA14*, as well as an arsenic, copper and silver resistance gene cluster (Figure 1).

Figure 1. Circular visualization of *K. pneumoniae* plasmids as compared to pIHIT34097 using BRIG [43]. Details of the origin and genes harbored by the plasmids are presented in Supplementary Table S1.

All of the 68 closely related *K. pneumoniae* isolates were of human origin (n = 60) or were retrieved from the environment (n = 8). Only IHIT34097 and BL714 (SRA ERS9590854) were of animal origin, both isolated from tortoises. Core genome MLST-based distance calculations revealed no clustering of isolates according to their geographical origin (Figure 2).

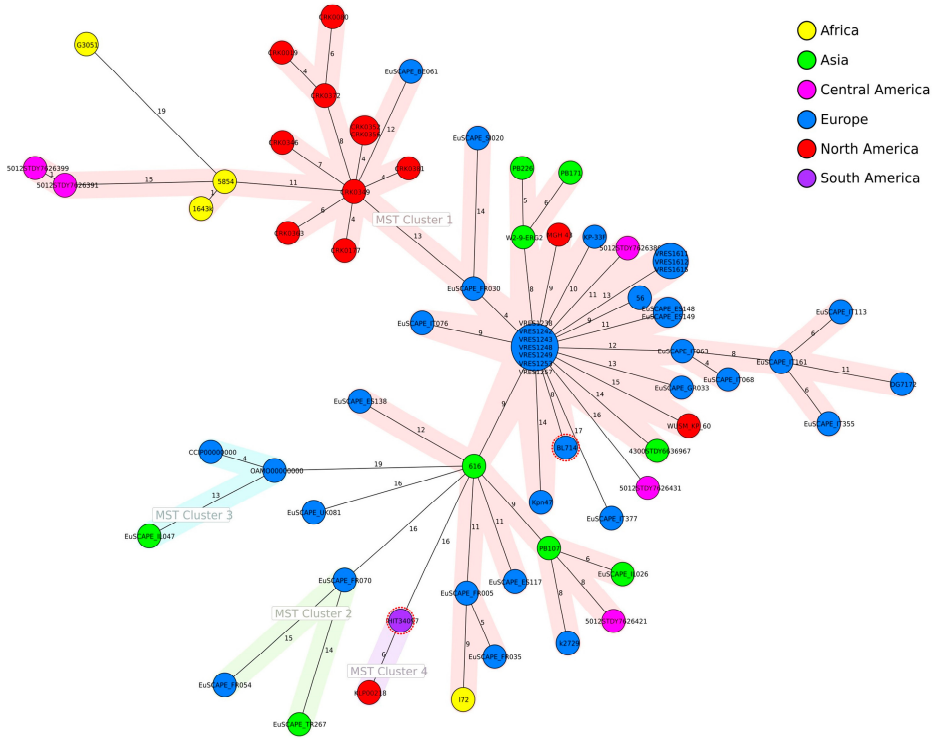


Figure 2. Minimum spanning tree (MST) based on allelic distances of 70 *K. pneumoniae* genomes (60 × human, 8 × environment, 2 × tortoise) determined by cgMLST analysis. The nodes are colored according to the geographical origin of the strains. The size of the nodes is proportional to the number of isolates. Numbers on branches indicate allele differences between core genomes. The nodes representing IHIT34097 and BL714, both isolated from tortoises, are marked with a dotted margin. Strains are assigned to MST clusters 1 to 4 with a threshold of 15 allele differences. Details of the origin and genes harbored by the strains are presented in Supplementary Table S1.

3.4. Phenotypic Analysis

3.4.1. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing of strain IHIT34097 revealed an MDR pattern, defined as non-susceptibility to at least one agent in three or more antimicrobial categories, according to Magiorakos et al. (2012) [44]. VITEK®2 testing confirmed an ESBL phenotype (Table 1).

Table 1. Antimicrobial susceptibility of *Klebsiella pneumoniae* strain IHIT34097, cultured from the feces of leopard tortoises.

