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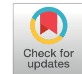
First detection of a plasmid located carbapenem resistant bla_{VIM-1} gene in *E. coli* isolated from meat products at retail in Belgium in 2015



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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

Carbapenemase-producing Enterobacteriaceae (CPE) confer resistance to antibiotics that are of critical importance to human medicine. There have only been a few reported cases of CPEs in the European food chain. We report the first detection of a carbapenemase-producing *Escherichia coli* (ST 5869) in the Belgian food chain. Our aim was to characterize the origin of the carbapenem resistance in the *E. coli* isolate. The isolate was detected during the screening of 178 minced pork samples and was shown to contain the carbapenemase gene bla_{VIM-1} by PCR and Sanger sequencing. Whole genome short and long read sequencing (MiSeq and MinION) was performed to characterize the isolate.

With a hybrid assembly we reconstructed a 190,205 bp IncA/C2 plasmid containing bla_{VIM-1} (S15FP06257_p), in addition to other critically important resistance genes. This plasmid showed only low similarity to plasmids containing bla_{VIM-1} previously reported in Germany. Moreover, no sequences existed in the NCBI nucleotide database that completely covered S15FP06257_p. Analysis of the bla_{VIM-1} gene cassette demonstrated that it likely originated from an integron of a *Klebsiella* plasmid reported previously in a clinical isolate in Europe, suggesting that the meat could have been contaminated by human handling in one of the steps of the food chain.

This study shows the relevance of fully reconstructing plasmids to characterize their genetic content and to allow source attribution. This is especially important in view of the potential risk of antimicrobial resistance gene transmission through mobile elements as was reported here for the of public health concern bla_{VIM-1}.

1. Introduction

Carbapenems are broad-spectrum β-lactam antimicrobials that play a crucial role in human infections caused by multidrug resistant bacteria, which cannot be treated by other therapeutic options. In clinical practice they are considered as one of the 'last resort' antimicrobials in such cases. Therefore, to maintain their effectiveness, the development and spread of resistance mechanisms against carbapenems have to be prevented. The main advantage of carbapenems is that they are relatively resistant to hydrolysis by most β-lactamases, including AmpC and extended-spectrum β-lactamases (ESBL). They are however inactivated by carbapenemases, which also confer resistance to β-lactams (Queenan and Bush, 2007; Roschanski et al., 2017a). Carbapenemase-producing Enterobacteriaceae (CPE) were usually isolated and restricted to humans in healthcare settings, especially in the acute and chronic care sectors. This is because carbapenems are not licensed for veterinary use in Europe. Nevertheless, during recent years, the presence of acquired carbapenemase-producing microorganisms from non-human origin, present in food-producing animals and other ecosystems, has been reported (Guerra et al., 2014; Roschanski et al., 2017a; Woodford et al., 2014). This increases concern about the potential spread of resistant organisms in the community. It also indicates other sources, such as food, companion animals or the environment, as vehicles for transmission to humans. This spread is even a higher risk if the resistance

Abbreviations: AMR, antimicrobial resistance; AST, antimicrobial susceptibility testing; BMD, broth microdilution method; CPE, Carbapenemase-producing Enterobacteriaceae; ECOFF, epidemiological cut-off values; ESBL, extended spectrum β-lactamase-producing isolates; ONT, Oxford Nanopore Technologies; SRA, Sequence Read Archive; ST, sequence type; TAE, 40 mmol/L Tris–HCl [pH 8.3], 2 mmol/L acetate, 1 mmol/L EDTA; WGS, whole genome sequencing

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genes are situated on mobile elements such as plasmids.

The first CPE detection in food-producing animals in Europe was in German swine and broiler farms (Fischer et al., 2013, 2012; Roschanski et al., 2017a). Isolates belonging to *Escherichia coli* and *Salmonella* Infantis species harbored plasmids containing the carbapenemase encoding bla_{VIM-1} gene. Later, other plasmids bearing bla_{VIM-1} were detected in German seafood (Roschanski et al., 2017b) and minced meat (Borowiak et al., 2017). The implication of livestock and food products from animal origin in the spread and transfer of zoonotic and foodborne CPE to humans, is a major risk factor and of major concern. Moreover, a transfer of CPE from humans to animals/animal products before the spread can occur, is also a concern. However, only scarce data exists on the presence of CPE in food-producing animals and food in Europe. Besides the occurrence in Germany, *Acinetobacter* producing *OXA-23* from horses were sporadically reported in Belgium (Smet et al., 2012) and also in dairy cows in France (Poirel et al., 2012). In Belgium, carbapenemase-producing *E. coli* have not yet been detected in livestock or other food components. However, a significant increased proportion of clinical CPE among hospitalized patients was seen in 2015 compared with 2012, i.e. 0.55% in 2015 vs 0.25% in 2012 ($p = 0.02$) (Huang et al., 2017). The majority of CPE among hospitalized patients belonged to *Klebsiella pneumoniae*, with OXA-48 like carbapenemases being the predominant enzymes (Huang et al., 2017). The risk of presence, sporadic or emergent, of CPE in food-producing animals, and therefore in the food chain, cannot be ignored. An EFSA opinion ("Scientific Opinion on Carbapenem resistance in food animal ecosystems,", 2013) on carbapenem resistance in food-producing animals recommended surveillance at both the national and international levels to avoid the spread of CPE strains in livestock. In Belgium, since 2014, presumptive β-lactam resistant *E. coli* isolates, ESBL/AmpC and carbapenemase-producing isolates, from livestock and food are monitored. This is done to identify potential reservoirs and prevent the spread of carbapenemase-producing *E. coli* into the food chain to avoid transmission to healthy humans and/or patients. In 2015, within the framework of the national antimicrobial monitoring of zoonotic and commensal bacteria, specific monitoring of ESBL/AmpC-producing *E. coli* from pork preparations at retail was performed. For this the procedures described in the Commission Implementing Decision 2013/652/EU were followed. A total of 178 minced pork samples were collected in Belgium at retail of which 28 tested positive for β-lactam resistant *E. coli* isolates. Subsequently, those isolates were subjected to antimicrobial susceptibility testing (AST) to determine their resistance profile as stated in the aforementioned Decision 2013/652/EU. One isolate exhibited resistance to meropenem, ertapenem, imipenen and temocillin. In this study, short and long read whole genome sequencing (WGS) technologies were used to confirm the presence of carbapenemase determinants, to localize the carbapenem encoding resistance gene, and to reconstruct the plasmid. The obtained bla_{VIM-1}-bearing plasmid was then further investigated through comparison with previously reported sequence data.

