



Emergence of Carbapenemase-Producing Hypervirulent *Klebsiella pneumoniae* in Switzerland

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ABSTRACT Increasing occurrence of multidrug-resistant (MDR) and hypervirulent (hv) *Klebsiella pneumoniae* (MDR-hvKp) convergent clones is being observed. Those strains have the potential of causing difficult-to-treat infections in healthy adults with an increased capacity for mortality. It is therefore crucial to track their dissemination to prevent their further spread. The aim of our study was to investigate the occurrence of carbapenemase-producing hvKp isolates in Switzerland and to determine their genetic profile. A total of 279 MDR carbapenemase-producing *K. pneumoniae* from patients hospitalized all over Switzerland was investigated, and a rate of 9.0% *K. pneumoniae* presenting a virulence genotype was identified. Those isolates produced either KPC, NDM, or OXA-48 and had been either recovered from rectal swabs, urine, and blood. A series of previously reported *K. pneumoniae* clones such as ST23-K1, ST395-K2, and ST147-K20 or ST147-K64 were identified. All the isolates defined as MDR-hvKp (4.7%) possessed the aerobactin and the yersiniabactin clusters. The ST23-K1s were the only isolates presenting the colibactin cluster and achieved higher virulence scores. This study highlights the occurrence and circulation of worrisome MDR-hvKp and MDR nonhypervirulent *K. pneumoniae* (MDR-nhv-Kp) isolates in Switzerland. Our findings raise an alert regarding the need for active surveillance networks to track and monitor the spread of such successful hybrid clones representing a public health threat worldwide.

KEYWORDS *Klebsiella pneumoniae*, hypervirulence, carbapenemase, multidrug resistance, convergent clones

Since its first clinical report, back in Taiwan 1986 (1), hypervirulent *Klebsiella pneumoniae* (hvKp) causing invasive infections have been progressively increasingly reported worldwide (2–4). These isolates are usually susceptible to almost all antibiotics (4, 5). The hvKp clinical pathotype is mainly associated with community-acquired infections, affecting healthy patients and causing diverse severe diseases, such as liver abscesses, endophthalmitis, and meningitis (6).

Intrinsically, *K. pneumoniae* exhibits a so-called opportunistic pathogenicity, causing invasive hospital-acquired infections among high-risk patients, corresponding to the “classical” *K. pneumoniae* (cKp) (7). Urinary tract infections, pneumonia, and bacteremia are some of the infections caused by cKp that may be multidrug-resistant (MDR), including carbapenemase producers (CpKp), which are nowadays widely disseminated around the globe (8, 9).

Although hvKp and CpKp populations are recognized for nonoverlapping clonal groups and sequence types (STs), respectively, circulating mainly in the community and hospitals (10), some of their virulence- and resistance-associated genes can be genetically transmitted and therefore exchanged between each other (11, 12). Hence, this worrisome evolution may lead to convergent clones of MDR hypervirulent *K. pneumoniae* (MDR-hvKp). These MDR-hvKps have the potential of causing very-difficult-to-treat infections in previously healthy adults (13, 14). These isolates have been detected

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in Argentina, China, Egypt, Japan, Switzerland, Taiwan, the United Kingdom, and the United States (14–22).

Although the MDR-hvKp population has been diversely distributed worldwide, the most prevalent STs are the KPC-producing *K. pneumoniae* ST11 and the dominant hvKp lineage ST23 (13, 23). The recent emergence of the latter in Europe led to a public alarm raised by the European Centre for Disease Prevention and Control (ECDC) (13).

It is therefore of utmost importance to track the spread of such clones to better trace their dissemination and reinforce measures to prevent their further spread. The goal of this study was to investigate the occurrence of carbapenemase-producing hvKp isolates in Switzerland and to determine their genetic profile.

RESULTS

Among the studied 279 carbapenemase-producing *K. pneumoniae* isolates, 58.4% were isolated from rectal swab, 21.5% from urine, 6.1% from respiratory tract, 3.9% from wounds, 3.6% from blood culture, and 6.5% from other biological sites. A total of 9.0% (25 of 279) MDR *K. pneumoniae* carrying important virulence genotype isolates were identified, distributed among 10 different Swiss cantons. The prevalence of such isolates along the years was 0%, 14%, 9.4%, and 6.1% for years 2017, 2018, 2019, and 2020, respectively. These isolates were recovered from rectal swabs (56.0%), urine (28.0%), blood culture (8.0%), tracheal fluids (4.0%), and punctures (4.0%). Antimicrobial susceptibility testing showed that 48%, 80%, and 100% of those isolates presented reduced susceptibilities to imipenem, meropenem, and ertapenem, respectively. A large proportion (84%) of the isolates was resistant to the fluoroquinolones ciprofloxacin and norfloxacin, as well as to aminoglycosides (52%, 84%, and 88% to amikacin, gentamicin, and tobramycin, respectively). Furthermore, 88% of them were resistant to sulfamethoxazole-trimethoprim. All phenotypic resistance profiles are shown in Table S1. The most prevalent carbapenemase identified was NDM-1 (44%), followed by KPC (KPC-3, 28%; KPC-41, 8%) and OXA-48 (20%). The extended-spectrum β -lactamase (ESBL) gene *bla*_{CTX-M-15} was detected in 64% of the isolates. Hence, co-occurrence of *bla*_{CTX-M-15} with different carbapenemase genes was frequently identified, being, respectively, 12%, 20%, and 32% for *bla*_{KPC-3}, *bla*_{OXA-48}, and *bla*_{NDM-1} genes. Complete data showing the corresponding resistomes are given in Table S1.

