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Detection of carbapenemase- and ESBL-producing *Klebsiella pneumoniae* from bovine bulk milk and comparison with clinical human isolates in Italy

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A R T I C L E I N F O

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A B S T R A C T

Klebsiella pneumoniae is the most common *Klebsiella* species infecting animals and is one of the causing agents of mastitis in cows. The rise of antimicrobial resistance in *K. pneumoniae*, particularly in strains producing extended-spectrum β-lactamases (ESBLs) and/or carbapenemases, is of concern worldwide. Recently (Regulation UE No 2022/1255), carbapenems and cephalosporins in combination with β-lactamase inhibitors have been reserved only to human treatments in the European Union.

The aim of this study was to investigate the role of cattle as carrier of human pathogenic carbapenem-resistant (CR) and ESBL-producing *K. pneumoniae*. On this purpose, a study involving 150 dairy farms in Parma province (Northern Italy) and 14 non replicate *K. pneumoniae* isolates from patients admitted at Parma University-Hospital was planned.

Four multidrug resistant (MDR) *K. pneumoniae* strains were detected from 258 milk filters collected between 2019 and 2021. One carbapenemase KPC-3-positive *K. pneumoniae* ST307 (0.4 %; 95 % CI – 0.07 - 2.2) was detected in milk filters. The isolate also harboured OXA-9, CTX-M-15 and SHV-106 determinants, together with genes conferring resistance to aminoglycosides (*aac(3′)-IIa, aph (3″)-Ib, aph (6)-Id*), fluoroquinolones (*oqxA, oqxB, qnrB1*), phosphonic acids (*fosA6*), sulphonamides (*sul2*), tetracyclines (*tet(A)6*) and trimethoprim (*dfrA14)*. One KPC-3-producing *K. pneumoniae* ST307 was identified also among the human isolates, thus suggesting a possible circulation of pathogens out of the clinical settings.

The remaining three bovine isolates were MDR ESBL-producing *K. pneumoniae* characterized by different genomic profiles: CTX-M-15, TEM-1B and SHV-187 genes (ST513); CTX-M-15 and SHV-145 (ST307); SHV-187 and DHA-1 (ST307). Occurrence of ESBL-producing *K. pneumoniae* in milk filters was 1.2 % (95 % CI 0.4–3.4). All the isolates showed resistance to aminoglycosides, 3rd-generation cephalosporins, and fluoroquinolones. Among the human isolates, two multidrug resistant ESBL-producing *K. pneumoniae* ST307 were found, thus confirming the circulation of this high-risk lineage between humans and cattle.

Our findings suggest that food-producing animals can carry human pathogenic microorganisms harboring resistance genes against carbapenems and 3rd-generation cephalosporins, even if not treated with such antimicrobials. Moreover, on the MDR *K. pneumoniae* farms, the antimicrobial use was much higher than the Italian median value, thus highlighting the importance of a more prudent use of antibiotics in animal productions.

Abbreviations: AMR, Antimicrobial Resistance; BPW, Buffered Peptone Water; CAZ, Ceftazidime; CDT, Combination Disc Test; CP, Carbapenemase-producing; CR, Carbapenem-resistant; CTX, Cefotaxime; DDD, Defined Daily Dose; DDDit, Defined Daily Dose Animal for Italy; EARS-Net, European Antimicrobial Resistance Surveillance Network; ECOFF, Epidemiological cut off; ESBL, Extended-Spectrum β-Lactamase; EU, European Union; EUCAST, European Committee on Antimicrobial Susceptibility Testing; MEM, Meropenem; MIC, Minimal Inhibitory Concentration; MDR, Multidrug-resistant; MLST, Multi Locus Sequence Typing; WGS, Whole genome sequencing; WWWTP, Wastewater treatment plant.

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1. Introduction

The rise of antimicrobial resistance (AMR) in *Klebsiella pneumoniae*, particularly in strains producing Extended-Spectrum β-Lactamases (ESBLs) and/or carbapenemases (Bialek-Davenet et al., 2014) is of concern. To strengthen the European One Health AMR surveillance approach, the European Antimicrobial Resistance Surveillance network in Veterinary medicine (EARS-Vet) was recently built and connected with the European Antimicrobial Resistance Surveillance Network (EARS-Net), which monitors AMR in invasive bacterial isolates from humans. The microorganisms covered in EARS-Vet also include carbapenem-resistant (CR) *K. pneumoniae* to tackle its occurrence in the animal compartment and to assess the risk of transmission of CR *K. pneumoniae* or CR genes between animals and humans (Mader et al., 2021). Carbapenems include imipenem, meropenem and ertapenem, which are "last line defence" antimicrobials in human infections caused by Gram-negative multi-resistant bacteria (Nordmann et al., 2011; Woodford et al., 2014). In 2022, Commission Implementing Regulation EU 2022/1255 included carbapenems as well as combinations of cephalosporins with β-lactamase inhibitors among the antimicrobial groups reserved for treatments in humans and prohibited for veterinary treatments.

At the time of writing, the β-lactamases data base ((Naas et al., 2017); www.bldb.eu) includes >7000 enzymes. Among β-lactamases, carbapenemases are enzymes capable of hydrolyzing carbapenems. Based on their structural properties, they are divided into different classes: Class A gathers KPC, IMI, GES and SME (serine-enzymes) (Bonomo, 2017); Class B enzymes (metallo-enzymes) includes the IMP, VIM and NDM families and show the highest carbapenemase activity (Nordmann et al., 2011); at last, class D (serine-enzymes) groups carbapenem-hydrolyzing oxacillinases (OXA) (Naas et al., 2017). Among the Extended-Spectrum β-Lactamases (ESBLs), the enzymes TEM, SHV, VEB, or CTX-M are the most frequently identified, as well as CMY, FOX, DHA, ACT, or MOX among the AmpC cephalosporinases (Bonomo, 2017). These genes are commonly located on mobile genetic elements and often coexist with additional resistance determinants active against other classes of antimicrobials such as aminoglycosides and fluoroquinolones (Marti et al., 2014).

