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HIGHLIGHTS

- Swiss national data about the carbapenemase-producing *K. pneumoniae* (CP-*Kp*) are scarce, especially for the human setting.
- We analyzed 285 *Kp* genomes (170 were CP-*Kp*) isolated in Switzerland from human and non-human sources.
- We identified 5 top STs for CP-*Kp*: ST512, ST258, ST101, ST11 and ST307.
- ST512, ST258, and ST101 strain were of human origin only.
- ST11 and ST307 strains were from both human and non-human settings.

Carbapenemase-producing *Klebsiella pneumoniae* strains in Switzerland: human and non-human settings may share high-risk clones

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Running title: Carbapenemase-producing *K. pneumoniae* in various Swiss settings

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ABSTRACT

Background: The spread of carbapenemase-producing *K. pneumoniae* (CP-*Kp*) strains belonging to high-risk sequence types (STs) is a concern. For Switzerland, national data about the molecular features (especially the STs) of the CP-*Kp* of human origin is not available, while in veterinary clinics ST11 and ST307 $bla_{\text{OXA-48}}$-possessing *Kp* strains have been recently reported.

Methods: We analyzed a collection of 285 *Kp* genomes (170 were CP-*Kp*) isolated in Switzerland from human and non-human sources during 2006-2020. Whole-genome sequencing, core genome phylogenies and public databases were implemented to present a detailed overview regarding their carbapenemases, STs, and plasmids.

Results: The top 5 STs were (main carbapenemase gene): ST512 ($bla_{\text{KPC-3}}$), ST258 ($bla_{\text{KPC-2}}$) and ST101 ($bla_{\text{OXA-48}}$) consisting of strains of human origin only, ST11 ($bla_{\text{OXA-48}}$) and ST307 ($bla_{\text{OXA-48}}$) strains isolated from human, animal, and environmental sources. However, during 2016-2020, the main STs for CP-*Kp* were ST11 (17.6%), ST307 and ST101 (both 14.7%), while ST258 (5.9%) and ST512 (4.4%) significantly declined. Most of the carbapenemase genes were carried on plasmids already described. Core genome analysis revealed that ST11 *Kp* of animal and human origins were closely-related, while those of ST307 were distant.

Conclusions: We described, for the first time, the features of the CP-*Kp* circulating in Switzerland among human and non-human settings. Our genomic analysis revealed that the emerging high-risk ST11 and ST307 lineages were often isolated from non-human settings. This study provided a baseline for further WGS-based One-Health surveillance of CP-*Kp* and emphasized the need of metadata to track dissemination routes between the different settings.

KEY WORDS: OXA-48, ST11, ST307, plasmids, animals, environment, carbapenemase

1. INTRODUCTION

Klebsiella pneumoniae (*Kp*) is an important pathogen that can favor the exchange of antimicrobial resistance genes (ARGs) among the Enterobacterales spreading in human, animal and environmental settings [1]. In particular, the rapid increase of carbapenem-resistant *Kp* (CR-*Kp*) strains of nosocomial origin and their emergence in non-human settings represent a public health concern [2, 3]. In fact, in these pathogens, carbapenem resistance can be due to the expression of class A (e.g., KPCs), class B (e.g., NDMs), and/or class D (OXA-48-like) carbapenemase *bla* genes that are associated to mobile genetic elements (MGEs; e.g., plasmids) horizontally transferable between different Enterobacterales species [4].

In Europe, carbapenemase-producing *Kp* (CP-*Kp*) strains belonging to successful high-risk clones have been reported as responsible for infection and/or colonization in humans. For instance, numerous recent studies have shown that the main sequence types (STs) and their associated *bla* carbapenemase genes are: ST512 *bla*$_{\text{KPC-3}}$-*Kp*, ST307 *bla*$_{\text{OXA-48/KPC-2/-3}}$-*Kp*, ST258 *bla*$_{\text{KPC-2}}$-*Kp*, ST101 *bla*$_{\text{KPC-2/-3}}$-*Kp*, and ST11 *bla*$_{\text{OXA-48/KPC-3}}$-*Kp* [5-8]. Although such data are still scarce for non-human settings, CP-*Kp* strains have been reported in pets [9, 10], wild animals [11, 12], and the environment [13-16].

With regard to Switzerland, national data about the STs of the CP-*Kp* of human origin is not available; only the *bla*$_{\text{NDM}}$-*Kp* (mostly of ST11 and ST147) have been reported in detail [17]. Furthermore, sporadic CP-*Kp* were found in wastewater [18, 19], while in pets and the nosocomial environment we recently identified ST11 and ST307 *bla*$_{\text{OXA-48}}$-*Kp* strains [3, 20, 21].

In this study, we analyzed a collection of CP-*Kp* isolated in Switzerland from human and non-human sources. Whole-genome sequencing (WGS), core genome phylogenies and public database information were implemented to present a detailed overview regarding their carbapenemase genes, STs, and plasmids circulating among the different settings.

2. MATERIALS AND METHODS

2.1 Isolation, species identification (ID) and antimicrobial susceptibility tests (ASTs)

From our strain collection of multidrug-resistant *Kp* (which included CP-*Kp*) isolated during 2013-2020 in Switzerland, we recovered 67 strains of human origin (see 3.1). The initial species ID was routinely obtained using the MALDI-TOF MS (Bruker). Accurate ID was achieved with WGS data using the Type (Strain) Genome Server (TYGS; https://tygs.dsmz.de/) and Kleborate v2.0.4 (https://github.com/katholt/Kleborate) with default parameters.