Among all isolates studied, five main groups could be identified with respect to their capsular types. Group 1 and 2 isolates possess the typical hypervirulent capsular types K1 and K2, respectively, and were thus named MDR-hvKp (4.7%); The remaining groups were considered MDR nonhypervirulent *K. pneumoniae* (MDR-nhv-Kp) strains (4.3%), since they are either common MDR clones that acquired a virulence plasmid or clones presenting with nontypically hypervirulent capsular types, as detailed below. Summarized and complete characteristics of all selected isolates are shown, respectively, in Table 1 and Table S1.

Group 1. Three MDR-hvKp were identified as belonging to the worldwide spread ST23, possessing the conserved capsular type K1 (ST23-K1) (24). They had a virulence score of 5 (presence of aerobactin, colibactin, and yersiniabactin) and carried the aerobactin cluster (*iucABCD_iutA*) along with the yersiniabactin cluster (*ybtAEPQSTUX*, *irp1*, *irp2*, and *fyuA*), the colibactin cluster (*clbABCDEFGHIJLMNOPQ*), and the ferric uptake system cluster (*kfuABC*). Two of those isolates additionally carried a truncated *rmpA2* mucoid regulator. The *magA* gene encoding a component of K1 capsule formation was detected in all ST23-K1 isolates. It is noteworthy that all those ST23-K1 hvKp produced the OXA-48 carbapenemase and had been recovered from rectal swabs (Table 1). All of them presented a plasmid 98% similar to pVir-CR-HvKP4 (MF437313.1) (15), which was detected here and which we named pKpST23_OXA-48 (221,352 bp; OP690161). A schematic representation of plasmid pKpST23_OXA-48 is shown in Fig. S1.

Group 2. Ten MDR-hvKps possessed the K2 capsular type, and all had a virulence score of 4 (presence of aerobactin and yersiniabactin, but no colibactin). Nine of them were ST395 (ST395-K2), and one was ST14 (ST14-K2). We detected the aerobactin cluster and yersiniabactin genes in all these isolates. Six strains possessed a truncated *rmpA2* gene, and the remaining four strains lacked this gene. Of note, a single ST14-K2 isolate possessed the ferric uptake cluster. The ST395-K2 isolates produced different

TABLE 1 Characteristics of carbapenemase-producing *K. pneumoniae* isolates from our collection^a