In cattle, *K. pneumoniae* is considered an opportunistic pathogen which is shed by feces and can cause mastitis (Kikuchi et al., 1995), belonging to the so called "environmental" group of mastitis-causing pathogens (Klaas and Zadoks, 2018). Detection of ESBL-producing *K. pneumoniae* in bovine mastitis is reported in different countries, including Italy (Locatelli et al., 2010), as well as the occurrence of carbapenemase-producing (CP) *K. pneumoniae* in China (NDM-5 *K. pneumoniae*), Lebanon (OXA-48 *K. pneumoniae*) and Brazil (KPC-2 *K. pneumoniae)* (Diab et al., 2017; He et al., 2017; Silva-Sanchez et al., 2021).

In humans, *K. pneumoniae* is an opportunistic pathogen which commonly colonises the gastrointestinal tract, the skin, and the respiratory tract of hospitalised patients, leading to urinary tract, lower respiratory tract, intra-abdominal and bloodstream infections. Most infections caused by *K. pneumoniae* are healthcare-associated and can spread rapidly in hospital settings, even causing outbreaks (ECDC (European Centre for Disease Prevention and Control), 2020). In Italy, *K. pneumoniae* ST258 producing KPC-2 or KPC-3 had an epidemic dissemination in the past decade (Giani et al., 2013). Recently, hospital outbreaks have been predominantly attributed to KPC-3 *K. pneumoniae* ST258, ST307 and ST512 (Calia et al., 2017; Rimoldi et al., 2017; Villa et al., 2017). From 2018 to 2021, a prolonged outbreak of NDM-1 *K. pneumoniae* ST147 has been reported in Tuscany region, Central Italy (Tavoschi et al., 2020).

Even before Commission Implementing Regulation EU 2022/1255 was in force, carbapenems were never licensed for veterinary use worldwide (OIE (World Organisation for Animal Health), 2022). Consequently, no maximum residue limits for carbapenems had been defined in the European Union (EU) and no legally 'safe' limits in meat or milk do exist. Nevertheless, food-producing animals could acquire CR *K. pneumoniae via* different routes mainly represented by environmental pollution from hospitals' sewage drains and wastewaters (Picão et al., 2013; Popa et al., 2021; Rocha et al., 2022; Zurfluh et al., 2017). Additionally, selective pressure from using other β-lactams such as 3rd generation cephalosporins could play an important role in developing CR microorganisms (Mollenkopf et al., 2018).

To investigate the role of cattle as carriers of CR *K. pneumoniae*, we planned a study in dairy farms located in Parma province, Northern Italy. Thanks to the cooperation with Parma University-Hospital, we could compare clinical human CR *K. pneumoniae* strains with the bovine isolates. Therefore, our study was focused on: *i)* the monitoring of CR *K. pneumoniae* in dairy cattle farms; *ii)* the genomic comparison of CR *K. pneumoniae* of bovine origin with CR *K. pneumoniae* clinical human strains; and *iii)* the evaluation of factors influencing CR emergence in dairy cattle.

2. Materials and methods

2.1. Sample collection

2.1.1. Bovine samples

A total of 258 milk filters were collected by the Competent Authority in 150 dairy cattle farms in Parma province from March 2019 to March 2021. The number of lactating cows per farm ranged from 13 to 1447, with a median number of 79 lactating animals. Sample collection varied between farms, mainly based on the number of lactating cows and farmers' willingness to cooperate. In most farms ($n = 124$), one filter was collected during the whole study period. Two filters were collected from 22 farms, and three, seven and eight filters were collected from one farm each, respectively. From farm N° 31 (1065 and 1038 lactating cows in 2020 and 2021, respectively) the number of collected filter was 66. Filters were stored in sterile bags, transported to the laboratory at refrigeration conditions (2–4 °C) and tested within 24–48 h.

2.1.2. Human isolates

The human strains were represented by 14 non replicate CR *K. pneumoniae* strains collected from patients admitted at the Renal Intensive Care Unit and the Renal Transplant Unit of the Parma University-Hospital (Italy) with a diagnosis of acute kidney injury or kidney transplant during the routine surveillance activity. The isolates were frozen at −80 °C and transported to the laboratory at refrigeration conditions (2–4 °C). Patients suffering from kidney diseases are a population at high risk for MDR-associated complications because of the coexistence of many factors increasing the risk of colonization and infection (Wang et al., 2019). The microorganisms were isolated in the four-year period 2017–2020 and were either resistant to meropenem ($n = 11$ isolates) or revealed Minimal Inhibitory Concentration (MIC) values higher than the epidemiological cut off (ECOFF) ($n = 4$ isolates) proposed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) in 2019. The strains were anonymised by the responsible of hospital surveillance. Following a One Health perspective, they were included in the present study to allow a comparison with the animal CR *K. pneumoniae* isolates detected in the same geographical area of Northern Italy.

2.2. Sample testing

Each milk filter was suspended in 100 ml of Buffered Peptone Water (BPW; Biolife Italiana, Italy) and incubated at 37 ± 1 °C for 18–22 h. After overnight incubation, *i)* 100 μl of the brothculture were streaked onto Mac Conkey agar (Oxoid) plates added with a 10-μg meropenem (MEM) disk and *ii)* 10 μl were streaked onto Brilliance CRE Agar (Oxoid), which provides presumptive identification of CR microorganisms. Plates were incubated aerobically at 35 ± 2 °C for 18–24 h.

Suspect *K. pneumoniae* colonies (blue colonies grown on Brilliance CRE Agar) and purple colonies grown in proximity of the MEM disk were subcultured onto Trypticase Soy Agar plates (TSA; Oxoid) at 37 ± 1 °C for 18–22 h to obtain pure cultures. One single, pure colony was picked and diluted in 5 ml of sterile saline solution, then the bacterial suspension was used to fill up the compartments of API® 20 E microsubstrate system (bioMérieux, Marcy l'Etoile, France) to obtain species identification. The test was performed following the manufacturer's instructions.

2.3. Antimicrobial susceptibility testing

K. pneumoniae isolates were analysed by the MIC test for meropenem (MEM), cefotaxime (CTX) and ceftazidime (CAZ) by using Sensititre EUVSEC3 plates (Thermo Scientific, East Grinstead, UK). Clinical categorization of the isolates was performed based on the (EUCAST, 2019) clinical breakpoints.

The isolates showing MIC values indicative of resistance to MEM (> 8 μg/ml; clinical breakpoint) were retested by the MIC dilution method proposed by EUCAST (EUCAST, 2019). Resistant strains were subjected to phenotypic confirmation of carbapenemase production by the Combination Disc Test (CDT) (KPC/Metallo-β-Lactamase Confirm Kit, code 98006, ROSCO Diagnostica, Taastrup, DK) following the manufacturer's instructions.