ASTs were performed using the broth microdilution GNX2F Sensititre™ panels (Thermo-Fisher). MICs for antibiotics were interpreted according to the 2019 European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (version 9.0).

2.2 WGS and bioinformatics

The 67 *Kp* strains were selected for short and long read WGS as previously described [22]. Briefly, all strains underwent Illumina WGS (NovaSeq 6000 sequencer; 2 × 150 bp paired-end reads). A subset of 23 strains were also sequenced with the MinION (Oxford Nanopore Technologies, ONT) instrument using the rapid barcoding library preps (SQK-RBK004) and FLO-MIN 106D R9 flow cells (File S1).

To generate *de novo* draft genome assemblies, the Illumina raw reads were first quality checked with FastQC v0.11.9 (https://github.com/s-andrews/FastQC) and MultiQC v1.11.dev0 (https://github.com/ewels/MultiQC). Read trimming and adaptor removal was done with Trimmomatic v0.36 (https://github.com/usadellab/Trimmomatic). Assemblies were generated with Unicycler v0.4.8 (https://github.com/rrwick/Unicycler) using the Illumina-only assembly pipeline.

Long reads from the ONT sequencing runs (n=23) were quality checked with NanoStat v2.0.4 (https://github.com/wdecoster/nanostat). Complete genome assemblies were generated *de novo* by combining Nanopore and Illumina reads using the hybrid assembly pipeline of

Unicycler; two plasmid sequences were circularized and completed with Flye assembler v2.8-b1674 (https://github.com/fenderglass/Flye) coupled with Pilon v1.22 (https://github.com/broadinstitute/pilon) (GenBank: CP083001.1 and CP083027.1). Assembly coverages were calculated with QualiMap v.2.2.2-dev (http://qualimap.conesalab.org/).

Illumina assemblies were screened *in silico* for ARGs with ResFinder v4.1 and PlasmidFinder v2.1 (50% threshold for minimum percentage identity), (http://www.genomicepidemiology.org/); Kleborate v2.0.4 was used to determine multi-locus sequence type (MLST), and virulence factors.

Genome annotation was performed with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). Insertion sequence (IS) elements were annotated according to ISfinder (https://isfinder.biotoul.fr/) using BLASTx. Integrons were confirmed with INTEGRALL (http://integrall.bio.ua.pt/). Circular BLASTn analyses were generated with BLAST Ring Image Generator v0.95 (https://github.com/happykhan/BRIG). Plasmid database comparisons were done with NCBI BLASTn and PLSDB v2020_11_19 (https://ccb-microbe.cs.uni-saarland.de/plsdb/) using "Mash distribution." Plasmid mapping of the Illumina assemblies (i.e., contigs) and reference plasmid sequences were done with minimap2 v2.17 (https://github.com/lh3/minimap2) set to 5% max divergence. Consensus sequences were generated with Geneious v11.1.5 (https://www.geneious.com/) with the parameters threshold= "100% identical"; no coverage= "N/X"; and "trim to reference sequence." Unless specified, all software analyses were done with default parameters.

2.3 Database search

To place our collection (n=67) into a larger perspective of strains circulating in Switzerland, we included 218 genomes from *Kp* strains isolated in Switzerland (as per BioSample description) from the publicly available NCBI assembly and the SRA databases (data retrieval date: January 14 to May 20, 2021). PubMed query: "*Klebsiella pneumoniae* Switzerland" and SRA query: "(*Klebsiella pneumoniae* Switzerland) AND "*Klebsiella*

pneumoniae"[orgn:_txid573]." Overall, the 285 Swiss *Kp* strains were isolated in Basel (n=173), Bern (n=75), Geneva (n=21), Zurich (n=6), St. Gallen (n=3), Lausanne (n=2), Fribourg (n=1), and 4 were not specified (see File S1).

For a broader comparison, 45 non-Swiss *Kp* genomes (human and non-human) from European countries surrounding Switzerland were included in the individual ST core-genome analyses (see below). Data were retrieved from NCBI (via PubMed; query "*Klebsiella pneumoniae*" "ST11" "ST101" "ST258" "ST307" "ST512") and from the European survey of CP Enterobacteriaceae (EuSCAPE) [23] (https://microreact.org/project/EuSCAPE_Kp) (File S1**)**. All additional genomes included in this study were assembled and screened for ARGs as described in the section above (2.2).

2.4 Core-genome analyses

A core-genome alignment of 285 *Kp* genomes from Switzerland was performed to estimate their epidemiological and phylogenetic distribution in human and non-human sources, the carbapenemase genes and STs. Likewise, core-genome analysis was individually conducted on the following STs: ST11 (n=27; 17 Swiss, 10 non-Swiss), ST101 (n=34; 25 Swiss, 9 non-Swiss), ST258 (n=35; 25 Swiss, 10 non-Swiss), ST307 (n=29; 19 Swiss, 10 non-Swiss), and ST512 (n=56; 50 Swiss, 6 non-Swiss). In brief, the core genome alignment was done with Parsnp v1.2 (https://github.com/marbl/parsnp) with recombination filter (parameter: -x) and with a reference genome chosen at random as previously described [22].