Strain	ST	K-type	Virulence score	Definition	<i>rmpABC</i>	<i>rmpA2</i>	<i>iucABCD_iutA</i>	<i>ybt</i>	<i>clb</i>	<i>kfu</i>	No. virulence genes	Virulence plasmids	Carbapenemase	Canton	Source	Date
N330	23	K1	5	hvKp	-	+, trunc	+	+	+	+	35	pST23_OXA-48	OXA-48	VD	Rectal swab	2018
N488	23	K1	5	hvKp	-	+, trunc	+	+	+	+	35	pST23_OXA-48	OXA-48	VD	Rectal swab	2019
N593	23	K1	5	hvKp	-	-	+	+	+	+	34	pST23_OXA-48	OXA-48	VD	Rectal swab	2019
N911	14	K2	4	hvKp	-	+, trunc	+	-	+	+	20	pKpCH_aerob	OXA-48	GE	Rectal swab	2019
N321	395	K2	4	hvKp	-	+, trunc	+	-	-	-	17	pKpCH_aerob	KPC-3	VS	Urine	2018
N346	395	K2	4	hvKp	-	-	+	-	-	-	16	pKpCH_aerob	KPC-41	VS	Rectal swab	2018
N447	395	K2	4	hvKp	-	+, trunc	+	-	-	-	17	pKpCH_aerob	KPC-3	VS	Blood culture	2018
N1052	395	K2	4	hvKp	-	-	+	-	-	-	16	pKpST147B-like	NDM-1	TI	Urine	2019
N570	395	K2	4	hvKp	-	+, trunc	+	+	-	-	17	pKpCH_aerob	KPC-3	VD	Tracheal fluid	2018
N732	395	K2	4	hvKp	-	-	+	+	-	-	16	pKpCH_aerob	KPC-3	VS	Rectal swab	2019
N793	395	K2	4	hvKp	-	+, trunc	+	+	-	-	17	pKpCH_aerob	KPC-3	BL	Urine	2019
N435	395	K2	4	hvKp	-	-	+	+	-	-	16	pKpCH_aerob	KPC-41	VD	Rectal swab	2018
N649	395	K2	4	hvKp	-	+, trunc	+	+	-	-	17	pKpCH_aerob	OXA-48	BE	Rectal swab	2019
N621	147	K20	3	nhv-Kp	+	+, trunc	+	-	-	-	9	pKpST147B-like	NDM-1	VD	Rectal swab	2019
N969	147	K20	3	nhv-Kp	+	+, trunc	+	-	-	-	9	pKpST147B-like	NDM-1	LU	Urine	2019
N829	147	K20	3	nhv-Kp	+	-	+	-	-	-	8	pKpST147B-like	NDM-1	LU	Urine	2019
N1259	147	K20	3	nhv-Kp	+	+, trunc	+	-	-	-	9	pKpST147B-like	NDM-1	LU	Urine	2020
N1498	147	K20	3	nhv-Kp	+	+, trunc	+	-	-	-	9	pKpST147B-like	NDM-1	SG	Urine	2020
N1712	147	K64	4	nhv-Kp	-	-	+	+	-	-	16	pKpST147B-like	NDM-1	ZH	Puncture	2020
N1071	147	K64	4	nhv-Kp	+	-	+	+	-	-	19	pKpST147B-like	NDM-1	GE	Rectal swab	2020
N1448	147	K64	4	nhv-Kp	+	+, trunc	+	+	-	-	20	pKpST147B-like	NDM-1	ZH	Rectal swab	2020
N1692	147	K64	4	nhv-Kp	-	-	+	+	-	-	16	pKpST147B-like	NDM-1	ZH	Blood culture	2020
N896	101	K17	4	nhv-Kp	-	+, trunc	+	+	-	+	20	pKpCH_aerob	KPC-3	GE	Rectal swab	2019
N1325	101	K17	4	nhv-Kp	-	-	+	+	-	+	19	pKpCH_aerob	KPC-3	GR	Rectal swab	2020
N608	16	K51	4	nhv-Kp	+	+, trunc	+	+	-	-	20	pKpST147B-like	NDM-1	VS	Rectal swab	2019

^a*rmpABC* and *rmpA2* are mucoid phenotype regulators. *iucABCD_iutA* is an aerobactin cluster, *ybt* is a yersiniabactin cluster, and *clb* is a colibactin cluster. *kfu* shows ferric iron uptake. The virulence plasmids used have the following accession numbers: pST23_OXA-48, OP690161; pKpCH_aerob, OP690162; and pKpST147, CP040726.1. -, absence; +, presence; BE, Bern; BL, Basel-Landschaft; GE, Geneva; GR, Graubünden; LU, Lucerne; SG, St-Gallen; TI, Ticino; VD, Vaud; VS, Valais; ZH, Zürich; hvKp, hypervirulent *K. pneumoniae*; nhv-Kp, nonhypervirulent *K. pneumoniae*; trunc, truncated.