The isolates resistant to CTX (> 2 μg/ml) and/or CAZ (> 4 μg/ml) were tested by CDT for phenotypic confirmation of ESBL production (ESBL Confirm Kit according to CLSI/EUCAST, code 98011, ROSCO Diagnostica) following the manufacturer's instructions.

E. coli ATCC 25922 was tested periodically as quality control microorganism.

2.4. Whole genome sequencing

K. pneumoniae isolates of bovine and human origin were tested by whole genome sequencing (WGS).

K. pneumoniae genomic DNA was extracted using the MagAttract HMW DNA Kit (Qiagen, Hilden, Germany). The purified DNA concentration and the quality parameter ratio at 260/280 were measured by BioSpectrometer fluorescence (Eppendorf). Whole genomes were sequenced on Illumina MiSeq platform (Nextera library, paired-end reads). The Unicycler v0.5.0 pipeline was used to quality check and *de novo* assemble reads into contigs. The pipeline includes SPAdes 3.14.0 as *de novo* assembler and provides Multi Locus Sequence Typing (MLST) profile.

SNP calling was performed on reads using the freely available snippy 4.0 pipeline with default setting (https://github.com/tseemann/snippy). An alignment of core SNPs was produced to infer a high-resolution phylogeny. A maximum likelihood (ML) tree was constructed using the iQTree program to analyse SNP differences between isolates. The phylogeny tree inferred was viewed using iTOL v4.2.3 software (https://itol.embl.de/). *Klebsiella pneumoniae* str. HS11286 (Ref Seq GCA_000240185.2) was used as references for SNP calling on *K. pneumoniae* newly sequenced reads.

Analysis of resistome of genomes was performed using ABRicate v1.0.1 (https://github.com/tseemann/abricate/). With this tool a BLAST search of genes included in the Resfinder database was performed on *de novo* assemblies of newly sequenced genomes (Center for Genomic Epidemiology, 2019).

De novo assemblies were annotated by PROKKA v1.14.6. The genetic environment of carbapenemase encoding genes was visualised by SnapGene (https://www.snapgene.com). Assembled sequences are available at NCBI (https://www.ncbi.nlm.nih.gov) under BioProject PRJNA587603.

3. Results

3.1. Klebsiella pneumoniae of bovine origin

3.1.1. Identification of β-lactamase-encoding genes

Four *K. pneumoniae* strains were detected from 258 milk filters. MIC was indicative of resistance to MEM in the isolate named FL 172 (64 μg/ml). Tested by CDT, the combination MEM and fenilboronic acid disk showed a ≥5 mm inhibition zone diameter increase *versus* the MEM disk, thus phenotypically confirming the production of carbapenemases. By WGS, the strain FL 172 was found to harbour KPC-3 and OXA-9 determinants, together with the ESBL genes CTX-M-15 and SHV-106. The isolate belonged to ST307 (Table 1). The KPC-3 gene was found located within ISKpn6 and ISKpn7 two insertion sequences used by Tn3 family transposases to mobilise included genes (Fig. 1). No further AMR genes were found in the same genetic environment of KPC-3 (Fig. 1). Occurrence of carbapenemase-producing (CP) *K. pneumoniae* in milk filters was 0.4 % (95 % CI – 0.07 - 2.2).

The other three *K. pneumoniae* isolates showed MIC_{MEM} values indicative of sensitivity to carbapenems (0.03 μg/ml) (Table 2). They belonged to ST513 and ST307 and carried the ESBL genes CTX-M-15, TEM-1B and SHV-187 (ST513), CTX-M-15 and SHV-145 (ST307) and SHV-187 plus the AmpC cephalosporinase DHA-1 (ST307). CDT phenotypically confirmed the production of β-lactamases. Occurrence of ESBL-producing *K. pneumoniae* in milk filters was 1.2 % (95 % CI 0.4–3.4).

3.1.2. Identification of other AMR genes

In the *K. pneumoniae* strains of bovine origin, a minimum of 9 to a maximum of 14 antimicrobial resistance genes other than carbapenemase- and β-lactamase-encoding genes were found. KPC-3 *K. pneumoniae* strain ST307 (strain FL 172) coharboured OXA-9, CTX-M-15, and SHV-106 determinants together with 10 other genes conferring resistance to aminoglycosides (*aac(3′)-IIa, aph (3″)-Ib, aph (6)-Id*), fluoroquinolones (*oqxA, oqxB, qnrB1*), phosphonic acids (*fosA6*), sulphonamides (*sul2*), tetracyclines (*tet(A)*) and trimethoprim (*dfrA14*) (Table 1). In ESBL-producing *K. pneumoniae* strains, in addition to the abovementioned classes of antimicrobials, resistant genes to macrolides (*mph(A)*) and phenicols (*catA1*) were identified (Table 2).

3.2. Klebsiella pneumoniae of human origin

3.2.1. Identification of β-lactamase-encoding genes

In the eleven phenotypically CR K. pneumoniae isolates (MICMEM range from 16 to 256 μg/ml), CDT confirmed the production of carbapenemases. WGS identified class A and B β-lactamase-encoding genes, namely KPC-3 (7 isolates; 63.6 %), KPC-2 (1 isolate; 9.1 %), and VIM-1 (3 isolates; 27.3 %). One K. pneumoniae strain (9.1 %) coharboured KPC-2 and VIM-1 genes. In addition, class D β-lactamase genes were harboured, namely OXA-1 (3 isolates; 27.3 %), OXA-9 (8 isolate; 72.7 %); one strain (9.1 %) carried both OXA-1 and OXA-9. ESBL genes (CTX-M-15, TEM—1B, SHV-12, SHV-27, SHV-28, SHV-106, SHV-187) were carried in different combinations (Table 1).

CP K. pneumoniae isolates belonged to different sequence types, namely ST554 (6; 54.5 %), ST37 (1; 9.1 %), ST258 (2; 18.2 %), ST307 (1; 9.1 %), and ST4525 (1; 9.1 %). KPC-2 and KPC-3 determinants were linked to specific ST, namely ST258 (KPC-2), ST307 (KPC-3) and ST554 (KPC-3). VIM-1 was carried by ST258, ST37 and ST4525 isolates (Table 1).