The core genome alignments were subjected to phylogenetic tree inference by maximum likelihood with IQ-TREE v2.1.2 (http://www.iqtree.org/). The best-fit DNA substitution models with ascertainment bias correction (parameter: +ASC) were chosen according to ModelFinder (included in IQ-TREE; parameter: -m MFP+ASC). Bootstrapping was done 1000 times with ultrafast bootstrap (UFBoot) (parameter: -bb) and the SH-aLRT test (parameter: -alrt).

The Illumina and complete hybrid assemblies are deposited in GenBank under BioProjects PRJNA758223 and PRJNA759850, respectively. Individual genome accessions and other metadata are available in File S1.

3. RESULTS AND DISCUSSION

The first objective of the present study was to define the molecular characteristics of the Swiss CP-*Kp* isolates circulating in humans. Moreover, following the recent report of ST11 and ST307 *bla*$_{OXA-48}$-*Kp* strains in hospitalized pets and veterinary clinic environment in Switzerland [3], human CP-*Kp* isolates were compared to those detected in other hosts/sources to study their possible exchange among the different settings.

3.1 ASTs and main genomic features

A total of 67 *Kp* clinical strains of human origin isolated in 2013 (n=5), 2017 (n=26), 2018 (n=30), and 2020 (n=6) were analyzed. All strains showed resistance to multiple classes of antibiotics, with 43% (n=29), 73% (n=49), 94% (n=63), 39% (n=26), and 8% (n=5) of them resistant to gentamicin, ciprofloxacin, cefotaxime, ertapenem, and colistin, respectively (Table S1). The majority of the strains carried extended-spectrum β-lactamase (ESBL) genes (mainly *bla*$_{CTX-M-15}$) together with additional ARGs. Twenty-six strains were ertapenem-resistant, of which 23 possessed carbapenemase genes (CP-*Kp*): 14 *bla*$_{OXA-48}$-*Kp*, 4 *bla*$_{NDM-1}$-*Kp*, 3 *bla*$_{OXA-232}$-*Kp*, and 2 *bla*$_{KPC}$-*Kp* (File S1).

3.2 Main STs and carbapenemase genes

To put our collection into a larger phylogenetic perspective and to identify potential clonal reservoirs of CP-*Kp* strains from other sources outside humans, we conducted a core-genome analysis that resulted in 93671 single nucleotide variants (SNVs) across 285 genomes (including our 67 strains) of Swiss origin. Overall, 170 CP-*Kp* and 115 non-CP-*Kp* genomes were analyzed. In particular, these strains were collected in 2006-2010 (CP-*Kp*, n=8; non-CP-*Kp*, n=6), 2011-2015 (CP-*Kp*, n=94; non-CP-*Kp*, n=17), and 2016-2020 (CP-*Kp*, n=68; non-CP-*Kp*, n=92) (File S1).

As shown in Figure 1, 5 major STs were identified [consisting of ≥6% of total STs (Table S2)] in the overall analyzed genomes: ST512 (CP-*Kp*, n=48; non-CP-*Kp*, n=2), ST258 (CP-*Kp*, n=25), ST101 (CP-*Kp*, n=19; non-CP-*Kp*, n=6), ST11 (CP-*Kp*, n=13; non-CP-*Kp*,

n=4), and ST307 (CP-*Kp*, n=10; non-CP-*Kp*, n=9); smaller clusters of previously defined hyperepidemic STs (e.g., ST395, ST15, ST147) were also noted [17, 24, 25]. However, considering only the genomes of strains collected more recently (i.e., during 2016-2020), the main STs for CP-*Kp* were (% considering the total of 68 CP-*Kp*): ST11 (17.6%), ST307 and ST101 (both 14.7%), ST258 and ST395 (both 5.9%), and ST512 (4.4%) (Table S1). Similar shifts in non-ST258/ST512 dominant lineages have been reported in other countries (e.g., the emergence of ST307 in Italy) [7, 26]. However, as these Swiss data derive from public datasets and not all CP-*Kp* from Switzerland have been sequenced and deposited, these differences might not be representative of the present epidemiological situation.

In our analysis, ST512, ST258, and ST101 consisted only of strains of human origin. In particular, ST512 and ST258 strains carried $bla_{\text{KPC-3}}$ and $bla_{\text{KPC-2}}$, respectively, whereas ST101 harbored $bla_{\text{OXA-48}}$. We also noted that the majority of CP-*Kp* strains of ST11 and ST307 possessed the $bla_{\text{OXA-48}}$ and consisted of isolates from human, animal, and environmental sources [3, 24, 27, 28] (Figure 1; Table S2).

To better study the population structure of the main lineages of CP-*Kp* spreading in Switzerland in the different settings, a detailed core-genome analysis was performed for the strains belonging to the main STs (ST11, ST307, ST101, ST258, and ST512). Furthermore, for epidemiological perspective, we included representative genomes (n=45) of CP-*Kp* strains isolated from other European countries.

3.3 ST11

The ST11 clone [Clonal Complex (CC) 258] is globally distributed and typically associated with CP-*Kp* carrying different carbapenemase genes (e.g., bla_{KPC}, bla_{NDM}, $bla_{\text{OXA-48}}$) of human origin responsible for nosocomial infections [4]. In non-human settings, ST11 CP-*Kp* strains have been reported, for example, in poultry in China [29], and in pets, wild birds, and water in Europe [10, 11, 14]. In Switzerland, these pathogens have been reported in the environment of veterinary clinics and hospitalized pets [3, 20, 21].