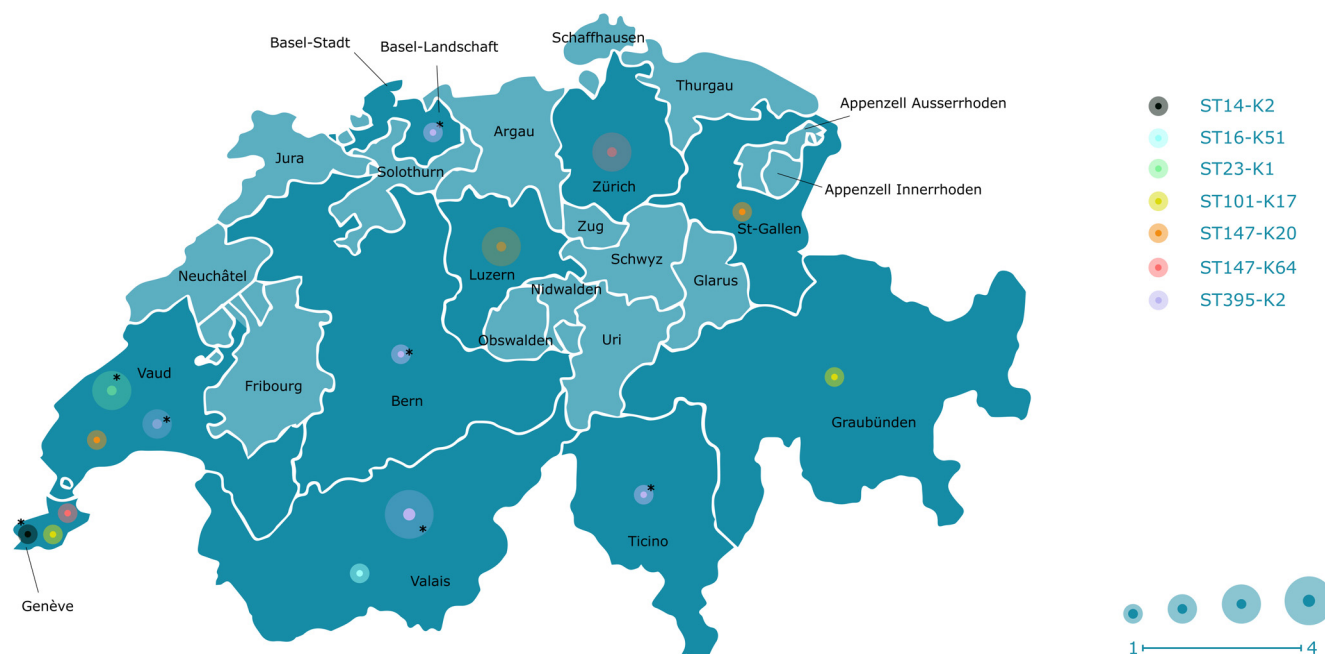


FIG 1 Cantons of Switzerland where the MDR-hvKp and MDR-nhv-Kp isolates were detected from January 2017 to December 2020. Cantons from which an MDR-hvKp or MDR-nhv-Kp isolate was detected are colored dark blue, and the ones where no isolate was detected are colored light blue. *, MDR-hvKp.

carbapenemases, namely, KPC-3 (55.6%, 5 of 9), KPC-41 (22.2%, 2 of 9), NDM-1 (11.1%, 1 of 9), or OXA-48 (11.1%, 1 of 9). The single ST14-K2 produced the OXA-48 carbapenemase. Isolates N911 and N1052 (respectively, ST14 and ST395) were the only isolates giving positive results to the string test. Acquisition and dissemination of the hypervirulence trait among K2-type producers was mainly due to a given plasmid identified here and named pKpCH_aerob (297,473bp; [OP690162](#)) (Fig. S2) that has 86.7% of similarity to plasmid pBA813_1 ([MK649825.1](#)) (25). The exception was only one ST395 isolate that had a pKpvST147B-like ([CP040726.1](#)) (26). Altogether, those MDR-hvKp possessing the K2 capsular type were detected from diverse biological sources and originated from six different Swiss cantons (Fig. 1).

Group 3. Five isolates presented a K20 capsular type, all belonging to ST147 (ST147-K20). All of them had a virulence score of 3 (presence of aerobactin, and absence of yersiniabactin- or colibactin-encoding genes). They carried the aerobactin cluster, and additionally, all but one had a complete *rmpACD* cluster, the remaining isolate having a truncation in the *rmpA* gene and an intact *rmpCD*. The former four isolates also presented the other mucoid phenotype regulator, namely, *rmpA2*, but in all cases, the gene was truncated. They all produced the NDM-1 carbapenemase and were recovered from urine (four isolates) and rectal swab (one isolate) from patients hospitalized in three Swiss cantons (Fig. 1). The occurrence of virulence genotype in those MDR-nhv-Kp isolates was related to the dissemination of a pKpvST147B plasmid ([CP040726.1](#)) (26).

Group 4. Four MDR-nhv-Kp ST147 exhibited a K64 capsular type. These four isolates had a virulence score of 4 and were positive for aerobactin and yersiniabactin clusters. Two of them possessed an additional *rmpACD* cluster, and one of these two had also a truncated *rmpA2* gene. They were all NDM-1 producers and had been recovered from blood culture, puncture, and rectal swabs in two cantons (Fig. 1). Likewise, they carried the aforementioned pKpvST147B plasmid (26).

Group 5. Three MDR-nhv-Kp strains belonged to ST16-K51 ($n = 1$), and ST101-K17 ($n = 2$). These isolates had a virulence score of 4. All of them possessed the aerobactin and yersiniabactin clusters. Additionally, the ST101 isolates had the ferric uptake cluster, and one of them also had a truncated *rmpA2* gene. They produced the KPC-3 carbapenemase and originated from rectal swabs collected in two cantons. The ST16

isolate originating from a rectal swab produced NDM-1 and also carried the whole mucoid phenotype regulator cluster *rmpACD* and a truncated *rmpA2* gene. The pKpVST147B plasmid was identified in the ST16 isolate and both ST101 strains possessed the pKpCH_aerob plasmid, first identified in this study.

DISCUSSION

MDR-hvKp isolates have been increasingly reported during the last decade, even if it is still difficult to evaluate whether this really corresponds to an emerging phenomenon due to the paucity of epidemiological data in relation with that subject. Although these isolates are now commonly identified in Asia (15, 16, 21), it is difficult to evaluate to what extent they have disseminated around the globe (14, 17, 20, 22). In Switzerland, a study reported the first two cases of severe multifocal infections caused by hypervirulent *K. pneumoniae* in 2017 (27), those two isolates being of wild-type phenotype in terms of antibiotic susceptibility.