2. Materials and methods

2.1. Bacterial isolates and resistance to carbapenems

Specific isolation of ESBL/AmpC/carbapenemase-producing *E. coli* from minced pork was performed based on the protocol defined by the EU reference laboratory on antimicrobial resistance (AMR) available for fresh meat (https://www.eurl-ar.eu/CustomerData/Files/Folders/21-protocols/277_esbl-ampc-cpeprotocol-version-meat-january2017-version4.pdf). Briefly, 25 g of minced meat was sampled and incubated on a non-selective overnight culture. Subsequently, one loop-full was inoculated in McConkey agar supplemented with cefotaxime (1 mg/l) (Bio-Rad 35 M142.02) for the detection of ESBL and AmpC-producing *E. coli*, and on CarbaSmart (bioMérieux) for the detection of carbapenemase-producing *E. coli*. The isolation media were incubated at 44 ± 0.5 °C/18–22 h and 37 ± 0.5 °C/18–22 h, respectively. Suspected colonies were purified as described in the protocol and species confirmation was performed by MALDI-TOF mass-spectrometry (Bruker Daltonics, Bremen, Germany).

Table 1
Minimum inhibitory concentration (MIC, mg/l) of the presumptive carbapenemase-producing *E. coli* derived from retail minced pork, Belgium 2015.

Antimicrobial	Cut-off (R > mg/l)[c]	MIC (mg/l)
Ampicillin[a]	8	**64**
Azithromycin[a]	16	16
Cefotaxime[a]	0.25	**4**
Ceftazidime[a]	0.5	**8**
Chloramphenicol[a]	16	8
Ciprofloxacin[a]	0.064	**8**
Colistin[a]	2	1
Gentamicin[a]	2	**8**
Meropenem[a]	0.125	**1**
Nalidixic Acid[a]	16	**128**
Sulphamethoxazole[a]	64	**1024**
Tetracycline[a]	8	2
Tigecycline[a]	1	0.5
Trimethoprim[a]	2	**32**
Cefepime[b]	0.125	**32**
Cefotaxime[b]	0.25	**64**
Cefotaxime/clavulanic[b] acid	0.25	**64**
Cefoxitin[b]	8	**64**
Ceftazidime [b]	0.5	**128**
Ceftazidime/clavulanic acid[b]	0.5	**128**
Ertapenem[b]	0.06	**0.5**
Imipenem[b]	0.5	**4**
Meropenem[b]	0.125	**2**
Temocillin[b]	32	**128**

MIC values in bold indicate resistance according to Decision 2013/652/UE.
[a] EUVSEC plate Trek diagnostics, Thermo Scientific.
[b] EUVSEC2 plate Trek diagnostics, Thermo Scientific.
[c] EUCAST Epidemiological cut-off values as described in the Decision 2013/652/UE.

2.2. Phenotypic determination of resistance profile

Antimicrobial susceptibility testing (AST) of the presumptive carbapenemase-producing *E. coli* isolate was done based on the broth microdilution method (BMD) (M100-S25 Performance Standards for Antimicrobial Susceptibility Testing; Twenty-fifth Informational Supplement, n.d.) using EUVSEC and EUVSEC2 sensititre plates (Trek diagnostics, Thermo Scientific). The MIC (minimum inhibitory concentration) (Table 1) was determined and interpreted by the application of the EUCAST (http://www.eucast.org) epidemiological cut-off values (ECOFF) as described in the Decision 2013/652/EU.

2.3. PCR detection of antibiotic resistance genes and Sanger sequencing

The presence of most of the carbapenemase encoding genes (bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{NDM}, bla_{OXA-48}) relevant in clinical isolates were verified by PCR, using primers previously described (Poirel et al., 2011). Amplification was carried out with the following thermal cycling conditions: 5 min at 94 °C and 35 cycles of amplification consisting of 60 s at 94 °C, 60 s at 52 °C, and 60 s at 72 °C, with 10 min at 72 °C for the final extension. DNA fragments were analyzed by electrophoresis in a 2% agarose gel at 100 V for 1 h in 1× TAE (40 mmol/l Tris–HCl [pH 8.3], 2 mmol/l acetate, 1 mmol/l EDTA) containing 0.05 mg/l ethidium bromide. Subsequently, the PCR products were purified and analyzed by Sanger sequencing, as outlined by the manufacturer's instructions (ABI3130XL, Applied Biosystems).

2.4. Characterization of the location of the AMR genes by whole genome sequencing (WGS)

Genomic DNA was extracted using the genomic Tip 100/G kit (Qiagen) following the manufacturer's instructions. The purity of the DNA was evaluated using Nanodrop 2000 (ThermoFisher Scientific). DNA was quantified using a Qubit 4.0 fluorometer (ThermoFisher Scientific). Fragment length and DNA integrity were analyzed with a 4200 Tapestation (Agilent Technologies).

Short read sequencing libraries were prepared using a Nextera XT DNA sample preparation kit (Illumina) and sequenced on an MiSeq instrument (Illumina) with a 250-bp paired-end protocol (MiSeq v3 chemistry) according to the manufacturer's instructions. Raw sequencing data was submitted to NCBI Sequence Read Archive (SRA) (Leinonen et al., 2011) and NCBI Genbank (Benson et al., 1993) under accession number PRJNA564835.

Raw MiSeq sequencing reads were trimmed using Trimmomatic (v0.32). First the Illuminaclip option was used to remove the Nextera adapter sequences. Then a sliding window approach of four bases and trimming when the Phred score dropped below 30 was employed. Lastly, the leading and trailing bases of a read were removed when the Phred dropped below a score of 3 (Bolger et al., 2014). The trimmed MiSeq reads were used in a *de novo* assembly generated with the bioinformatics webtool Assembler 1.2 (Larsen et al., 2012).