Antimicrobial [1]	Interpretation	MIC (mg/L)
Amoxicillin/Clavulanic acid	I	=16/8
Cephalexin *	R	>16
Cefovecin *	R	>4
Chloramphenicol	S	=4
Doxycycline *	R	>1
Enrofloxacin *	R	>2

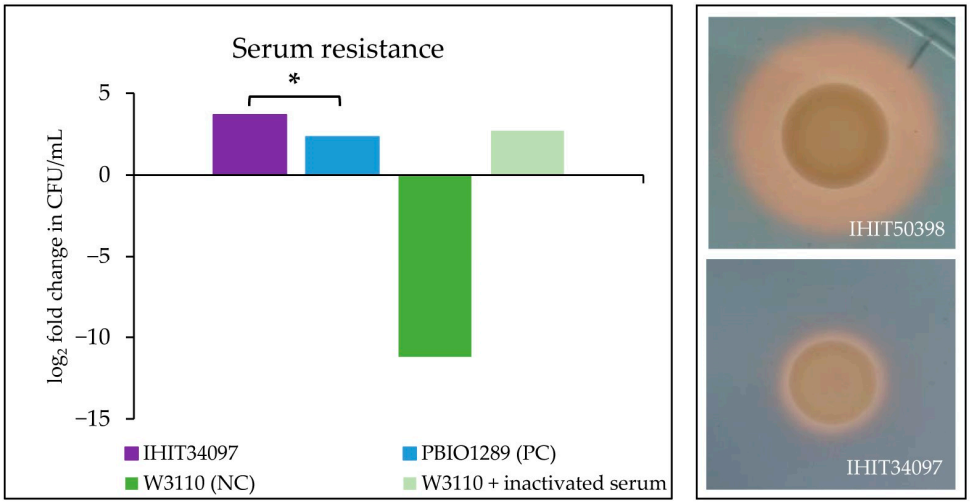
Table 1. *Cont.*

Antimicrobial [1]	**Interpretation**	**MIC (mg/L)**
Florfenicol *	I	=8
Gentamicin	R	>4
Pradofloxacin *	R	>1
Trimethoprim/Sulfamethoxazole	R	>2/38
Tetracycline	NS	>4
Antimicrobial [2]	**Interpretation**	**MIC (mg/L)**
ESBL	Positive	
Ampicillin/Sulbactam	R	≥32
Piperacillin/Tazobactam	NS	=16
Cefuroxim	R	≥64
Cefuroxim-Axetil	R	≥64
Cefpodoxim	R	≥8
Cefotaxim	R	≥64
Ceftazidim	R	=32
Imipenem	S	≤0.25
Meropenem	S	≤0.25
Gentamicin	R	≥16
Ciprofloxacin	R	≥4
Tigecycline	S	≤0.5
Fosfomycin	S	≤16
Nitrofurantoin	S	=32
Trimethoprim/Sulfamethoxazole	R	≥320
Antimicrobial [3]	**Interpretation**	**MIC (mg/L)**
Temocillin	NS	≤4
Ceftazidim/Avibactam	S	=0.25
Ceftolozan/Tazobactam	S	≤0.25
Imipenem/Relebactam	S	≤0.25
Meropenem/Vaborbactam	S	≤0.5
Eravacyclin	S	0.25
Colistin	I	0.5

[1] Companion Animal Layout E1-319-100 (Merlin Micronaut-S software Version 6.00, Merlin Diagnostika GmbH, Bornheim-Hersel, Germany); [2] AST N430 and [3] AST XN24 (VITEK®2 system, v9.03.3, bioMérieux, Nürtingen, Germany); R—resistant, I—intermediate, NS—non-susceptible, S—susceptible; minimum inhibitory concentrations (MIC) were determined using standardized broth dilution methodology based upon the recommendation of the Clinical and Laboratory Standards Institute (CLSI). As there are no defined clinical breakpoints for reptiles, results were interpreted according to CLSI (M100 ED33:2023) and European Committee on Antimicrobial Susceptibility Testing (EUCAST; Version 14.0, 2024) breakpoint tables for human isolates. Where breakpoints for human isolates were not provided, CLSI breakpoints for other animal species according to VET01S ED7:2024 have been applied as indicated by an asterisk (*).