Following the evolution timeline and confirming the silent spread of convergent MDR-hvKp isolates in the country, Blanc et al. (19) recently reported the occurrence of an ST23-K1 OXA-48-producing *K. pneumoniae* in Switzerland. This isolate was obtained from the rectal microbiota of a young male patient hospitalized at the University Hospital of Lausanne (Vaud Canton) in 2018. The present study confirmed that *K. pneumoniae* possessing an hypervirulence genotype are quite commonly either infecting or colonizing patients in Switzerland, being identified all over the country, since 2018. Hence, we detected one MDR-hvKp ST23-K1 isolate from 2018 and two from 2019. Noticeably, the frequency of detection identified during the years in our collection argues for a significant increasing trend, gathering different clonal backgrounds. Among those, there were isolates producing either the K1 or the K2 capsular type.

The capsular type K1 is the main serogroup causing hvKp infections in Taiwan (28, 29). K1-type strains often belong to ST23, being for that reason considered so-called relatively monophyletic (24). Noticeably, the ST23-K1 isolates identified in this study were all very similar to the ST23 isolate previously reported in Switzerland (19), being also OXA-48 producers, and carrying the colibactin, aerobactin, and yersiniabactin clusters, as well as the *rmpA2* gene (except in isolate N593) (19). However, this gene was found to be truncated in two other isolates in our collection. They were all positive for the *magA* chromosomal gene, involved in capsule biosynthesis of K1 isolates (24, 29). The three ST23-K1 identified in our study had the highest virulence score (virulence score of 5) among all the other 22 isolates. This finding is in line with data from the ECDC report establishing that the majority (82.4%) of the isolates from clade ST23-K1 had a virulence score of 5 (13). Additionally, the *kfuABC* cluster was also identified in the ST23 strains of our collection. This cluster has been associated with increased virulence by enabling lineages to use iron from diverse human and environmental sources (30).

Those ST23-K1 isolates carried a plasmid detected in our study that we named pKpST23_OXA-48. This genetic mobile element carried the aerobactin cluster, a truncated *rmpA2* gene, and a series of resistance genes, including *bla*_{CTX-M-15}, *sul2*, *bla*_{TEM-17}, and *qnrB1*. Moreover, this plasmid has two replicons, namely, IncFIB and IncHI1B. Interestingly, this plasmid had a lower similarity with commonly ST23- or K1-associated plasmids such as plasmids pSGH10 (86%; CP025081.1) (31) and pK2044 (87%; AP006726.1) (32) compared to the 98% of similarity with plasmid pVir-CR-HvKP4 (MF437313.1) (15). The latter is a pLVPK-like plasmid that had been identified in a ST11 carbapenem-resistant hvKp responsible for causing a fatal outbreak in a Chinese Hospital (15). Noteworthy, as opposed to the aforementioned plasmid detected in China, the ST23-K1 detected in our study (all from rectal swabs) was negative for the salmochelin-encoding *iroE* gene. Considering the salmochelin cluster has been proven to be associated with invasive *K. pneumoniae* infections (8, 33) and enhancement of the bacterial growth (34), it might be speculated that those isolates lacking this gene would be less virulent.

As opposed to K1, K2-type MDR-hvKp isolates are not strongly linked to a particular sequence type (ST). Nevertheless, ST65 and ST86 strains are often associated with the K2

serotype (35), but none of them were identified in our study. Two different sequence types were detected within the K2 isolates, confirming the molecular heterogeneous characteristics of this group. One of those isolates corresponded to an OXA-48-producing ST14-K2 isolate. Although ST14 is not often related to K2 in *K. pneumoniae* isolates, Fursova et al. (36) reported cases of severe systemic infections associated with these organisms that evolved to fatal outcomes. According to our findings, the ferric iron uptake system was also detected in the isolates from the mentioned study, as well as in other studies reporting ST14-K2 isolates (37, 38). Interestingly, the ST14-K2 isolate identified in our study was the only one carrying the aerobactin cluster compared to those identified in Russia, Japan, and Algeria (36–38).

ST395-K2 strains were the most frequent MDR-hvKp strains identified in our study. All the nine isolates presented the yersiniabactin and the aerobactin clusters. This combination of features enables bacteria to increase iron acquisition from host transport proteins, enhances the ability to replicate and survive in different biological sites, and finally causes invasive infections (39, 40). Of note, one of the ST395-K2 isolates identified in our study was recovered from a blood culture and produced the KPC-3 carbapenemase. It exhibited a similar virulence profile compared to hvKp isolates with same ST and capsular serotype identified in Russia (41). In that latter study, results from a mouse lethality assay showed that the MDR hvKp-ST395-K2 isolates harboring *rmpA2*, aerobactin, yersiniabactin, and additional virulence genes displayed a higher lethality in the model compared with the ones lacking *rmpA2* and aerobactin but harboring yersiniabactin genes (41). In contrast, four ST395-K2 isolates of our study were negative for the *rmpA2* mucoid phenotype regulator, and the remaining five had truncations in the gene, making them unable to upregulate capsule production (42). This finding might explain the relatively high percentage of strains belonging to this group originating from biological surveillance sources (e.g., 44.4%, 4 of 9 from rectal swabs) rather than from infections.