A long-read sequencing library was prepared with the 1D native barcoding genomic DNA (with EXP-NBD103 and SQK-LSK108) protocol from Oxford Nanopore Technologies (ONT). The optional addition of control (CS) DNA and DNA repair was omitted, whereas the optional step of shearing to 8 kb with Covaris G-tubes was included. The long-read sequencing library pool consisting of 9 barcodes (including other samples not relevant for this study) was loaded onto a R9.4 MinION flowcell (FLO-MIN106) and sequenced for 48 h. Basecalling was performed with Guppy (version 3.1.5) with the DNA trimming option enabled that removes low quality bases before the adapter sequence. Subsequently, the long reads were checked with NanoStat and NanoPlot to have a general overview of the read quality. The adapter, barcode and chimeric sequences were removed with Porechop (version 0.2.4). Low quality (< q7) and short (< 300 bp) reads were removed with NanoFilt (version 2.0.0) (De Coster et al., 2018). Both the short (MiSeq) and long (MinION) sequencing reads were used to perform a hybrid assembly with Unicycler (v0.4.8) with default settings. The following tools are included in the Unicycler pipeline: SPAdes (version 3.11.1), Miniasm (version 0.3), Racon, makeblastdb (version 2.7.1+), tblastn (version 2.7.1+), bowtie2 (version 2.3.4.3), Samtools (version 1.5), Java (version 1.8) and Pilon (1.22) (Wick et al., 2017).

Plasmid sequences were visualized in a circular plot using BLAST Ring Image Generator (BRIG) version 0.95 (Alikhan et al., 2011). Additionally, BLAST results (generated with ncbi-blast-2.7.1+) of plasmid sequences against different plasmids of interest available in the NCBI nucleotide database, were visualized with BRIG. All plasmid sequences were aligned to the *de novo* assembly with ProgressiveMauve (version 2.4.0) to check for recombination (Darling et al., 2004).

Gene annotation was performed with Prokka (version 1.13.7) (Seemann, 2014). For prediction of resistance genes, plasmid typing, chromosomal mutations and serotyping, the web versions of ResFinder 3.1 (Zankari et al., 2012), PlasmidFinder 2.0 (Carattoli et al., 2014), PointFinder 3.1 (Zankari et al., 2017) and SerotypeFinder 2.0 (Joensen et al., 2015) were used on the assembled contigs. The thresholds for sequence identity and coverage length were set to 90% and 60%, respectively. For detection of the sequence type, SRST2 (Inouye et al., 2014) using the MiSeq reads was performed with the *E. coli* MLST Warwick database (https://enterobase.warwick.ac.uk/species/index/ecoli) (Alikhan et al., 2018).

3. Results

3.1. Isolation and phenotypic characterization of ESBL/AmpC/carbapenemase positive E. coli

In Belgium, according to the European Decision 2013/652/EU, isolation of presumptive ESBL/AmpC and carbapenemase-producing *E. coli*, and monitoring of antimicrobial susceptibility, is mandatory for isolates from livestock and meat since 2014. Within the Belgian annual program of AMR monitoring in zoonotic and commensal bacteria, specific monitoring of ESBL/AmpC/carbapenemase-producing *E. coli* was done for 178 minced pork samples. Out of these samples, 28 (15.73%) tested positive for the presence of β-lactamase producing *E. coli*. Performing AST for all isolates demonstrated that all except one exhibited an ESBL phenotype (i.e. 27 out of 178, or 15.16% with ESBL phenotype), and one isolate exhibited a carbapenem resistant phenotype (0.56%). Here, we report the data of the carbapenemase-producing *E. coli*. The isolate was resistant to ampicillin, cefotaxime, ceftazidime, cefepime, cefoxitin, ciprofloxacin, gentamicin, nalidixic acid, sulphamethoxazole, trimethoprim, meropenem, ertapenem, imipenen and temocillin, but not resistant to azithromycin, colistin, tetracycline and tigecycline (Table 1).

3.2. Identification of $bla_{VIM\text{-}1}$ by PCR

After phenotypic testing, the most common β-lactamase encoding genes were tested by single PCR including $bla_{CTX\text{-}M}$, bla_{SHV}, bla_{TEM}, $bla_{OXA\text{-}10}$, $bla_{CMY\text{-}2}$, $bla_{ACC\text{-}1}$ and those encoding for carbapenemases, namely bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{NDM}, $bla_{OXA\text{-}48}$. The amplicons of detected genes for bla_{VIM}, bla_{SHV} and bla_{CMY} were Sanger sequenced, identifying respectively the $bla_{VIM\text{-}1}$, $bla_{SHV\text{-}5}$ and $bla_{CMY\text{-}13}$ variants.

3.3. Further characterization of the E. coli isolate including detection of AMR genes and their location using hybrid de novo assembly