In our analysis, core-genome alignment of ST11 strains resulted in 8073 SNVs across 27 genomes (79.8% alignment) (Figure 2A; File S2). The majority of the Swiss ST11 *Kp* strains clustered in a single group (n=10) of CP-*Kp* isolated during 2018-2019 from animal and environmental sources [3]. Such strains possessed the *bla*$_{\text{OXA-48}}$ and almost identical β-lactamase and virulence profiles (0-11 ΔSNVs). Three other CP-*Kp* strains of human origin were related to each other: 2 almost identical *bla*$_{\text{OXA-48}}$-*Kp* (403420-11 and kpneu028; 18 ΔSNVs) isolated in 2011 and 2017 and 1 *bla*$_{\text{KPC-2}}$-*Kp* (KP05-2017) detected in 2017. The latter was imported to Switzerland from a Brazilian patient and co-harbored the 16s rRNA methyltransferase *rmtG* gene [27].

Notably, in 11 of 12 Swiss CP-*Kp*, the *bla*$_{\text{OXA-48}}$ was carried in identical IncL plasmids (100% query coverage and 100% identity with the reference plasmid p2-4906.28-OXA48, GenBank: CP083038.1; File S4). We also noted that in comparison to other European isolates, no clonal links to the Swiss isolates were observed (Figure 2A).

3.4 ST307

The ST307 (CC307) CP-*Kp* strains have been associated with a variety of carbapenemase genes (e.g., *bla*$_{\text{KPC-2/-3}}$, *bla*$_{\text{OXA-48/-181}}$, *bla*$_{\text{NDM-1}}$), and are linked to nosocomial human infections [7]. In non-human settings, ST307 CP-*Kp* have been reported, for instance, in chimpanzees in Senegal, in wastewater in Romania, in a well in Italy, and in pets in Switzerland [3, 16, 30, 31].

In our study, the ST307 core-genome alignment resulted in 710 SNVs across 29 genomes (75.1% alignment) (Figure 2B; File S2). Several highly identical Swiss strains of animal and environmental origin clustered into two small groups consisting of non-CP-*Kp* and *bla*$_{\text{OXA-48}}$-*Kp* strains showing 3-9 and 16-81 ΔSNVs, respectively. One group contained the non-CP-*Kp* strains D32, C23 and i7 (including the outgroup t12) identified among pets in households and veterinary clinic environment [28]. The second group was composed of the *bla*$_{\text{OXA-48}}$-*Kp* AR142_2b and 18KM2445b strains identified in an infection site and gut of

hospitalized pets, respectively [3]. The CP-*Kp* carried identical *bla*$_{\text{OXA-48}}$ in IncL plasmids (100% query coverage and 99.98% identity with p2-4906.28-OXA48 File S4). Other Swiss *bla*$_{\text{OXA-48}}$-*Kp* were of human origin and more closely related to each other (n=6; 2-34 ΔSNVs), as from those from pet environment suggesting independent origin. They also all carried *bla*$_{\text{OXA-48}}$ in IncL plasmids (94-100% query coverage and 99.97-99.99% identity with p2-4906.28-OXA48; File S4).

Two Swiss strains carrying other carbapenemase genes were also identified. The *bla*$_{\text{KPC-3}}$-*Kp* N311 obtained from a patient previously hospitalized in Sicily was related (72 ΔSNVs) to a non-CP-*Kp* strain (EuSCAPE_IT400) also originating from Italy [24]. The *bla*$_{\text{KPC-3}}$ was likely carried in a plasmid similar to plasmid p2 from strain KPN_KPC_HUG_07 [IncFII(K)/FIB(pQil)] (GenBank: CP019774.1; 98% query coverage and 99.34% identity; File S4). The second human CP-*Kp* strain (402962-16) carried *bla*$_{\text{NDM-1}}$, but had no clonal links with other Swiss or international strains.

3.5 ST101

The ST101 (CC258) CP-*Kp* strains mostly harbor *bla*$_{\text{KPC-2}}$ and *bla*$_{\text{OXA-48}}$ and are associated with hospital-acquired infections in humans [32]. Non-human ST101 CP-*Kp* strains have not been reported in Switzerland, while in Romania and Ireland, *bla*$_{\text{OXA-48}}$-*Kp* were found in hospital wastewater and in recreational seawater, respectively [15, 33].

Our core-genome phylogenetic analysis for the ST101 *Kp* strains resulted in 4750 SNVs across 34 genomes (75.9% alignment) (Figure 3A; File S2). The majority of Swiss genomes were associated to human isolates with the exception of one ST101 non-CP-*Kp* strain recovered from poultry [34]. The CP-*Kp* primarily carried *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{KPCs}}$ (n=23 and n=3, respectively). In 19 Swiss CP-*Kp*, 18 *bla*$_{\text{OXA-48}}$ were associated with IncL (n=3), Inc/R/FIA(HI1) (n=4), and IncM1 (n=2) replicon-type plasmids, as well as with 9 non-typeable plasmids. Likewise, the other Swiss strain (6712.08, isolated in 2018) carried *bla*$_{\text{KPC-3}}$ on an IncR/FII(K)/FIB(K) plasmid (File S4). Interestingly, this Swiss strain was related to 2

other *bla*$_{KPC-2/-3}$-*Kp* (EuSCAPE_IT201 and EuSCAPE_IT042; 40-70 ΔSNVs, respectively) collected in Rome and Sanremo (Italy) in 2013 from hospital-acquired bloodstream infections [23].