ST4525 is a novel ST-type with the following allelic profile: gapA (2); infB (9); mdh (2); pgi (1); phoE (13); rpoB (214); tonB (16). This allelic profile differs from ST37 by only one nucleotide within rpoB gene: T at position 100 of allele rpoB_1 (ST37) is substituted by a C in allele rpoB_214 (ST4525) (Fig. 1).

The remaining three K. pneumoniae isolates showed MICMEM values ranging from 0.03 to 2 μg/ml and did not carry carbapenemase-

Table 1
MIC values against MEM (meropenem), CTX (cefotaxime), and CAZ (ceftazidime), MLST and β-lactamase-encoding genes in bovine and human carbapenemase-producing *K. pneumoniae* isolates.

Source	ID code	MIC_{MEM} μg/ml	MIC_{CTX} μg/ml	MIC_{CAZ} μg/ml	MLST	*bla* genes	Other AMR genes
Cattle	FL 172	64	4	8	307	KPC-3, OXA-9, CTX-M-15, SHV-106	aac(3′)-IIa, aph(3″)-Ib, aph(6)-Id, dfrA14, fosA6, oqxA, oqxB, qnrB1, sul2, tet(A),
Human	NEF 48	16	4	8	37	VIM-1, OXA-1, CTX-M-15, SHV-12	ant(3″)-Ia, aph(3′)-XV, aph(3″)-Ib, aph(6)-Id, armA, catA1, catB2, catB3, dfrA5, dfrA14, fosA6, mph(A), mph(E), msr(E), oqxA, oqxB,sul1, sul2
Human	NEF 87	32	4	8	258	KPC-2, OXA-9, SHV-187	aac(6′)-Ib, aadA2, catA1, dfrA12, fosA6,oqxA, oqxB, mph(A), sul1
Human	NEF 88	16	4	8	258	KPC-2, VIM-1, OXA-9, SHV-12	aac(6′)-Ib, aadA2, catA1, dfrA12, mdf(A), mph(A), oqxA, oqxB, sul1
Human	NEF 116	64	4	8	554	KPC-3, OXA-9, SHV-182	aac(6′)-Ib, aadA2, aph(3′)-Ia, dfrA12, fosA6,mph(A), oqxA, oqxB,sul1
Human	NEF 123	16	4	8	4525	VIM-1, OXA-1, CTX-M-15, SHV-12	ant(3″)-Ia, aph(3′)-XV, aph(3″)-Ib, aph(6)-Id, catB2, catB3, dfrA14, fosA6, mph(A), oqxA, oqxB, sul1, sul2,
Human	NEF 130	>256	4	8	554	KPC-3, OXA-9, SHV-182	aac(6′)-Ib, aadA2, aph(3′)-Ia, catA1, dfrA12, fosA6, mdf(A), mph(*A*)c, oqxA, oqxB, sul1
Human	NEF 131	>256	4	8	554	KPC-3, OXA-9, SHV-182	aac(6′)-Ib, aadA2, aph(3′)-Ia, catA1,dfrA12, fosA6, mdf(A), mph(A), oqxA, oqxB, sul1
Human	NEF 196	64	4	8	554	KPC-3, OXA-9, SHV-182	aac(6′)-Ib, aadA2, aph(3′)-Ia, catA1, dfrA12, fosA6, mdf(A), mph(A), oqxA, oqxB, sul1,
Human	NEF 197	64	4	8	554	KPC-3, OXA-9, SHV-182	aac(6′)-Ib, aadA2, aph(3′)-Ia, oqxA, oqxB, mdf(A), mph(A), fosA6, catA1, sul1, dfrA12
Human	NEF 297	32	4	8	554	KPC-3, OXA-9, SHV-182	aac(6′)-Ib, aadA2, aph(3′)-Ia, dfrA12, catA1, fosA6, mph(A), oqxA, oqxB, sul1
Human	2 NEF 120	>256	4	8	307	KPC-3, OXA-1, OXA-9, SHV-106, CTX-M-15, TEM-1B	aac(3′)-IIa, aac(6′)-Ib-cr,aph(3″)-Ib, aph(6)-Ia, dfrA14, oqxA, oqxB, qnrB1, sul2, tet(A)

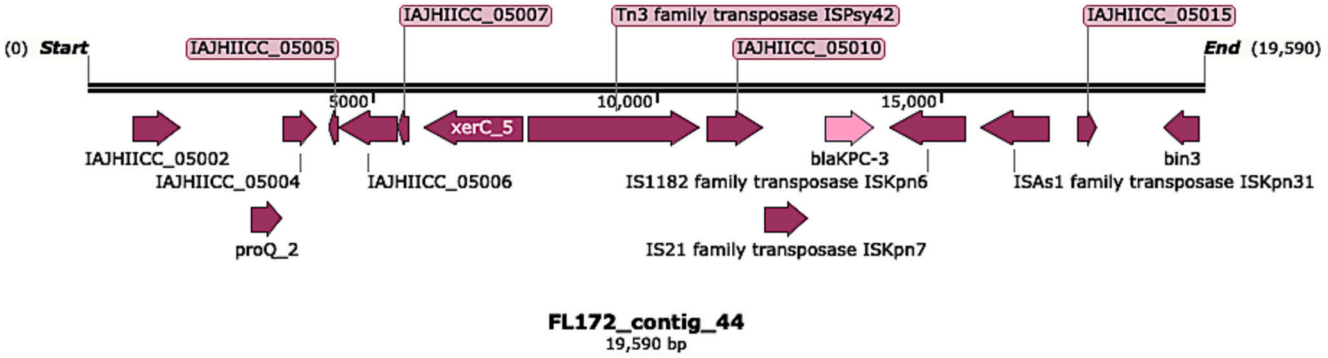

Fig. 1. Genetic environment of *bla*KPC-3 gene in FL172.

Table 2
MIC values against MEM (meropenem), CTX (cefotaxime), and CAZ (ceftazidime), MLST and β-lactamase-encoding genes in bovine and human ESBL-producing *K. pneumoniae* isolates.