The genomic DNA extracted from the carbapenemase-producing *E. coli* isolate was used for short-read MiSeq (Illumina) sequencing. Sequencing data analysis of the short read data using the ResFinder and PlasmidFinder databases demonstrated the presence of several AMR genes and an IncA/C2 plasmid replicon (Table 2). However, the resulting assembly from the MiSeq data was very fragmented, with the AMR genes spread out over multiple contigs. This made it difficult to localize them (plasmid or chromosome). Therefore, additional long read MinION (ONT) sequencing was performed using the same DNA extract. The obtained long reads were combined with the MiSeq sequencing reads to construct a *de novo* hybrid assembly that contained one circular contig (S15FP06257_p) with a coverage of 95× with MiSeq reads and 25× with MinION reads. The contig represented a 190,205 bp plasmid harboring several AMR genes (Fig. 1.A and B). In this plasmid, besides the presence of the carbapenemase $bla_{VIM\text{-}1}$ gene, other β-lactamase encoding genes ($bla_{SHV\text{-}5}$, $bla_{CMY\text{-}13}$), genes coding for aminoglycosides (*aadA1*, *aadA24*, *aac(6′)-Ib3*, *aac(6′)-Il*, *aac(3)-I*), fluoroquinolones (*aac(6′)Ib-cr*), macrolides (*mph(B)*), sulphonamide (*sul1*) and trimethoprim (*dfrA1*), were detected as well. In the chromosome, only the *mdf(A)* gene could be detected, encoding a multidrug efflux pump. In addition, chromosomal mutations in the *gyrA* (S83L and D87N), *parC* (S80I) and *parE* (S458A) genes were detected, conferring resistance to nalidixic acid. These genes and mutations correspond with the phenotypical AMR from Table 1 and with the data solely obtained from MiSeq reads. Furthermore, the presence of a plasmid type IncA/C2 replicon was identified in the plasmid (Table 2). Then annotation was performed to fully characterize S15FP06257_p. The *intI1* gene indicated the presence of integrons in the plasmid. The plasmid also confers narrow-spectrum resistance to inorganic mercury by its *mer* operon (Osborn et al., 1997). The sequence type of the *E. coli* isolate harboring the plasmid was found to be sequence type (ST) 5869 and the serotype

Table 2
ResFinder, PlasmidFinder and PointFinder output of the hybrid assembly of S15FP06257 isolated from the Belgian food chain.

ResFinder[a]

Resistance gene	Identity (%)	Coverage (%)	Query/HSP	Location	Position in contig	Accession no.
Aminoglycoside						
aadA1	99.75	100	792/792	Plasmid	49179..49970	JQ414041
aadA1	100	100	792/792	Plasmid	61964..62755	JX185132
aadA24	94.54	91.54	780/714	Plasmid	7478..8191	AM711129
aac(3)-I	92.26	100	465/465	Plasmid	47671..48135	AJ877225
aac(6′)-Ib3	100	100	555/555	Plasmid	47041..47595	X60321
aac(6′)-Il	100	100	459/459	Plasmid	6317..6775	U13880
β-lactam						
$bla_{\text{CMY-13}}$	100	100	1146/1146	Plasmid	41737..42882	AY339625
$bla_{\text{SHV-5}}$	100	100	861/861	Plasmid	55023..55883	X55640
$bla_{\text{VIM-1}}$	100	100	801/801	Plasmid	5423..6223	Y18050
Fluoroquinolone						
aac(6′)-Ib-cr	99.61	100	519/519	Plasmid	47077..47595	EF636461
MLS - macrolide, lincosamide and streptogramin B						
mdf(A)	98.46	100	1233/1233	Chromosome (contig 6)	26683..27915	Y08743
mph(B)	100	100	909/909	Plasmid	17269..18177	D85892
Sulphonamide						
sul1	100	100	840/840	Plasmid	50475..51314	U12338
sul1	100	100	840/840	Plasmid	8698..9537	U12338
Trimethoprim						
dfrA1	99.79	100	474/474	plasmid	62848..63321	X00926
dfrA1	99.79	100	474/474	plasmid	6918..7391	X00926

PlasmidFinder[a]

Replicon	Identity (%)	Coverage (%)	Query/HSP	Location	Position in contig	Accession no.
IncA/C2	100	100	417/417	Plasmid	86377..86793	JN157804

PointFinder[a]

Mutation	Resistance	Nucleotide change	Amino acid change	Location	PMID
gyrA p.S83L	Nalidixic acid ciprofloxacin	TCG → TTG	S → L	Chromosome	8891148
gyrA p.D87N	Nalidixic acid ciprofloxacin	GAC → AAC	D → N	Chromosome	12654733
parC p.S80I	Nalidixic acid ciprofloxacin	AGC → ATC	S → I	Chromosome	8851598
parE p.S458A	Nalidixic acid ciprofloxacin	TCG → GCG	S → A	Chromosome	28598203

Query = length of the gene/replicon in the database, HSP = the length of the alignment between the gene/replicon and S15FP06257_p.

[a] ResFinder, PlasmidFinder and PointFinder analyses were performed both on MiSeq assemblies and on hybrid assemblies, giving the same AMR genes, plasmid replicon and mutations. The analysis based on MiSeq assemblies could not make the distinction between whether a gene is located on a plasmid or on the chromosome.

O184:H23.

3.4. Comparison of the $bla_{\text{VIM-1}}$ containing plasmid with previously reported data

The Belgian $bla_{\text{VIM-1}}$-bearing plasmid (S15FP06257_p) sequence was compared to the sequences of other $bla_{\text{VIM-1}}$-bearing plasmids reported previously in *E. coli* and *Salmonella* Infantis isolated from the German food chain (Fischer et al., 2013, 2012; Roschanski et al., 2017b, 2017a) (Fig. 1.A). The $bla_{\text{VIM-1}}$ plasmid detected in the Belgian food chain showed almost no similarity to the plasmids detected in Germany. While the plasmids from the German food chain also contained a class 1 integron harboring $bla_{\text{VIM-1}}$ (Tn3-like transposon), the variable regions in the integron were different compared to S15FP06257_p (Tn3-like transposon) (Fig. 1.A). We also compared our plasmid sequence with all sequences of the NCBI nucleotide database (Fig. 1.B). While the plasmid from the Belgian isolate shares its backbone with many other IncA/C2 plasmids, the $bla_{\text{VIM-1}}$ gene was not present in any of these plasmids. Even though ST5869 has been reported in Chinese swine (Enterobase, P0461_T EC), S15FP06257_p showed only low similarity to plasmid sequences of Chinese isolates present in the NCBI nucleotide database (Supplementary information). Moreover, these plasmids from Chinese isolates also did not contain the $bla_{\text{VIM-1}}$ gene.

Therefore, only the class 1 integron containing $bla_{\text{VIM-1}}$ (7000 bp) was BLASTed against the NCBI nucleotide database. This resulted in matching sequences from clinical *Klebsiella* integrons/plasmids and clinical *Enterobacter* plasmids. In Fig. 2, the high similarity (with 4 bp difference) between the class 1 integrons of the plasmids S15FP06257_p and pPBH01 is visualized. The latter was detected in 2004 in a clinical *Klebsiella* isolate from a French university hospital (Kassis-Chikhani et al., 2006).