No clonal links were found between the human Swiss *bla*$_{OXA-48}$-*Kp* strains and those from other European countries; moreover, ST101 CP-*Kp* strains were not found in non-human Swiss sources (Figure 3A). However, we were surprised that two *bla*$_{OXA-48}$-*Kp* strains found in hospital wastewater in Romania in 2018 (22bac and 23bac) were identical to those found in humans in Switzerland [15]. Furthermore, 2 human (P0517 and P0518) and one poultry non-CP/ESBL-producing *Kp* strain (F0025) detected in Switzerland in 2017 were identical to each other (0-9 ΔSNVs) [34]. Therefore, non-CP ST101 *Kp* isolates should be closely-monitored as they could potentially acquire carbapenemase-carrying MGEs, such as the highly promiscuous IncL *bla*$_{OXA-48}$-harboring plasmid.

3.6 ST258

The globally successful ST258 clone (CC258) largely comprises *bla*$_{KPC}$-*Kp* strains which have been responsible for nosocomial outbreaks in humans [4]. In non-human sources, ST258 CP-*Kp* have not been reported in Switzerland. However, they have been identified in other European countries, such as in wastewater and river water [16, 31, 35]. Sporadic reports from non-European countries include *bla*$_{KPC-2}$-*Kp* from American crows and in bovine mastitis in Mexico [36, 37].

Core-genome analysis of our ST258 *Kp* resulted in 1151 SNVs across 35 genomes (74.7% alignment) and consisted entirely of strains carrying *bla*$_{KPC-2}$ (n=28) or *bla*$_{KPC-3}$ (n=7) (Figure 3B; File S2). The Swiss *bla*$_{KPC-2}$-*Kp* were associated with IncFII(K)/FIB(pQil)/FIB(K) (n=15), as well as with 5 non-typeable plasmids, while the *bla*$_{KPC-3}$-*Kp* were located in IncFII(K)/FIB(pQil) plasmids (n=4; 1 was non-typeable) (File S4). Most ST258 CP-*Kp* strains (n=25) formed distinct clusters together with other human CP-*Kp* from Switzerland. However, the Swiss *bla*$_{KPC-2}$-*Kp* strain 6711.43 was related to *bla*$_{KPC-2}$-*Kp* strains isolated from

wastewater (RADAR74, RADAR113) and a human (RADAR87) in 2018/2019 in Romania (51-60 ΔSNVs; Figure 3B) [16]. This finding underlines the potential of ST258 *bla*$_{KPC}$-*Kp* strains to spread in non-human settings, including those in Switzerland.

3.7 ST512

The emerging ST512 clone (CC258), a single locus variant of ST258, has been reported in many *bla*$_{KPC}$-*Kp* outbreaks worldwide [4]. In non-human settings, CP-*Kp* of this lineage have been reported in wastewater and animals in Algeria and Italy, respectively [38, 39].

The core-genome phylogeny of our ST512 *Kp* resulted in 294 SNVs across 56 genomes (69.0% alignment). All *Kp* strains were strictly of human origin (Figure S1) consisting of 54 CP-*Kp* carrying *bla*$_{KPC-3}$ and 2 non-CP-*Kp* Swiss isolates. The *bla*$_{KPC-3}$ in the Swiss CP-*Kp* (n=48) was associated with IncFII(K)/FIB(pQil) (n=46) and with 2 non-typeable plasmids (File S4). As for ST258, ST512-*Kp* seems to be also exclusively a human strain and to specifically favor *bla*$_{KPC}$-harboring plasmids.

Despite ST512 CP-*Kp* strains from non-human sources have not been reported in Switzerland and Europe, such pathogens have been sporadically reported in other geographic areas (e.g., bat guano in Algeria [38]). Therefore, despite the apparent low risk of ST512 CP-*Kp* spreading in non-human settings in Switzerland, surveillance should be conducted irrespective of the risks.

3.8 Plasmid-borne carbapenemase genes

The 23 CP-*Kp* deriving from our collection of 67 strains were further analyzed using short and long read WGS to characterize the *bla*$_{OXA-48}$ (n=14), *bla*$_{KPC-2}$- and *bla*$_{KPC-3}$- (both n=1), *bla*$_{NDM-1}$- (n=4), and *bla*$_{OXA-232}$-harboring (n=3) plasmids (File S1).

3.8.1 *bla*$_{OXA-48}$-carrying plasmids

As anticipated, most of the analyzed *bla*$_{OXA-48}$-*Kp* Swiss strains carried the gene on IncL plasmids of 61.6-64.3kb; however, IncR/FIA(HI1) (n=3; 94.9kb) and IncM1 (n=3; 67.1-74.7kb) plasmids were also identified. In all 3 plasmid types, the *bla*$_{OXA-48}$ was flanked by

IS*10A* (one disrupted by IS*26*) and ΔIS*1R* elements characteristic of Tn*1999* transposon [40] (Figure 4).