A novel pKpCH_aerob plasmid was detected in most of the K2-type isolates from our collection. This virulence plasmid first described here possesses two replication sites: IncFIB and IncHI1B. It carries the aerobactin cluster and a truncated *rmpA2* gene, as well as some resistance genes such as *sul1*, *catA1*, and *ant(2'')Ia*. This plasmid is 86.7% similar to the pBA813_1 (MK649825.1) detected in a ST2096-K64 *K. pneumoniae* in India (25). As opposed to the plasmid detected in the Indian isolate, the ST14-K2 and ST395-K2 isolates identified in our study lacked the salmochelin cluster. The pBA813_1 plasmid also carries the *sul1* resistance gene, but in contrast to pKpCH_aerob, it harbors other acquired resistance genes such as *bla*_{CTX-M-15}, *bla*_{OXA-232}, and *bla*_{TEM-54}, among others (25). Surprisingly, one ST395-K2 isolate had a plasmid similar to the pKpVST147 virulence plasmid, which is mainly associated with ST147 *K. pneumoniae* isolates (26).

The well known MDR lineage ST147 was detected in our study presenting two different serotypes: the hypervirulent-associated K20 and the MDR-related K64. Few studies had detected MDR-hvKp ST147-K20 isolates so far. A study performed in Russia identified NDM-1-producing MDR-hvKp ST147-K20 isolates presenting *rmpA*, *rmpA2*, and the aerobactin cluster but lacking the yersiniabactin genes (41). The data shown in the Russian study are similar to the virulome profile of our ST147-K20 isolates, all of them being negative to the *ybt* cluster. Unfortunately, the limitation of our study is that no *in vivo* virulence assay (e.g., *Galleria mellonella* as an infection model) has been performed; thus, the ST147-K20 isolates identified in our study have been defined as MDR-nhv-Kp. Another study performed in the United Kingdom reported an NDM-producing ST147 *K. pneumoniae* carrying the *rmpA*, *rmpA2*, aerobactin, and yersiniabactin genes; however, this isolate had a K35 capsular type (43).

The ST147 isolates of K64 are commonly recognized as classical *K. pneumoniae* (cKp), but they have also recently been described as hybrid MDR-hvKp due to virulence plasmids acquisition (26). Here, we described four NDM-1-producing MDR-Kp belonging to ST147-K64 that all carried the aerobactin and the *ybt* clusters, with two of them possessing the two mucoid phenotype regulators often associated with hypervirulence, namely, *rmpACD* and *rmpA2*. Capsular type K64 isolates have been recently shown to possess an hypervirulence phenotype in the *G. mellonella* model (44); thus, we might also consider that the ST147-K64 isolates from our study could be regarded

as hypervirulent. Of note, isolates belonging to the ST147-K64 lineage that were responsible for a large regional outbreak in Italy carried a similar virulence profile (*ybt*, *iuc*, *rmpACD*, and *rmpA2*) compared to our isolates (44). That latter study showed that the virulence potential of that lineage was variable after infection using *G. mellonella* as an experimental model (44). A multicenter analysis conducted in China showed that ST11-K64 carbapenem-resistant *K. pneumoniae* was the most prevalent hypervirulent profile, being confirmed by an *in vivo* infection model (45). Furthermore, the same capsular type K64 and ST11 profile was reported in a fatal bacteremia in Brazil (46), reinforcing that the K64 isolates could be hypervirulent and transmissible.

The ST147 isolates detected in our study had a virulence plasmid that shared ca. 98.5% of nucleotide identity with plasmid pKpvST147B (CP040726.1) described by Turton et al. (26), which was first detected from a rectal swab of a patient in the United Kingdom (26). Here, we identified such pKpvST147B plasmid among different NDM-producing MDR-nhv-Kp ST147 strains disseminated across the country. We found evidence that this plasmid was commonly associated with virulence factors in our strains, such as (i) the aerobactin cluster, which was carried by this plasmid in all ST147 isolates, and (ii) the mucoid phenotype regulators, being identified in 77.8% (7 of 9) of our isolates. Di Pilato et al. (44) recently demonstrated that hybrid pKpvST147B-like plasmids are mobilizable by conjugation, highlighting their ability to spread among successful MDR clones of *K. pneumoniae*. Indeed, the emergence of these plasmids has been noticed not only in *K. pneumoniae* ST147, but also ST307, ST11, ST15, ST16 (present study), ST395 (present study), and ST383 since 2017 in different countries, suggesting that the distribution of these elements carrying virulence and resistance genes may be wider than expected (22, 26, 47–49).