4. Discussion

We present the first confirmed detection of the presence of a carbapenemase encoding $bla_{\text{VIM-1}}$ gene on a plasmid conferring carbapenem resistance in *E. coli* isolated from minced pork in the Belgian food chain. Previous studies in Belgium at the farm level reported the presence of ESBL and AmpC encoding genes in *Salmonella* isolates from healthy pigs and chickens (Cloeckaert et al., 2007), and in pig feces and tonsils at slaughter (Van Damme et al., 2017). Moreover, the national monitoring program organized by the Federal Agency for the Safety of

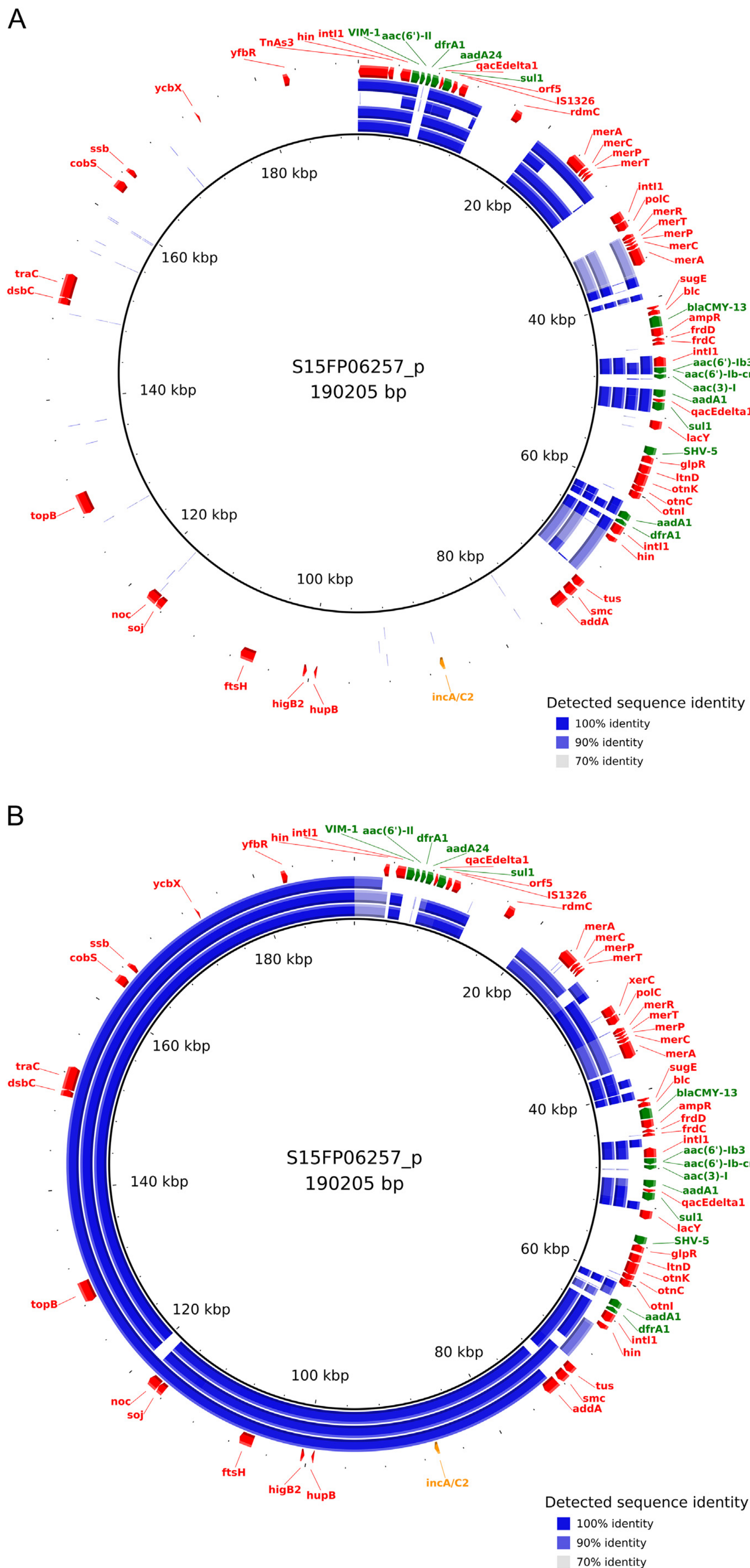

Fig. 1. Annotation of the Belgian bla_{VIM-1} plasmid S15FP06257_p and comparison with other plasmids. The inmost black ring always represents the bla_{VIM-1} plasmid S15FP06257_p isolated from the Belgian food chain, which was used as the reference for the BLAST analysis for both panels, and the outmost ring represents the annotation based on Prokka. A. bla_{VIM-1} plasmids detected in food in other European countries. Inner ring: pRH-R178 from *E. coli* isolated from a German pig farm (accession HG530658.1). Second ring: pRH-R27B from *Salmonella* Infantis isolated from the same German pig farm (accession LN555650.1). Third ring: pE-124-4 from *E. coli* isolated from German seafood (accession MG182343.1). Outer ring: pSE15-SA01028 from *Salmonella* Infantis isolated from German minced pork (accession: NZ_CP026661.1). B. Plasmids in the NCBI nucleotide database that showed high similarity to the Belgian bla_{VIM-1} plasmid. However, all these plasmids lack the bla_{VIM-1} gene. Inner ring: pECO-c85f from clinical *E. coli* isolated in the USA (accession CP026405.1). Middle ring: pMG252 from clinical *E. coli* isolated in the USA (accession MK638972.1). Outer ring: RCS46_p from clinical *E. coli* isolated in France (accession LT985249.1). The blue color represents shared regions according to the sequence identity as labelled in the legend, green features represent antibiotic resistance genes, the orange feature represents the plasmid replicon, and red features represent all other genes. Visualization of the BLAST analysis and annotation was performed with BRIG. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