Source	ID code	MIC_{MEM} μg/ml	MIC_{CTX} μg/ml	MIC_{CAZ} μg/ml	MLST	*bla* genes	Other AMR genes
Cattle	FL 98	0.03	4	8	513	CTX-M-15, TEM—1B, SHV-187	aph(3″)-Ib, aph(6)-Id, dfrA1, fosA5, oqxA, oqxB, qnrB1, sul2, tet(A)
Cattle	FL 133	0.03	4	8	307	SHV-187, DHA-1	aadA2, aph(3″)-Ib, aph(6)-Id, catA1, dfrA12, mph(A), oqxA, oqxB, qnrB1, qnrS1, sul1, sul2, tet (A)
Cattle	FL 212	0.03	4	8	307	CTX-M-15, SHV-145	aadA2, aph(3″)-Ib, aph(6)-Id, dfrA12, fosA6, mph(A), oqxA, oqxB, qnrB1, qnrB4, qnrS1, sul1, sul2, tet(A)
Human	NEF 199	2	4	8	277	SHV-27	fosA6, mdf(A), oqxA, oqxB
Human	NEF 261	0.03	4	8	307	CTX-M-15, SHV-28	aac(3′)-IIa, aadA1, ant(3″)-Ia, aph(3″)-Ib, fosA6, fosA7, mph(E), mdf(A)
Human	NEF 278	0.06	4	8	307	CTX-M-15, TEM—1B, SHV-106	aac(3′)-IIa, aac(6′)-Ib, aph(3″)-Ib, aph(6)-Id, dfrA14, mdf(A), oqxA, oqxB, qnrB1, sul2, tet(A)

encoding genes. They were found to be CTX and CAZ resistant and CDT confirmed the production of β-lactamases. The ESBL-producing K. pneumoniae belonged to ST277 and ST307 and carried SHV-27 gene (ST277) as well as CTX-M-15, TEM—1B, SHV-106 (ST307) and TEM—1B, SHV-28 determinants (ST307) (Table 2).

3.2.2. Identification of other AMR genes

Apart from carbapenemase- and β-lactamase-encoding genes, a

minimum of 9 up to a maximum of 18 antimicrobial resistance genes were found in human *K. pneumoniae* isolates. The most common detected genes were associated to resistance against aminoglycosides, β-lactams, macrolides, fluoroquinolones, phosphonic acids, phenicols, trimethoprim and sulphonamides (Table 1). In the ESBL *K. pneumoniae* strains, 4 to 11 other antimicrobial resistance genes were found, mainly against aminoglycosides and fluoroquinolones (Table 2).

As detailed in Table 1, KPC-2 *K. pneumoniae* ST258 isolates carried other β-lactamase producing genes, namely OXA-9 and SHV-187 (NEF 87 isolate) or OXA-9 and SHV-12 (NEF 88 isolate). Apart from carbapenemases and β-lactamases, their resistome profile consisted of 9 genes conferring resistance to aminoglycosides (*aac(6′)-Ib, aadA2*), β-lactams, chloramphenicol (*catA1*), fluoroquinolones (*oqxA, oqxB*), fosfomycin (*fosA6*) (NEF 87 only), macrolides (*mph(A)*), major facilitator superfamily (*mdf(A)*) (NEF 88 only), sulphonamides (*sul1*), and trimethoprim (*dfrA12*). The six KPC-3 *K. pneumoniae* ST554 isolates also carried OXA-9 and SHV-182 determinants, together with 10 to 12 other genes indicative of resistance to aminoglycosides (*aac(6′)-Ib, aadA2, aph(3′)-Ia*), macrolides (*mphA*), sulphonamides (*sul1*), trimethoprim (*dfrA12*), chloramphenicol (*catA1*), fluoroquinolones (*oqxB, oqxA*), fosfomycin (*fosA6*) and major facilitator superfamily (*mdf(A)*). VIM-1 *K. pneumoniae* ST37 isolate coharboured OXA-1, CTX-M-15 and SHV-12 determinants and its resistome consisted of 22 genes; in addition to β-lactams, genes conferring resistance to aminoglycosides (*ant(3″)-Ia,aph(6)-Id, aph(3″)-Ib, aph(3′)-XV) armA*), macrolides (*mph(E), mph(A), msr(E)*), sulphonamides (*sul1, sul2*), trimethoprim (*dfrA14, dfrA5*), chloramphenicol (*catA1, catB2, catB3*), fluoroquinolones (*oqxB, oqxA*), and fosfomycin (*fosA6*) were found.

The novel VIM-1 *K. pneumoniae* ST4525 (NEF 123) was characterized by a resistome of 17 genes, which included the β-lactamase genes CTX-M-15, SHV-12, OXA-1 and genes for resistance to aminoglycosides (*ant (3″)-Ia, aph(3′)-XV, aph(3″)-Ib, aph(6)-Id),* fluoroquinolones (*oqxA, oqxB)*, macrolides (*mph(A)*), *fosfomycin (fosA6),* chloramphenicol *(catB2, catB3),* sulphonamides *(sul1, sul2),* and trimethoprim *(dfrA14)* (Table 1).

Among the ESBL *K. pneumoniae* isolates, one ST307 strain harboured SHV-106 and TEM-102 determinants, and the other carried OXA-1, CTX-M-15, TEM-1B and SHV-106 encoding genes. *K. pneumoniae* ST277 strain carried SHV-27. Apart from β-lactamase-encoding genes, their resistome profiles consisted of 4 to 11 genes and showed a great diversity (Table 2).

3.3. Subtyping of K.pneumoniae of bovine and human origin

Based on SNP calling, a phylogenetic tree of all *K. pneumoniae* genomes was inferred (Fig. 2). As expected, genomes clustered according to their ST-Type. Interestingly four clusters gathered genetically similar isolates. The first group included ST307 strains namely FL 172, 2NEF 120 and NEF 261. FL 172 of bovine origin and 2NEF 120 of human origin were CP *K. pneumoniae* whereas NEF 261 of human origin was carbapenem sensitive and ESBL producer. This cluster showed SNPs pairwise distance from 133 to 156 SNPs suggesting the genetic similarity of these strains. The second cluster gathered NEF 48 strain belonging to ST37. A third cluster included ST258 strains namely NEF 87 and NEF 88 isolated the same day from two different patients. Both carbapenemase producers and of human origin, they differed by 51 SNPs. The fourth cluster gathered ST5424 *K. pneumoniae* strains namely NEF 297, NEF 130, NEF 131, NEF 116, NEF 196, NEF 187. All carbapenemase producers and of human origin, they were isolated from July 2017 to August 2018 and they showed pairwise SNP distance from 7 to 59 SNPs, suggesting their close genetic relatedness probably due to a common ancestor.