3.4.2. Mucoid Phenotype, Siderophore Production and Serum Resistance

The isolate did not demonstrate a hypermucoviscous phenotype, as confirmed by the negative string test. However, it did exhibit a serum-resistant phenotype (Figure 3a). Siderophore production was detected by the qualitative CAS plate assay based on the formation of a halo. For better comparability, the halo formation of IHIT34097 is shown in Figure 3b together with the internal *iucABCD* positive *K. pneumoniae* control strain IHIT50398 (liver, pig, Germany, 2022), which displayed a very broad halo. Both isolates additionally harbored the siderophore production genes *entABCEF*, *fepABCDG*, *fes*, *ybdA*, and *iutA*. IHIT34097 revealed a small halo of 9.5 mm surrounding a 7.5 mm wide colony, indicating comparatively low siderophore production, possibly due to the absence of *iucABCD* genes, which encode for aerobactin synthesis [1,45].



(a) Serum resistance assay (b) CAS plate assay

Figure 3. Results of phenotypic virulence-associated assays. (**a**) Survival in 50% human serum after 0 and 4 h of incubation compared to serum-resistant strain PBIO1289 and serum-sensitive strain W3110 revealed a serum-resistant phenotype of IHIT34097. The serum-sensitive strain W3110 mixed with heat-inactivated serum was added to validate the bactericidal effect of the serum. Results are given as a $\log_2$ fold change in CFU/mL. IHIT34097 showed a significantly greater increase in growth after 4 h of incubation compared to the positive control PBIO1289 (Fisher exact * $p < 0.00001$; the result is significant at $p < 0.05$). (**b**) Qualitative siderophore production of IHIT34097 revealed a small halo of 9.5 mm and therefore positive siderophore production (*entABCEF*, *fepABCDG*, *fes*, *ybdA*, and *iutA*). For better comparability, *iucABCD* positive strain IHIT50398 is also shown, indicating an increased siderophore production as defined by halo comparison (16.5 mm halo, 8.5 mm colony).

4. Discussion

Initial isolation of CTX-M-15-producing *K. pneumoniae* ST307 strains from mammals in Europe was documented in cats, dogs, and horses between 2012 and 2014 [18,19,21]. The first occurrence of ST307 *K. pneumoniae* from a reptile in Europe, namely the UK, was reported by Foster et al. (2020). In 2019, *K. pneumoniae* strain BL714 was isolated from the lung tissue of an African spurred tortoise (*Centrochelys sulcata*) deceased due to pneumonia [24]. The isolation of the CTX-M-15-producing *K. pneumoniae* ST307 from leopard tortoises imported from Ecuador to Germany presented in this study dates back to 2014. This proves an earlier introduction of this strain to reptiles in Europe than previously assumed.

The isolation of ESBL-producing *Enterobacteriaceae* from turtles has been reported previously from several sources. In free-living turtles, there are reports on the isolation of a temoneira (TEM)-236 and sulfhydryl variable (SHV)-12 ESBL-producing strain of *Citrobacter* (*C.*) *freundii* isolated from an injured loggerhead sea turtle (*Caretta caretta*) in Italy between 2016 and 2019 [46]. Also, Goldberg et al. (2019) reported CTX-M-15-producing strains of *C. freundii* and *Enterobacter hormaechei* isolated from the infected wrists of a captured green sea turtle (*Chelonia mydas*) in Brazil in 2016 [47]. In 2012, Cortés-Cortés et al. (2016) analyzed cloacal swabs from 71 adult turtles sheltered in a herpetarium in Puebla, Mexico, previously kept as pets (n = 67) or collected from household environments (n = 4). In 5.6% of the turtles CTX-M-producing *E. coli* were detected [48]. In addition, Hossain et al. (2020) analyzed fecal swabs from 49 turtles randomly collected from pet shops in Seoul, South Korea. *Klebsiella* sp. was present in 32.7% of the samples, of which 18.8% were CTX-M-positive [49]. Morrison et al. (2020) detected CTX-M-producing strains of mainly *E. coli*, but also *Citrobacter* and *Enterobacter* in turtle meat (frozen soft-shell turtles and a dried carapace) imported into Canada from China and Thailand in 2015 [50].