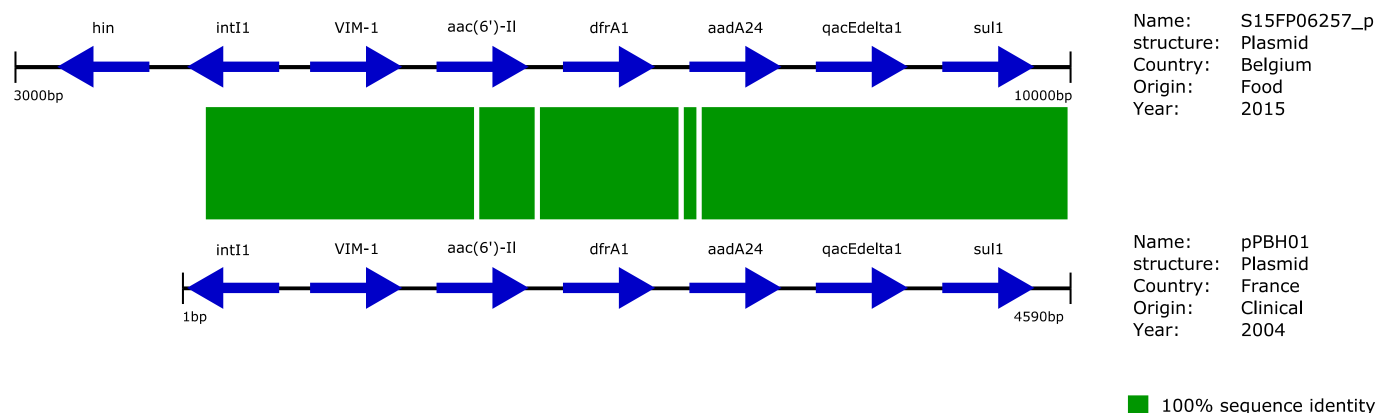

Fig. 2. Comparison between class 1 integron from plasmid S15FP06257_p isolated from the Belgian food chain and a clinical *Klebsiella* plasmid (pHBH01) (accession: AJ870988.1) (Kassis-Chikhani et al., 2006). The green bars indicate regions with a perfect match between both sequences. In total, only four bases were different between the shared regions of both sequences. The alignment was performed with MauveProgressive. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the Food Chain and performed by the National Reference Laboratory for AMR, detected commensal *E. coli* and *Salmonella* producing ESBL and/or AmpC in poultry and pig carcasses and meat derived thereof on an annual basis ("The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2015", 2017). To the best of our knowledge, a carbapenemase-producing *E. coli* has never been reported in neither livestock nor food within Belgium.

European reports on carbapenemase positive food and/or livestock isolates are limited to Germany. In this country the presence of an *E. coli* harboring bla_{VIM-1} isolated from retail seafood, *S.* Infantis and *E. coli* from pig-production farms, broiler farms and minced meat, were previously reported (Borowiak et al., 2017; Fischer et al., 2013, 2012; Roschanski et al., 2017a, 2017b). Although the German food isolate plasmids share the bla_{VIM-1} gene with the Belgian plasmid, their overall sequences and even their integrons are substantially different. Moreover, WGS indicated that the Belgian isolate carrying the bla_{VIM-1} plasmid belongs to ST 5869, differing from the aforementioned German *E. coli* clones (ST 10 and 88). We found only the IncA/C2 plasmid replicon, which differs from the IncHI2 and IncY plasmid replicons detected in Germany. Moreover, there were no sequences in the NCBI nucleotide database that completely covered the plasmid sequence detected in Belgium. Sequences with the highest sequence similarity came from IncA/C2 plasmids that however all lacked the bla_{VIM-1} gene. Analysis of the flanking regions of the bla_{VIM-1} gene of the Belgian food plasmid suggested that this gene likely originates from an integron found mainly in plasmids from clinical *Klebsiella* and *Enterobacter* isolates from France and Greece. This integron was also found in some isolates from USA and Egypt. For one of the plasmids of the clinical *Klebsiella* isolates only sequence information of its integron was publicly available (Kassis-Chikhani et al., 2006). Nevertheless, other reported data exist that strengthen the possibility of relationship with the Belgian bla_{VIM-1} plasmid. Pulsed-field gel electrophoresis indicated that the *Klebsiella* plasmid was larger than 150 kb. In addition, PCR results showed that it also contained the bla_{SHV-5} resistance gene. These are two characteristics shared with the Belgian bla_{VIM-1} plasmid. Moreover, the clinical French *Klebsiella* plasmid was highly similar to a plasmid detected in *Klebsiella* isolates from a hospital in Greece (Giakkoupi et al., 2003; Miriagou et al., 2003). Although similarly only the integron sequence was publicly available, PCR results for an *ampC* gene derived from *Citrobacter freundii* were described. This PCR amplicon showed high similarity (98%) with a *Klebsiella pneumoniae* plasmid fragment (Genbank accession: X91840.1). We also detected this high similarity (98%) between the *Klebsiella pneumonia* plasmid fragment (Genbank accession: X91840.1) and the bla_{CMY-13} gene present in the Belgian plasmid. Due to these similarities not found in other plasmids of the NCBI nucleotide database, it is likely that these clinical plasmids have a close relationship with S15FP06257_p. However, this hypothesis can only be confirmed by comparison of the Belgian plasmid and the clinical plasmids over their full length.

Based on these findings, the sporadic presence of the carbapenemase-producing *E. coli* containing a plasmid with bla_{VIM-1} could be due to import of contaminated meat at retail or through human contamination during manipulation at any stage of the food chain. Despite the fact that it is very likely that the bla_{VIM-1} gene originated from a clinical setting, its exact origin cannot be determined at this moment. Although the sequence type (ST 5869) of the *E. coli* isolate harboring bla_{VIM-1} corresponded with that previously reported in swine in China (Enterobase, P0461_T EC), the plasmid sequence showed only low similarity to Chinese sequences present in the NCBI database. It therefore appears unlikely that S15FP06257_p was directly transferred from China.