K. pneumoniae ST101-K17 isolates have been reported in Italy and Brazil (50–52). Virulence genotype of the different ST101-K17 isolates published in these studies includes the yersiniabactin cluster and ferric uptake system, but in contrast with our findings, the aerobactin cluster was lacking. Aerobactins have been associated with a significant increase in survival in serum, human ascites, and systemic and pulmonary infection in mouse models (40). These data support the hypothesis that aerobactin is an important virulence determinant among hvKp, enabling such isolates to cause systemic infection. As mentioned above, we did not perform an *in vivo* virulence test to further characterize our isolates; thus, ST101-K17 isolates identified in our study have been defined as MDR-nhv-Kp.

Isolates producing the K51 capsular type are rarely described. ST16-K51 nonhypervirulent *K. pneumoniae* isolates that were described in Thailand originated from biological sources from three different hospitals (53). In our study, a single NDM-1-producing MDR-nhv-Kp ST16-K51 was identified. The isolates from Thailand had only the yersiniabactin cluster, while the one described in our study possesses the aerobactin, yersiniabactin, and mucoid phenotype regulatory clusters. This isolate harbored a plasmid sharing 99% of nucleotide identity with the pKpvST147B virulence plasmid, known to be associated with the ST147 lineage (26). This finding therefore highlights that this given virulence plasmid might be disseminating within different strain backgrounds.

Phenotypic detection of hypermucoviscosity through the string test has been used to putatively define hypervirulence (54). However, the accuracy of using such a test to extrapolate any correlation with the hvKp phenotype infections remains highly questionable. Reports in the literature addressing this issue indicated that such association might range from 51% to 98% (54, 55). Another recent study reported a sensitivity of 28.7% of the string test compared with the presence of the *iucA* gene by PCR (56).

On the other hand, positive string test results have also been reported among “classical” *K. pneumoniae* phenotypes (57). Recently, Dey et al. (58) reported a *K. pneumoniae* isolate recovered from a patient’s solid cystic lesion that presented a hypermucoviscous phenotype; however, this isolate lacked key virulence determinants such as aerobactin, yersiniabactin, and salmochelin biosynthesis clusters, as well as the *rmpACD* and *rmpA2* genes, which are associated with enhanced capsule synthesis and hypermucoviscosity. Among our collection of 13 MDR-hvKp isolates, only a single ST14-K2 and a single ST395-K2 turned positive for the string test, although both were negative for the *rmpACD* cluster,

and one (ST14) had a truncated *rmpA2* gene. These data also suggest a more critical analysis when dealing with hypervirulence definition.

Interestingly, all our isolates that carried the *rmpA2* mucoid phenotype regulator (60%, 15 of 25) had truncations after, in average, 50.3% length of the intact amino acid sequence, generating a probable loss of protein function. This plasmid-borne mucoid regulator seems to be independent of *rmpA* and produces a RmpA2 protein that can help the K2 capsule serotype biosynthesis interacting with its promoters (59). As mentioned above, 60% (6 of 10) of the K2 isolates had *rmpA2*, but it was always truncated. In fact, truncations in the mucoid phenotype regulators seems to be common, and a significant proportion of the recorded *rmpA* or *rmpA2* genes carries frameshift mutations arising from insertion or deletion of nucleotides at a poly(G) sequence, causing loss of function (60).

Conclusion. This study including isolates recovered during a 4-year period in Switzerland confirms the occurrence and dissemination of worrisome convergent carbapenemase-producing and hypervirulent *K. pneumoniae* isolates. Our findings highlight the importance of an active surveillance network to track and monitor the evolution of those successful hybrid clones. These strains represent a substantial public health threat, and strict monitoring seems indeed crucial to prevent large-scale dissemination and potential outbreaks at the source of very-difficult-to-treat infections and increased mortality/morbidity. We might now consider that screening of carbapenemase genes could be associated with screening of virulence genes when dealing with MDR *K. pneumoniae* isolates. In fact, a new paradigm could be to consider the identification of hvKp strains (and therefore of the responsible virulence markers) as quite systematic when dealing with such MDR strains, with corresponding information being eventually reported to clinicians. Along with the accurate identification of resistance patterns, such systematic screening of virulence traits might be considered useful and help the clinician when a patient develops uncommon clinical and infectious signs.

MATERIALS AND METHODS

Strain collection. A total of 413 carbapenem-resistant *K. pneumoniae* (CrKp) were received at the Swiss National Reference Center for Emerging Antibiotic Resistance (NARA) between 2017 and 2020. From these isolates, 279 (67.6%) were identified as carbapenemase-producing *K. pneumoniae* (CpKp) and were initially included in the study. They had been recovered from different sources, being mostly urine, blood cultures, rectal swabs, and respiratory tract specimens.