As imported reptile products are usually niche market foods, they are often not routinely targeted by national surveillance programs [50]. In 2007, the European Food Safety Authority (EFSA) analyzed the public health risks associated with the consumption

of reptile meat, including turtle meat. The assessment highlighted the lack of data regarding the presence of bacterial species and drug residues of veterinary medicines in farmed reptiles and reptile meat, apart from the well-documented high contamination rates of *Salmonella* [51,52]. Although imports of reptile meat into the European Union increased by more than 50% between 2007 and 2017, data on biological hazards are still limited [53]. Turtle farming for meat production focuses mainly on freshwater turtle species, particularly the Chinese softshell turtle (*Pelodiscus sinensis*) in Asia and the diamondback terrapin (*Malaclemys terrapin*) in the USA [52]. As the leopard tortoise is a protected terrestrial species that is not typically farmed for meat production, it is likely that the leopard tortoises from which IHIT34097 was isolated were imported for the pet market [54]. With respect to Ecuador as the country of origin, it cannot be ruled out that the information in the shipping documents was incorrect. Although breeding facilities for the restoration of giant tortoises on the Galapagos Islands have been reported in Ecuador, to the authors' knowledge, there are no sources to support the existence of breeding facilities for leopard tortoises native to Africa in South America [55].

With proper hygiene measures (e.g., hand washing after reptile contact), there is, like in the well-documented cases of pet turtle-associated human salmonellosis, a negligible zoonotic risk. The same holds true also for the aforementioned findings of CTX-M-producing *Enterobacteriaceae* in pet turtles. Among the plethora of sources for AMR bacteria, it is not surprising that imported reptiles also represent potential reservoirs for ESBL-producing *Enterobacteriaceae* [49,56,57]. While reptiles are still popular pets, the isolation of MDR isolates like IHIT34097 represents yet missing mosaic pieces in the complete picture of ESBL-producing *Enterobacteriaceae* in reptiles in general and CTX-M-15-producing *K. pneumoniae* ST307 in particular.

In Europe, some of the most commonly used antimicrobials in reptile medicine appear to be fluoroquinolones and third-generation cephalosporins [58]. Additionally, the use of doxycycline, trimethoprim/sulfamethoxazole, and amikacin is recommended. In Ecuador, there are no official reports of the use of antimicrobial substances in most species, but the majority of the antibiotics can be acquired without prescription [59]. According to Nieto-Claudin et al. (2021), antimicrobials often used for farm animals and small animals are penicillin, oxytetracycline, gentamicin, erythromycin, and streptomycin [59]. To address this data gap, a five-year plan to establish a national surveillance system for AMR in humans, animals and the environment was implemented by the government in 2019. Also, the use of products containing colistin in animals was prohibited [60]. As our sample originates from farm-bred animals, the tortoises may have been treated with antimicrobials by the breeder. Unfortunately, no data was available to the authors. Another possibility for obtaining antimicrobial resistance is contact with AMR bacteria from the environment. In recent years, the contamination of the environment with AMR genes caused by agricultural production, solid waste disposal, and wastewater management has attracted increasing attention [61]. Wild animals or farm animals can then serve as reservoirs, and AMR bacteria can be reintroduced to humans [62]. Exposure to urban-impacted coastal waters is suggested as the reason for the isolation of human-associated bacteria in wild sea turtles, serving as bioindicators for human pollution in marine habitats [46,47]. Several studies have highlighted the burden of AMR genes in giant tortoises (*Chelonoidis* spp.) on the Galapagos archipel. They found between 34.0% and 35.7% of MDR isolates in the microbiome of these turtles, as well as high rates of tetracycline resistance [59,63,64].