The combination of resistance determinants conferring resistance to β-lactams and carbapenems coupled with resistance to other critically important antimicrobials such as fluoroquinolones, aminoglycosides and macrolides, as detected in the isolate is worrisome. However, this finding remains an exceptional case and since 2015 no such cases have been detected in any food matrix or food producing animal in the Belgian food chain. This means that the spread of this plasmid within the food chain is not successful and remains exceptional. The observation that all these AMR genes were found on a mobile genetic element is however a concern for public health, since it increases the risk of resistance transfer. Although we employed short-read WGS as a valuable tool for confirming the resistance mechanisms and finding new resistance determinants, it was impossible to fully characterize the plasmid and determine the localization (chromosome or plasmid) of each AMR gene without employing long-read sequencing. By combining the accurateness of short reads with the ability to span repetitive regions and thereby scaffold entire plasmids enabled by long reads, it was possible to fully reconstruct the bla_{VIM-1} plasmid. This approach will be beneficial in improving plasmid tracking in the food chain. Therefore, as WGS is increasingly being adopted for AMR characterization, it would be beneficial to include our employed strategy involving long sequencing reads if the presence of a plasmid is suspected. This strategy would allow to comply with the recommendations of the European Legislation (European Commission, 2013) for monitoring carbapenemase-producing *E. coli* to follow unexpected events and prevent their further dissemination.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

References

Alikhan, N.-F., Petty, N.K., Ben Zakour, N.L., Beatson, S.A., 2011. BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. BMC Genomics 12, 402. https://doi.org/10.1186/1471-2164-12-402.

Alikhan, N.-F., Zhou, Z., Sergeant, M.J., Achtman, M., 2018. A genomic overview of the population structure of Salmonella. PLoS Genet. 14, e1007261. https://doi.org/10.1371/journal.pgen.1007261.

Benson, D., Lipman, D.J., Ostell, J., 1993. Gen Bank. Nucleic Acids Res. 21, 2963–2965. https://doi.org/10.1093/nar/21.13.2963.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170.

Borowiak, M., Szabo, I., Baumann, B., Junker, E., Hammerl, J.A., Kaesbohrer, A., Malorny, B., Fischer, J., 2017. VIM-1-producing Salmonella Infantis isolated from swine and minced pork meat in Germany. J. Antimicrob. Chemother. 72, 2131–2133. https://doi.org/10.1093/jac/dkx101.

Carattoli, A., Zankari, E., García-Fernández, A., Voldby Larsen, M., Lund, O., Villa, L., Møller Aarestrup, F., Hasman, H., 2014. In silico detection and typing of plasmids using plasmid finder and plasmid multilocus sequence typing. Antimicrob. Agents Chemother. 58, 3895–3903. https://doi.org/10.1128/AAC.02412-14.

Cloeckaert, A., Praud, K., Doublet, B., Bertini, A., Carattoli, A., Butaye, P., Imberechts, H., Bertrand, S., Collard, J.-M., Arlet, G., Weill, F.-X., 2007. Dissemination of an extended-spectrum-beta-lactamase blaTEM-52 gene-carrying IncI1 plasmid in various Salmonella enterica serovars isolated from poultry and humans in Belgium and France between 2001 and 2005. Antimicrob. Agents Chemother. 51, 1872–1875. https://doi.org/10.1128/AAC.01514-06.

Darling, A., Mau, B., Blattner, F.R., P.N., 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Res. 14, 1394–1403. https://doi.org/10.1101/gr.2289704.

De Coster, W., D'Hert, S., Schultz, D.T., Cruts, M., Van Broeckhoven, C., 2018. NanoPack: visualizing and processing long-read sequencing data. Bioinformatics 34, 2666–2669. https://doi.org/10.1093/bioinformatics/bty149.

European Commission, 2013. Commission Implementing Decision of 12 November 2013 on the monitoring of antimicrobial resistance in zoonotic and commensal bacterial. Off. J. Eur. Union 303, 26–39 (652/EU).

Fischer, J., Rodriguez, I., Schmoger, S., Friese, A., Roesler, U., Helmuth, R., Guerra, B., 2012. Escherichia coli producing VIM-1 carbapenemase isolated on a pig farm. J. Antimicrob. Chemother. 67, 1793–1795. https://doi.org/10.1093/jac/dks108.

Fischer, J., Rodríguez, I., Schmoger, S., Friese, A., Roesler, U., Helmuth, R., Guerra, B., 2013. Salmonella enterica subsp. enterica producing VIM-1 carbapenemase isolated from livestock farms. J. Antimicrob. Chemother. 68, 478–480. https://doi.org/10.1093/jac/dks393.

Giakkoupi, P., Xanthaki, A., Kanelopoulou, M., Vlahaki, A., Miriagou, V., Kontou, S., Papafraggas, E., Malamou-Lada, H., Tzouvelekis, L.S., Legakis, N.J., Vatopoulos, A.C., 2003. VIM-1 metallo-beta-lactamase-producing Klebsiella pneumoniae strains in Greek hospitals. J. Clin. Microbiol. 41, 3893–3896. https://doi.org/10.1128/JCM.41.8.3893-3896.2003.

Guerra, B., Fischer, J., Helmuth, R., 2014. An emerging public health problem: acquired carbapenemase-producing microorganisms are present in food-producing animals, their environment, companion animals and wild birds. Vet. Microbiol. 171, 290–297. https://doi.org/10.1016/j.vetmic.2014.02.001.

Huang, T.D., Bogaerts, P., Berhin, C., Hoebeke, M., Bauraing, C., Glupczynski, Y., 2017. Increasing proportion of carbapenemase-producing Enterobacteriaceae and emergence of a MCR-1 producer through a multicentric study among hospital-based and private laboratories in Belgium from September to November 2015. Eurosurveillance 22, 30530. https://doi.org/10.2807/1560-7917.ES.2017.22.19.30530.

Inouye, M., Dashnow, H., Raven, L.-A., Schultz, M.B., Pope, B.J., Tomita, T., Zobel, J., Holt, K.E., 2014. SRST2: rapid genomic surveillance for public health and hospital microbiology labs. Genome Med. 6, 90. https://doi.org/10.1186/s13073-014-0090-6.