4. Discussion

4.1. Carbapenemase - and ESBL-producing K. pneumoniae in dairy cattle

K. pneumoniae is by far the most common *Klebsiella* species infecting animals (Brisse and Duijkeren, 2005). It is also the most common *Klebsiella* species to cause mastitis in dairy cattle (Erskine et al., 2002; Roberson et al., 2004) and its ability to damage bovine mammary epithelial cells has been demonstrated (Cheng et al., 2020).

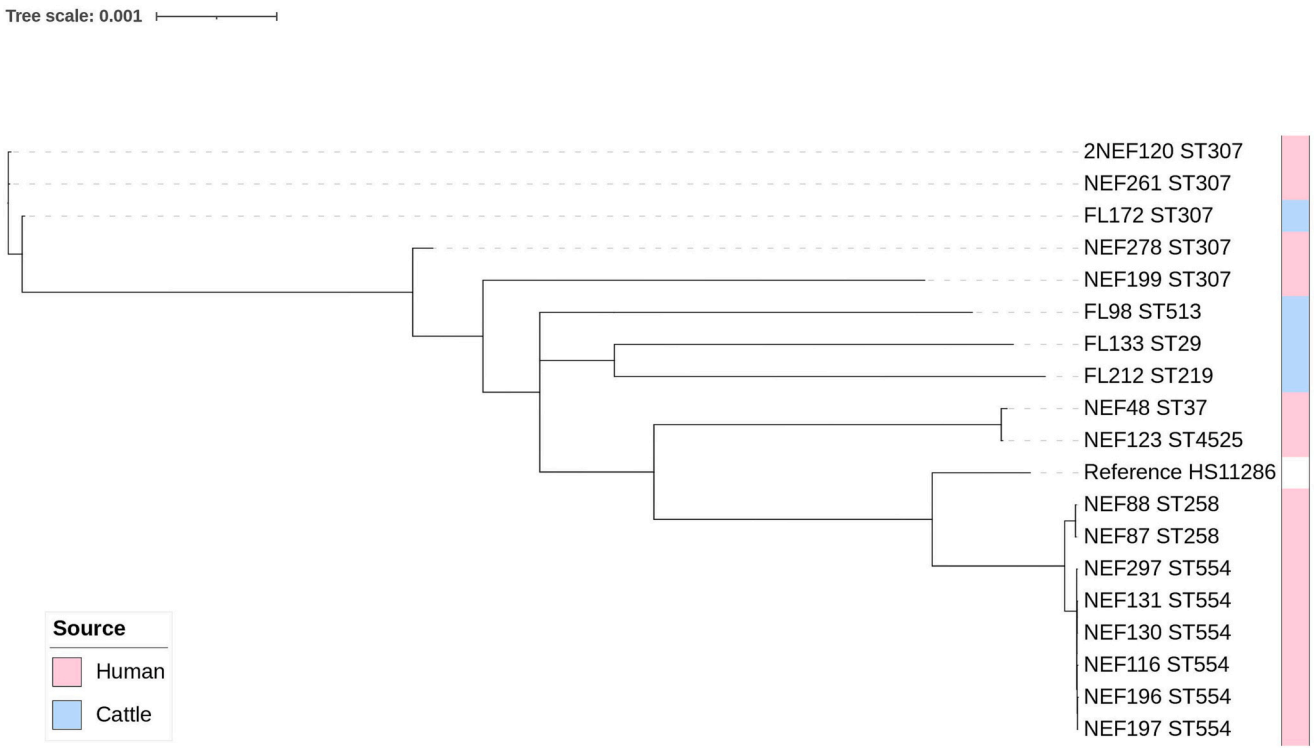

Fig. 2. Core SNPs Maximum Likelihood tree including the reference *K. pneumoniae* HS11286 (Ref Seq GCF_000240185.1).

To the best of our knowledge, this is the first report of a KPC-3-producing *K. pneumoniae* ST307 strain detected in a bovine dairy farm in Italy. The KPC-3 strain (FL 172) carried other β-lactam resistance genes encoding for a class D carbapenemase (OXA-9) and ESBLs (CTX-M-15, SHV-106), together with determinants of resistance to aminoglycosides, fluoroquinolones, phosponic acids, tetracyclines, sulfonamides and trimethoprim. Such a MDR strain shared several traits in common with the human clinical KPC3-encoding ST307 isolate (2NEF 120) of the study, as well as with the high-risk clone KPC-3 *K. pneumoniae* ST307 distributed worldwide (Fuentes-Castillo et al., 2021).

In Italy, antimicrobial consumption at farms may be voluntary registered in a national platform called "ClassyFarm", which allows farm risk categorization on the basis of biosecurity, animal welfare, animal health and production parameters, type of feed, antimicrobial consumption, and post-mortem lesions at slaughter (www.classyfarm.it). ClassyFarm monitoring system processes data collected by the competent authority during official controls, and those resulting from food business operator own-checks. Consumption of antimicrobials at farms can be evaluated by the antimicrobial use and by an additional indicator that considers the antimicrobial consumption of the entire biomass of animals reared at farm. This indicator is called "Defined Daily Dose Animal (DDDA)", which is the assumed average dose in mg of antimicrobials per kg animal per species per day and which is based on DDD ("Defined Daily Dose") in humans (EMA (European Medicine Agency), 2015; Jensen et al., 2004). DDDAs are established for cattle, pigs and broilers and are based on the average (arithmetic mean) of all observations of veterinary medicinal products for animal species, substance and route/form of administration, following the formula $Average = (a_1 + a_2 + a_3 + a_4 \ldots)/n$. In Italy, specific DDDAit (Defined Daily Dose Animal for Italy) were developed, rather than adopting the DDDvets used by other European countries, due to the fact that DDDvets are often based on dosages not used in Italy. At national level, the median rate per year of antimicrobial consumption in dairy cattle is expressed as DDDAit/biomass and can be compared to the rate of antimicrobial consumption in the different bovine dairy farms.