The *bla*$_{\text{OXA-48}}$-harboring IncL plasmids are hyperepidemic and globally disseminated in Enterobacterales [8, 41]. In Switzerland, they were already reported in *Kp* strains of human (ST101) [42, 43], wastewater (ST437) [19], veterinary environment and hospitalized pet origins (ST11, ST307) [3]. The same *bla*$_{\text{OXA-48}}$-harboring IncL plasmids have also been found in other Enterobacterales worldwide [8]

Less common, *bla*$_{\text{OXA-48}}$ was hosted in hybrid plasmids of the IncR/FIA(HI1A) replicon type where it was co-carried along multiple ARGs, including *bla*$_{\text{CTX-M-15}}$ (Figure 4B). This plasmid was similar to the *Kp* plasmid p101_srb identified in Serbia (GenBank: MN218814.1; 90% query coverage and 99.76% identity). Similarly, in the IncM1 plasmids (Figure 4C), *bla*$_{\text{OXA-48}}$ was also co-carried along *bla*$_{\text{CTX-M-14b}}$, and other ARGs. This plasmid was identical to pRIVM_C017036_1 (GenBank: CP068874.1; 100% query coverage and 100% identity) found in *bla*$_{\text{OXA-48}}$-*Kp* from the Netherlands [44].

3.8.2 *bla*$_{\text{KPC-2/-3}}$-carrying plasmids

The *bla*$_{\text{KPC-2/-3}}$ were located in 2 different hybrid plasmids, each possessing 3 replicons: p1-6711.43-KPC2 [IncFII(K)/FIB(K)/FIB(pQil), 211.3kb] and p1-6712.08-KPC3 [IncFII(K)/FIB(K)/R, 140.9kb). In both plasmids, *bla*$_{\text{KPC-2/-3}}$ were associated to the archetypal Tn*4401* transposon (Figure S2) [45].

In p1-6711.43-KPC2 (Figure S2A), the *bla*$_{\text{KPC-2}}$ was associated with *bla*$_{\text{OXA-9}}$ and *bla*$_{\text{TEM-1A}}$. This plasmid was similar to pJYC02A (GenBank: CP022923.1), a *bla*$_{\text{KPC-2}}$-associated MGE carried by an ST307 *Kp* strain involved in a tertiary care hospital outbreak in South Korea [46] (93% query coverage; 99.90% identity). Similarly, p1-6712.08-KPC3 co-carried the *bla*$_{\text{OXA-9}}$ and *bla*$_{\text{TEM-1A}}$ and additional ARGs (e.g., *armA*) (Figure S2B). Both *bla*$_{\text{KPC-2}}$- and *bla*$_{\text{KPC-3}}$-harboring plasmids were comparable to many international *Kp* hybrid plasmids containing similar ARG combinations (BLASTn analysis; data not shown).

3.8.3 *bla*$_{NDM-1}$-carrying plasmids

Three diverse replicons were identified for the *bla*$_{NDM-1}$-harboring plasmids: IncFII(pKPX1) (n=1; 137.9kb), IncFIB(pQil) (n=1; 54.1kb), and IncFIB(pNDM-Mar)/HI1B(pNDM-Mar) (n=2; 342.4-342.7kb). In p2-5208.51-NDM1 [IncFII(pKPX1)] (Figure S3A), *bla*$_{NDM-1}$ was uncommonly associated with IS*3000* and ΔIS*3000* elements [47], whereas the remaining plasmids were associated with ΔIS*Aba125* (disrupted by IS*Spu2*) (Figure S3B and S3C, respectively).

The *bla*$_{NDM-1}$ was carried along various ARGs, such as the *rmtF* 16S rRNA methyltransferase gene (conferring high-level resistance to aminoglycosides) in plasmid p2-5208.51-NDM1 (Figure S3A). Despite being significantly larger (250.4kb *vs.* 137.9kb), this plasmid was similar (94% query coverage; 99.99% identity) to a *bla*$_{NDM-1}$-containing plasmid (pKPX-1) isolated from an ST11 *Kp* strain detected in Taiwan from a Swedish patient [48] (GenBank: AP012055.1).

In p4-7008.20-NDM1 [IncFIB(pQil)] and p1-7011.62-NDM1 [IncFIB(pNDM-Mar)/HI1B(pNDM-Mar)] (Figure S3B and S3C, respectively), the *bla*$_{NDM-1}$ was also co-carried with *bla*$_{CTX-M-15}$ (associated to IS*26*) and additional ARGs. p4-7008.20-NDM1 was highly identical to pM321-NDM1 (100% query coverage; 99.99% identity) from a *Kp* isolated in Myanmar (GenBank: AP018834.1), while p1-7011.62-NDM1 resembled pKPN1481-1 (84% query coverage; 99.98% identity) from a *Klebsiella variicola* strain identified in the United States (GenBank: CP020848.1).

3.8.4 *bla*$_{OXA-232}$-carrying plasmids

Three identical *bla*$_{OXA-232}$-carrying plasmids of the CoIKP3 replicon type were identified (Figure S4). The *bla*$_{OXA-232}$ was located on a small 6.1kb plasmid without further ARGs. This *bla*$_{OXA-48-like}$ variant was already identified in identical non-conjugative plasmids [49], and has been increasingly reported worldwide in both *E. coli* and *K. pneumoniae* strains [8]. In

Switzerland, the first *bla*$_{\text{OXA-232}}$-harboring plasmid was reported in 2017 in ST231 *Kp* strains co-possessing *rmtF* rRNA methylase gene and *bla*$_{\text{CTX-M-15}}$ [50].