Antimicrobial susceptibility testing. Antibiotic susceptibility testing was performed by disk diffusion and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (61). Multidrug resistance (MDR) was defined as proposed by Magiorakos et al. (62).

Carbapenemase detection. Screening of the isolates for carbapenemase genes was performed by PCR for the *bla*_{KPC}, *bla*_{NDM}, *bla*_{IMP}, *bla*_{VIM}, and *bla*_{OXA-48} genes (63–66), and the corresponding amplicons were then sequenced (Microsynth AG, Switzerland). Sequence analysis was performed using MEGA software 11.0.11 (67) and the nucleotide Basic Local Alignment Search Tool (BLAST, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Hypermucoviscosity detection. The string test was performed as described by Fang et al. (54). Briefly, after an overnight incubation at 37°C on a 5% sheep blood agar, the colonies were stretched using a standard bacteriologic loop. Positive strains were defined as those forming a viscous string >5 mm in length.

Virulence features. A set of virulence features was selected to screen the carbapenemase-producing collection for hvKp by using a PCR approach. The focus was made on the mucoid phenotype regulatory genes *rmpA* and *rmpA2* (68, 69), the aerobactin siderophore cluster genes *iucABCD*, *iutA* (69–71); the yersiniabactin siderophore cluster was detected using the *ybtA*, *ybtS*, and *ybtU* genes (72, 73); and the genotoxin colibactin cluster was detected using the *clbABNQ* genes (74), the ferric iron uptake gene *kfu* (30), and the capsular types K1, K2, K5, K20, K54, and K57 (28, 54, 75). The gene *magA* was detected to double check the presence of K1 (69).

Hypervirulence definition. Preselection of hvKp candidates among CpKp was performed using the virulence score based on the presence of the genes encoding yersiniabactin (*ybt*), colibactin (*clb*), and aerobactin (*iuc*) proposed by Lam et al. (76). Using this scheme, virulence scores vary from 0 to 5. Isolates with a score equal to 0 to 2 were not further considered in this study, and those with scores of 4 to 5 were included in the study. Additionally, isolates with virulence score of 3 simultaneously presenting the molecular biomarker *rmpACD* and *rmpA2* genes (mucoid phenotype regulators) were also included (77). Finally, those strains presenting the typical hypervirulent capsular type K1 and K2 were named MDR-hvKp. The remaining isolates were considered MDR nonhypervirulent *K. pneumoniae* (MDR-nhv-Kp) strains; they were either common MDR clones that acquired a virulence plasmid or present non-typically hypervirulent capsular types.

Whole-genome sequencing (WGS). Genomic DNA paired-end libraries were generated using the Nextera sample preparation kit (Illumina, Inc., USA). The libraries were sequenced using the Illumina

MiSeq platform (Illumina, <https://www.illumina.com>) with 2×150 bp paired-end reads. Raw sequence data were previously submitted to the National Center for Biotechnology Information's Sequence Read Archive (BioProject no. [PRJNA890192](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA890192)). Reads were assembled into contigs using a SPAdes (78) tool-based pipeline called Shovill (<https://github.com/tseemann/shovill>). Sequence types, the presence of acquired resistance genes, species, and plasmid replicons were detected using, respectively, MLST version 2.0, ResFinder version 4.1 (79), KmerFinder version 3.2 (80), and PlasmidFinder 2.1, on the Center for Genomic Epidemiology platform (<https://www.genomepidemiology.org/>); the presence of virulence-associated features, including capsular type, and the virulence scores were confirmed using Kleborate tool (<https://github.com/katholt/Kleborate>); and contigs were annotated using Prokka (81).

Two isolates representing the well known hypervirulent associated K1 (ST23;_isolate N330) and K2 (ST395;_isolate N447) capsular types were selected for long-read sequencing as described elsewhere (82). Briefly, genomic DNA was extracted using QIAamp DNA minikit (Qiagen, Valencia, CA, USA) and sequenced using the MinION Mk1C (Oxford Nanopore Technologies, Oxford, UK). The libraries were prepared using a one-dimensional chemistry ligation sequencing kit (SQK-LSK109, Oxford Nanopore Technologies). Sequencing was performed in a R9.4.1 Flongle flow cell (FLO-FLG001, Oxford Nanopore Technologies). The sequences were first assembled using a Canu tool (83). Finally, trimmed long-read sequences and short-read sequences were used to construct hybrid assemblies with UniCycler tool (84).

Worldwide-disseminated virulence plasmid sequences were downloaded from GenBank to generate a plasmid reference database for mapping analyses. CLC Genomics Workbench 20.0.4 Qiagen Aarhus A/S (Qiagen, Redwood City, CA, USA) was used to confirm the presence of the plasmids by mapping sequences against the reference database. Then the Mauve Multiple Genome Alignment tool (85) was used to reorder and analyze plasmids sequence matches and manually mitigate against false-positive sequences. Finally, we used $>95\%$ coverage and identity to define significant matches.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.02 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.8 MB.

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