As shown in Table 3, the DDDAit/biomass in the KPC-3 *K. pneumoniae* ST307 (FL 172) - positive farm (Farm 31) was nearly three times higher than the national median value registered in 2020 (6814 *vs* 2500). In 2020, the antimicrobials used at farm included aminopenicillins, aminoglycosides, 1st and 2nd generation cephalosporins, lincosamides, macrolides, penicillins, phenicols, polymyxins and sulphonamides (data retrieved from farm antimicrobial use documentation). Genomic testing of FL 172 strain revealed the presence of several AMR genes, thus supporting the hypothesis that selection pressure could have influenced the emergence of MDR. In the same farm, an ESBL-MDR *K. pneumoniae* isolate (FL 98) carrying CTX-M-15, TEM—1B, and SHV-187 was found to harbour additional resistance genes to most of the antimicrobials used, plus resistance determinants to phosphonic acids, tetracyclines and trimethoprim. Noteworthy, despite the occurrence of ESBL-encoding *K. pneumoniae,* 3rd generation cephalosporins were not used for antimicrobial treatments in Farm 31. Similar findings for ESBL-producing *K. pneumoniae* in absence of 3rd and 4th generation cephalosporins use were reported in Japan (Taniguchi et al., 2021).

To justify the occurrence of KPC-3-producing *K. pneumoniae* ST307 in a dairy farm, in absence of carbapenem use, some suggestion could come from literature. For example, KPC-2-encoding *K. pneumoniae* ST258 were isolated both from a hospital and a close river in Croatia (Brkic et al., 2017), thus suggesting the role of the aquatic environment in the dissemination of clinical isolates. The AMR contamination rate of water compartment was investigated also in Italy, where *K. pneumoniae* ST307 and ST258 isolates detected in a well and a wastewater treatment plant (WWTP) resulted KPC-2 and KPC-3 producers, respectively. In addition, different CTX-M-, SHV-, DHA-type positive *Enterobacteriaceae* were identified from wells, streams, and WWPTs (Caltagirone et al., 2017). As well-known, in aquatic environments the prevalence of AMR bacteria originating from anthropogenic sources, such as hospital and municipal effluents, is growing globally (Baquero et al., 2008; Bouki et al., 2013) thus contributing to their dissemination to the animal compartment. Nevertheless, also the use of antimicrobials, whose resistant genes are located on mobile genetic elements along with carbapenemase encoding genes, can improve the co-selection of a MDR phenotype, including CR, without the use of carbapenems (De Oliveira et al., 2020).

In milk filters, prevalence of CP- *K. pneumoniae* was 0.2 % (95 % CI – 0.07 - 2.2), and prevalence of ESBL-encoding *K. pneumoniae* was 1.2 % (95 % CI 0.4–3.4). Due to the lack of a standardized sampling protocol among farms, prevalence at farm level could not be evaluated. The disproportion between 66 samples collected at one farm (Farm 31) and, for example, individual samples collected from 124 other farms precludes any consideration of *K. pneumoniae* prevalence at farm level. Nevertheless, the flip side of the coin of this uneven sampling plan is that the collection of multiple samples from a farm has made it possible to isolate a rare microorganism, that we would have probably lost if we had only tested one sample. In fact, since milk could not be collected from cattle affected by mastitis (Regulation CE 853/2004), the probability of milk contamination by *K. pneumoniae* is rather low.

One consideration in common to the four *K. pneumoniae* strains detected in milk is their MDR. Such a wide range of antimicrobial resistances in *K. pneumoniae* isolates from bovine milk was reported in Brazil (Tartor et al., 2021), Japan (Taniguchi et al., 2021) and China (Yang et al., 2021). In our isolates, apart from the β-lactamase genes, determinants for aminoglycoside modifying enzyme [*aph(3″)-I* and *aph (6)-I*], sulphonamide modifying enzyme (*sul2*), tetracycline efflux pump (*tetA*) and fluoroquinolone efflux pump (*oqxA, oqxB*) were found in MDR isolates, thus posing a threat to consumers.

4.2. Carbapenemase- and ESBL-producing K. pneumoniae in human patients

K. pneumoniae infections are commonly healthcare-associated, involving patients with impaired immune system (ECDC (European Centre for Disease Prevention and Control), 2020). In 2019, antimicrobials under surveillance in invasive *K. pneumoniae* isolates in Europe include 3rd generation cephalosporins, carbapenems, fluoroquinolones

Table 3
bla genes and MIC_{MEM} values of *K. pneumoniae* strains isolated from dairy farms and comparison of antimicrobial consumption both at farms and at national level as registered in the ClassyFarm system.

ID strain	*bla* genes	MIC_{MEM} (μg/ml)	Farm progressive N° (date of sampling)	N° and proportion of lactating cows /N° of animals	Rate of antimicrobial consumption (year) [a]	National median antimicrobial consumption (year) [a]
FL 98	$bla_{CTX\text{-}M\text{-}15}$ $bla_{TEM\text{-}1B}$ $bla_{SHV\text{-}106}$	0.03	31 (15.06.2020)	1065 (63.5 %) / 1676	6.814 (2020)	2.500 (2020)
FL 133	$bla_{SHV\text{-}187}$ $bla_{DHA\text{-}1}$	0.03	2 (22.09.2020)	199 (58.7 %) / 339	3.070 (2020)	2.500 (2020)
FL 172	$bla_{KPC\text{-}3}$ $bla_{OXA\text{-}9}$	64	31 (02.10.2020)	1065 (63.5 %) / 1676	6.814 (2020)	2.500 (2020)
FL 212	$bla_{CTX\text{-}M\text{-}15}$ $bla_{SHV\text{-}145}$	0.03	114 (16.03.2021)	281 (51.9 %) / 541	7.810 (2021)	1.170 (2021)

Legenda: expressed as DDDit/kg.

and aminoglycosides. The highest mean resistance percentage was reported for 3rd generation cephalosporins (31.3 %), followed by fluoroquinolones (31.2 %), aminoglycosides (22.3 %) and carbapenems (7.9 %). In Italy, higher resistance proportions for the four antimicrobial classes were reported, with resistance to carbapenems in invasive *K. pneumoniae* quoted at 28.5 % in 2019 and 29.5 % in 2020 (ECDC, 2020; (WHO Regional Office for Europe/European Centre for Disease Prevention and Control (ECDC), 2022)). In 2020, *K. pneumoniae* invasive isolates tested in Italy showed combined resistance to 3rd generation cephalosporins, carbapenems, fluoroquinolones and aminoglycosides (WHO Regional Office for Europe/European Centre for Disease Prevention and Control (ECDC), 2022). These data are in accordance with our results, since all human CP *K. pneumoniae* strains tested were carrying genomic determinants for resistance to 3rd generation cephalosporins, fluoroquinolones and aminoglycosides but also to macrolides, phosponic acids, sulphonamides and trimethoprim. In addition, all but one isolates (90 %) were harboring resistance genes to phenicols, leading to severe limitations in treatment options for serious infections caused by MDR *K. pneumoniae*.