4. CONCLUSIONS

In this work, we analyzed our collection of CP-*Kp* strains along with publicly available genomes to give, for the first time, insights into the major STs and carbapenemase-bearing plasmids as well as possible core genome-based relatedness between strains circulating in Switzerland in humans, animals, food, and the environment. However, the link between the different sources could not be made due to the lack of metadata.

We identified 5 main high-risk STs (ST11, ST307, ST101, ST258, ST512) which were associated primarily to the *bla*$_{\text{KPC-2/-3}}$ and *bla*$_{\text{OXA-48}}$ (Figure 1); such genes were also steadily carried by hyperepidemic plasmids (Figure 4, Figures S2-S4). Overall, our results - especially those referring to the period 2016-2020 - were consistent to those recently reported from neighboring European countries (e.g., Italy, France and Germany) [26, 51-54].

Nevertheless, our genomic analysis revealed that the emerging high-risk ST11 and ST307 lineages in Switzerland were often isolated from non-human settings. In particular, the majority of ST11 *bla*$_{\text{OXA-48}}$-*Kp* strains were frequently identified in hospitalized pets and veterinary environment and less frequently in humans, while the ST307 *bla*$_{\text{OXA-48}}$-*Kp* were spreading only in humans and pets. In both STs, *bla*$_{\text{OXA-48}}$ was carried by the classic ~63kb IncL international plasmid which could be also hosted by additional non-ST11/ST307 *K. pneumoniae*, such as ST147 and ST437 strains from wastewater [19, 25], and in multiple STs of human origin (Figure 1; File S1).

The use of WGS methods is crucial for rapid detection and monitoring of the spread and mechanisms of emerging zoonotic diseases [55]. However, it is important to know that for accuracy, this methodology must rely on public databases (e.g., from national reference labs). Unfortunately, as we experienced, the scarcity of deposited genomes into public databases may bias the results of surveys. Nevertheless, although the present study was not an epidemiological study, but a pure observational analysis with a set of available strains, it provided new insights into lineages circulating in different settings in Switzerland. These

results can serve as a basis for future WGS-based surveillances of clinically significant CP-*Kp* in the different settings in Switzerland. However, we emphasize the importance to also collect metadata linked to each CP-*Kp* strain to analyze the possible routes of transmission/exchange between the human and non-human domains.

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ETHICAL APPROVAL

Not required.

COMPETING INTERESTS

None declared.

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LEGEND TO THE FIGURES

Figure 1. Core genome phylogeny of publicly available *K. pneumoniae* genomes isolated in Switzerland (n=285) consisting of 115 non-CP-*Kp* and 170 CP-*Kp*. The core genome alignment resulted in a total of 93671 SNVs across all genomes (42.2% alignment). In the figure we show: main STs by colored clades; Sample names and other STs highlighted by isolation source ("Sources"); and carbapenemases by colored circles in the branches. Bootstrap support values are shown as black squares on branches only for those with SH-aLRT and UFboot support of ≥80% and ≥ 95%, respectively. The tree scale represents the average number of nucleotide substitutions per site. See File S1 for metadata corresponding to this analysis. Genomes with a black star correspond to high-risk clones of *bla*$_{\text{OXA-48}}$-*Kp* isolated from the veterinary setting [3]. Genomes with a red star correspond to representative human strains from a clonal dissemination between human and companion animals in a veterinary hospital in Switzerland [42].

Figure 2. A) Core genome phylogeny of *K. pneumoniae* ST11 genomes isolated in Switzerland (n=17) and epidemiologically representative genomes from other European countries (n=10). The core genome alignment resulted in a total of 8073 SNVs across all 27 genomes (79.8% alignment). **B)** Core genome phylogeny of *K. pneumoniae* ST307 genomes isolated in Switzerland (n=19) and epidemiologically representative genomes from other European countries (n=10). The core genome alignment resulted in a total of 710 SNVs across all 29 genomes (75.1% alignment).

In the figure we show: sample name and year highlighted by isolation source ("Sources"); country of isolation by colored circles in the branches. The following genomic features are represented by their presence (black square), absence (white square), and best-matching (black square with asterisk): carbapenemases ("CP"), β-lactamase families, and virulence factors; integrative conjugative elements ("ICE*kp*"), capsular polysaccharide ("K") and

lipopolysaccharide ("O"). Delta SNVs (ΔSNVs) represent core genome similarities between two or more genomes. Bootstrap support values are shown as black squares on branches only for those with SH-aLRT and UFboot support of ≥80% and ≥ 95%, respectively. The tree scale represents the average number of nucleotide substitutions per site. See File S2 for SNVs and identity matrices corresponding to this analysis.