Fortunately, IHIT34097 demonstrated susceptibility or intermediate susceptibility to all antimicrobial agents in the reserve group according to the World Health Organization (WHO) AWaRe (access, watch, reserve) classification of 2023.

In 2019, an outbreak of an extensively drug-resistant *K. pneumoniae* ST307 lineage associated with hypervirulence was recorded in four medical facilities in north-eastern Germany, adding to the growing number of global reports of known *K. pneumoniae* high-risk clones (e.g., ST11, ST147, and ST307) developing an MDR and hypervirulent phenotype [1,65]. While convergent MDR and hypervirulent *K. pneumoniae* (hvKp) have been documented in

animals, to the authors' knowledge, the documentation of MDR hvKp ST307 is limited to human isolation [19]. In order to monitor the further spread of the hvKp pathotype, we conducted phenotypic and genotypic tests specific to hvKp. Screening for the genotoxin colibactin encoding gene island *pks* was also conducted. It is closely related to the hvKp pathotype, as most *pks* positive isolates belong to the main hypervirulent sequence type ST23 and serotype K1 [66]. Furthermore, the acquisition of colibactin is assumed to be a critical event in the increase in hvKp within the CG23-I sublineage, contributing to its subsequent global spread. In hvKp, *pks* is often found on the mobile genetic element ICE*Kp10* alongside genes for microcin E492, the combination of which is thought to confer a strong colonization advantage over other *Enterobacteriaceae* in the presence of salmochelin, enabling cellular uptake of microcin E492 [67]. However, our isolate did not harbor *pks* or the genes for microcin E492 or salmochelin. Furthermore, IHIT34097 did not exhibit phenotypic characteristics of a hypervirulent pathotype, such as hypermucovisity or indication of extensive production of siderophores, nor did it possess genotypic markers like *peg344*, *iucA*, *rmpA*, and *rmpA2*.

The $bla_{\text{CTX-M-15}}$ gene was located in close proximity to IS*Ecp1*, both harbored by the IncF plasmid. IS*Ecp1* is recognized for its efficient mobilization of $bla_{\text{CTX-M}}$ genes within the *Enterobacteriaceae* family [3,68]. Furthermore, the isolate harbors mutations in *gyrA* (S83L) and *parC* (S80I) within the quinolone resistance region, placing it within a lineage of widespread global distribution [2,18,19,69]. In support of this finding, core genome MLST-based distance calculations with closely related *K. pneumoniae* ST307 strains revealed no clustering according to geographic origin, while the closest related plasmids detected by single nucleotide polymorphism (SNP)-based analysis originated from South Africa, the UK, Israel, and Canada. Previous isolates with similar plasmids have also been recovered from South America; however, there are no reports of isolates originating from Ecuador. Additionally, the IncF plasmid is associated with the AMR genes $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$, *aac(6′)-Ib-cr*, *qnrB1*, *oqxAB*, *sul2*, *dfrA14*, *catB3*, and *fosA* [2,42,70].

This study highlights the role of imported reptiles as a possible source of ESBL-producing *Enterobacteriaceae*, including CTX-M-15-producing *K. pneumoniae* ST307, posing a potential threat to human and animal health. Since 2017, the WHO Global Priority Pathogens List has classified ESBL-producing *K. pneumoniae* as a critical public health threat as it compromises the treatment of infectious diseases [71]. Given the putative transmission of *K. pneumoniae* to humans, inadequate hygiene measures when handling reptiles can lead to a risk of human infection [49].

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/applmicrobiol4020054/s1. Table S1: Plasmids and *K. pneumoniae* genomes with high similarity to pIHIT34097 and to IHIT34097; Table S2: Bridging PCRs.

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