Joensen, K.G., Tetzschner, A.M.M., Iguchi, A., Aarestrup, F.M., Scheutz, F., 2015. Rapid and easy in silico serotyping of Escherichia coli isolates by use of whole-genome sequencing data. J. Clin. Microbiol. 53, 2410–2426. https://doi.org/10.1128/JCM.00008-15.

Kassis-Chikhani, N., Decré, D., Gautier, V., Burghoffer, B., Saliba, F., Mathieu, D., Samuel, D., Castaing, D., Petit, J.-C., Dussaix, E., Arlet, G., 2006. First outbreak of multidrug-resistant Klebsiella pneumoniae carrying blaVIM-1 and blaSHV-5 in a French university hospital. J. Antimicrob. Chemother. 57, 142–145. https://doi.org/10.1093/jac/dki389.

Larsen, M.V., Cosentino, S., Rasmussen, S., Friis, C., Hasman, H., Marvig, R.L., Jelsbak, L., Sicheritz-Ponten, T., Ussery, D.W., Aarestrup, F.M., Lund, O., 2012. Multilocus sequence typing of total-genome-sequenced bacteria. J. Clin. Microbiol. 50, 1355–1361. https://doi.org/10.1128/JCM.06094-11.

Leinonen, R., Sugawara, H., Shumway, M., 2011. The sequence read archive. Nucleic Acids Res. 39, D19–D21. https://doi.org/10.1093/nar/gkq1019.

M100-S25 Performance Standards for Antimicrobial Susceptibility Testing; Twenty-fifth Informational Supplement, Twenty-Fif. ed, n.d.

Miriagou, V., Tzelepi, E., Gianneli, D., Tzouvelekis, L.S., 2003. Escherichia coli with a self-transferable, multiresistant plasmid coding for metallo-beta-lactamase VIM-1. Antimicrob. Agents Chemother. 47, 395–397. https://doi.org/10.1128/AAC.47.1.395-397.2003.

Osborn, A.M., Bruce, K.D., Strike, P., Ritchie, D.A., 1997. Distribution, diversity and evolution of the bacterial mercury resistance (mer) operon. FEMS Microbiol. Rev. 19, 239–262. https://doi.org/10.1111/j.1574-6976.1997.tb00300.x.

Poirel, L., Walsh, T.R., Cuvillier, V., Nordmann, P., 2011. Multiplex PCR for detection of acquired carbapenemase genes. Diagn. Microbiol. Infect. Dis. 70, 119–123. https://doi.org/10.1016/j.diagmicrobio.2010.12.002.

Poirel, L., Berçot, B., Millemann, Y., Bonnin, R.A., Pannaux, G., Nordmann, P., 2012. Carbapenemase-producing Acinetobacter spp. in Cattle, France. Emerg. Infect. Dis. 18, 523–525. https://doi.org/10.3201/eid1803.111330.

Queenan, A.M., Bush, K., 2007. Carbapenemases: the versatile beta-lactamases. Clin. Microbiol. Rev. 20, 440–458. https://doi.org/10.1128/CMR.00001-07.

Roschanski, N., Friese, A., von Salviati- Claudius, C., Hering, J., Kaesbohrer, A., Kreienbrock, L., Roesler, U., 2017a. Prevalence of carbapenemase producing Enterobacteriaceae isolated from German pig-fattening farms during the years 2011–2013. Vet. Microbiol. 200, 124–129. https://doi.org/10.1016/j.vetmic.2015.11.030.

Roschanski, N., Guenther, S., Vu, T.T.T., Fischer, J., Semmler, T., Huehn, S., Alter, T., Roesler, U., 2017b. VIM-1 carbapenemase-producing Escherichia coli isolated from retail seafood, Germany 2016. Eurosurveillance 22. https://doi.org/10.2807/1560-7917.ES.2017.22.43.17-00032.

Scientific Opinion on Carbapenem resistance in food animal ecosystems, 2013. EFSA J. 11. https://doi.org/10.2903/j.efsa.2013.3501.

Seemann, T., 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30, 2068–2069. https://doi.org/10.1093/bioinformatics/btu153.

Smet, A., Boyen, F., Pasmans, F., Butaye, P., Martens, A., Nemec, A., Deschaght, P., Vaneechoutte, M., Haesebrouck, F., 2012. OXA-23-producing Acinetobacter species from horses: a public health hazard? J. Antimicrob. Chemother. 67, 3009–3010. https://doi.org/10.1093/jac/dks311.

The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2015, 2017. EFSA J. 15. https://doi.org/10.2903/j.efsa.2017.4694.

Van Damme, I., Garcia-Graells, C., Biasino, W., Gowda, T., Botteldoorn, N., De Zutter, L., 2017. High abundance and diversity of extended-spectrum beta-lactamase (ESBL)-producing Escherichia coli in faeces and tonsils of pigs at slaughter. Vet. Microbiol. 208, 190–194. https://doi.org/10.1016/j.vetmic.2017.08.009.

Wick, R.R., Judd, L.M., Gorrie, C.L., Holt, K.E., 2017. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput. Biol. 13, 1–22. https://doi.org/10.1371/journal.pcbi.1005595.

Woodford, N., Wareham, D.W., Guerra, B., Teale, C., 2014. Carbapenemase-producing Enterobacteriaceae and non-Enterobacteriaceae from animals and the environment: an emerging public health risk of our own making? J. Antimicrob. Chemother. 69, 287–291. https://doi.org/10.1093/jac/dkt392.

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., Aarestrup, F.M., Larsen, M.V., 2012. Identification of acquired antimicrobial resistance genes. J. Antimicrob. Chemother. 67, 2640–2644. https://doi.org/10.1093/jac/dks261.

Zankari, E., Allesøe, R., Joensen, K.G., Cavaco, L.M., Lund, O., Aarestrup, F.M., 2017. PointFinder: a novel web tool for WGS-based detection of antimicrobial resistance associated with chromosomal point mutations in bacterial pathogens. J. Antimicrob. Chemother. 72, 2764–2768. https://doi.org/10.1093/jac/dkx217.