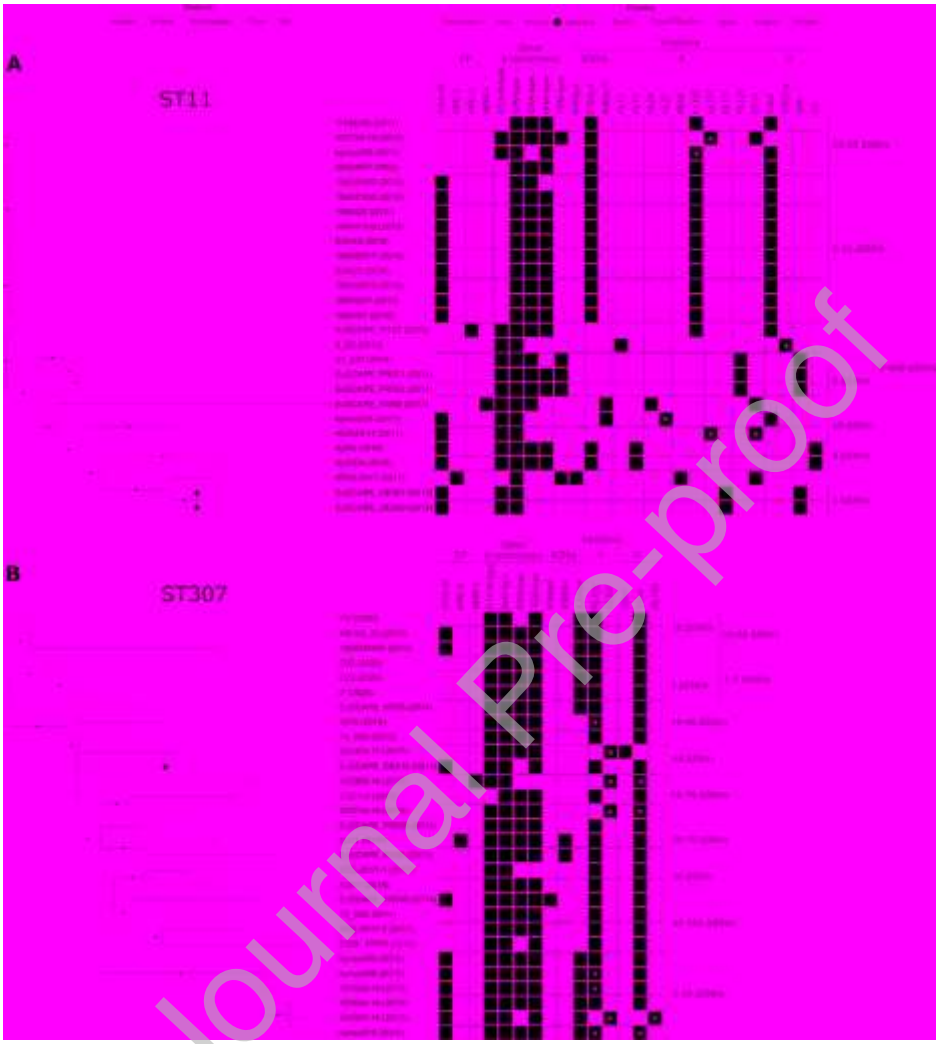

Figure 3. A) Core genome phylogeny of *K. pneumoniae* ST101 genomes isolated in Switzerland (n=25) and epidemiologically representative genomes from other European countries (n=9). The core genome alignment resulted in a total of 4750 SNVs across all 34 genomes (75.9% alignment). **B)** Core genome phylogeny of *K. pneumoniae* ST258 genomes isolated in Switzerland (n=25) and epidemiologically representative genomes from other European countries (n=10). The core genome alignment resulted in a total of 1151 SNVs across all 35 genomes (74.7% alignment).

In the figure we show: sample name and year highlighted by isolation source ("Sources"); country of isolation by colored circles in the branches. The following genomic features are represented by their presence (black square), absence (white square), and best-matching

(black square with asterisk): carbapenemases ("CP"), β-lactamase families, and virulence factors; integrative conjugative elements ("ICE*Kp*"), capsular polysaccharide ("K") and lipopolysaccharide ("O"). Delta SNVs (ΔSNVs) represent core genome similarities between two or more genomes. Bootstrap support values are shown as black squares on branches only for those with SH-aLRT and UFboot support of ≥80% and ≥ 95%, respectively. The tree scale represents the average number of nucleotide substitutions per site. See File S2 for SNVs and identity matrices corresponding to this analysis.

Figure 4. Circular BLASTn comparisons of all *K. pneumoniae* bla_{OXA-48}-carrying plasmids in our collection (n=14). **A)** Comparison of p2-4906.28-OXA48 (reference) against 7 other plasmid sequences of the same IncL replicon sequence type. **B)** Comparison of p1-6604.68-OXA48 (reference) against 2 other plasmid sequences of the same IncR/FIA(HI1) replicon

sequence types. **C)** Comparison of p2-6710.71-OXA48 (reference) against 2 other plasmid sequences of the same IncM1 replicon sequence type. **D)** Plasmid mapping results of all *bla*$_{OXA-48}$-positive genomes (n=51) against each individual reference plasmid from our collection (A, B, and C); see File S4 for plasmid mapping percent identity and coverage. Plasmids and their similarities are represented by the colored rings. The CDS/genes and IS elements of interest are represented by colored arrows (red: *bla*$_{OXA-48}$; blue: other ARGs; yellow: IS elements; orange: replicon genes [rep* corresponds to a CDS coding for a replication initiation protein]; green: replicon sequence site), with corresponding annotations (red: *bla*$_{OXA-48}$; blue: other ARGs; green: replicon sequence site). The IS annotations are listed in File S3. In the plasmid maps (A, B, C) we show the reference plasmid name, the replicon(s) sequence type, and the GenBank accession (in blue); to the right of the plasmid maps (A, B, C) we show GC content, GC skew, plasmid name, size, and GenBank accession for the comparison plasmids.