Keeping in mind that the human strains were not tested for clinical purposes but only to be compared with the bovine isolates, their sequencing revealed that most CP *K. pneumoniae* isolates carried KPC-3, in accordance with other Italian studies (Calia et al., 2017).

4.3. KPC-3 K. pneumoniae ST307 in cattle and humans

The genomic comparison of the KPC-3-producing *K. pneumoniae* ST307 strain isolated from a dairy farm (FL 172; Farm 31) with the KPC-3-producing *K. pneumoniae* ST307 strain of human origin (2NEF 120) revealed that they both harboured KPC-3, OXA-9, CTX-M-15 and SHV-106 genes, plus nine resistant determinants against aminoglycosides, fluoroquinolones, sulfonamides, trimethoprim, and tetracyclines. The isolates were detected in August 2020 (2NEF 120) and October 2020 (FL172). No information of an outbreak of KPC-3 *K. pneumoniae* ST307 was notified from the hospital.

Furthermore, two ESBL-producing *K. pneumoniae* strains belonging to the high-risk lineage ST307 (FL 133, FL 212) carrying a wide resistome against β-lactams, quinolones, aminoglycosides, macrolides, phenicols, phosphonic acids, sulfonamides, and tetracycline were found in milk filters, thus confirming the occurrence of MDR ESBL-producing *K. pneumoniae* ST307 in bovines.

Since two ESBL-producing *K. pneumoniae* ST307 harboring CTX-M-15, TEM-1B and SHV-106 (NEF 278) and TEM-102 and SHV-106 (NEF 261) were identified among the human clinical isolates, the occurrence of ESBL-producing *K. pneumoniae* ST307 in both compartments in the same geographical area was confirmed. This finding is in accordance with the international epidemiological landscape of *K. pneumoniae* ST307, whose wide distribution is attributed to the human-environment-animal interface (Fuentes-Castillo et al., 2021).

In recent years, MDR CP-*K. pneumoniae* lineages have emerged worldwide (Struve et al., 2015; Wyres et al., 2019). Among them, CP-*K. pneumoniae* ST307 carrying transferable resistance-conferring genes against carbapenems and newer-generation cephalosporins is a candidate for becoming one of the most clinically relevant clones (Villa et al., 2017; Wyres et al., 2019). Several outbreaks of MDR *K. pneumoniae* ST307 in clinical settings have been reported internationally (Baek et al., 2020; Boonstra et al., 2020; Heiden et al., 2020; Kim et al., 2017). In Italy, *K. pneumoniae* ST307 is extensively reported from human clinical samples, with high proportions of both KPC-3 and KPC-2 producing strains and lower proportions of VIM-1 and OXA-48 isolates (Loconsole et al., 2020; Magi et al., 2021).

As well known, antimicrobials and AMR pathogens could be released from human hospital settings to a multitude of ecological niches, such as water and soil (Wyres and Holt, 2018). These niches could act as resistance hotspots where AMR genes are disseminated and new AMR bacteria are created due to horizontal gene transfer (Berglund, 2015; Colombo et al., 2017). This human-environment interface could include a range of plant species, insects, birds, reptiles, and many different mammals in which AMR microorganisms can be either commensals or potential pathogens (Wyres and Holt, 2018). In the area of the study, a river (Parma River) flows from the town of Parma to the village where KPC-3 *K. pneumoniae* ST307-positive farm is located. The distance between the hospital and the farm is ~15 Km and some irrigation channels branch off from the river close to Farm 31. Even if the environmental sampling was not included in the study and, therefore, we have no evidence of KPC-positive *K. pneumoniae* contamination of the river, the surrounding farmland, or the animal forage, this hypothesis is intriguing. Furthermore, since neither carbapenems nor 3rd generation cephalosporins were administered to the cows, environmental spreading of KPC and ESBL *K. pneumoniae* from human sources could be a plausible event.

5. Conclusions

The spread of carbapenemase- and ESBL-producing *K. pneumoniae* has become a major threat for healthcare facilities worldwide and some clones are emerging globally, as the strains belonging to the high-risk clone ST307. Interestingly, in our study the first detection of KPC-3-producing *K. pneumoniae* ST307, as well as ESBL-producing *K. pneumoniae* ST307 and ST513, are reported in bovine bulk milk in Italy. Since registration of antimicrobial treatments at the positive farms excluded the use of carbapenems, which are banned in veterinary treatments in Europe, as well as the use of newer-generation cephalosporins in dairy cattle, our findings suggest that animals can carry opportunistic pathogens which harbour resistance genes against carbapenems and 3rd generation cephalosporins even if not treated with such antimicrobials. A plausible suggestion is that KPC-3 and ESBL-*K. pneumoniae* contamination in dairy cattle could have had an environmental origin. Furthermore, resistance to carbapenems and β-lactams in bovine *K. pneumoniae* isolates was associated to resistance to several antimicrobials, including aminoglycosides, macrolides, fluoroquinolones, sulphonamides, and tetracyclines in a MDR scenario, thus reflecting the excessive use of antimicrobials at some farms included in the study.

The crucial message of our study is that raw milk could be a potential threat to food safety and public health as regards MDR microorganisms. At last, even if carbapenems and combination of cephalosporins with β-lactamase inhibitors are only reserved for human treatments in the European Union, monitoring of carbapenemase- and ESBL-producing bacteria in milk and other food of animal origin should be encouraged. Indeed, since defence of human health starts with knowledge, the link between carbapenemase- and ESBL-producing *K. pneumoniae* and milk deserves further investigation.

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Declaration of competing interest

On behalf of the authors of the manuscript entitled “Detection of carbapenemase- and ESBL-producing *Klebsiella pneumoniae* from bovine bulk milk and comparison with clinical human isolates in Italy” I do declare we have no conflict of interest.

Data availability

The data that has been used is confidential.

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