Molecular epidemiology of cefotaxime-resistant *Escherichia coli* from dairy farms in South West England identifies a dominant plasmid encoding CTX-M-32

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Running heading: Cefotaxime-resistant *E. coli* from dairy farms

Abstract

Objectives: The objective of this study was to identify the mechanisms of cefotaxime resistance (CTX-R) in 1226 *Escherichia coli* from 4581 environmental samples collected on 53 dairy farms over a 2-year period in South West England and to characterise a $bla_{\text{CTX-M-32}}$-producing plasmid, pMOO-32, found to be widely distributed.

Methods: CTX-R isolates were identified using MIC breakpoint agar plates. β-lactamase genes of interest (GOIs) were detected by PCR. WGS was performed and analysed using the Center for Genomic Epidemiology platform. A plasmid-specific multiplex PCR was designed to indicate the presence of plasmid pMOO-32.

Results: Amongst 1226 CTX-R isolates, PCR identified $bla_{\text{CTX-M}}$ group 1 (549 isolates), $bla_{\text{CTX-M}}$ group 9 (100 isolates), bla_{CMY} (12 isolates), bla_{DHA} (1 isolate) and no GOI (566 isolates). WGS analysis of 184 representative isolates identified $bla_{\text{CTX-M}}$ (131 isolates; encoding CTX-M-1, -14, -15, -32 and the novel variant, CTX-M-214), $bla_{\text{CMY-2}}$ (6 isolates), $bla_{\text{DHA-1}}$ (one isolate) and presumed AmpC-hyperproduction in 46 isolates that were PCR negative for GOIs. A highly conserved plasmid was identified in 73 isolates, representing 27 *E. coli* STs. This ~220 kb IncHI2 plasmid carrying $bla_{\text{CTX-M-32}}$ was designated pMOO-32, was found to be stable in cattle and human transconjugant *E. coli* even in the absence of selective pressure, and was found by multiplex PCR to be present on 26/53 study farms.

Conclusions: β-lactamases capable of conferring resistance to third generation cephalosporins were evident on 47/53 farms within this study. This was largely because of the widespread dissemination of an IncHI2 plasmid carrying $bla_{\text{CTX-M-32}}$.

Introduction

Third generation cephalosporin (3GC)-resistant *Escherichia coli* have been increasingly reported in both animal and human populations, and are considered pathogens of major concern for humans.[1, 2] 3GCs, such as cefotaxime and ceftazidime, have been listed by the World Health Organisation (WHO) as "highest-priority critically important antimicrobials" (HP-CIAs) because of their importance for human health.[3] Resistance to 3GCs in *E. coli* can be caused by a number of mechanisms but is primarily attributed to the acquisition of ESBLs and/or plasmid-mediated AmpCs (pAmpCs).[4] Plasmids encoding ESBLs frequently harbour additional resistance genes and so can present a significant therapeutic challenge.[5] In recent years the promotion and implementation of the 'One Health' approach in antimicrobial resistance by the WHO has emphasised the importance of surveillance in both animal and human populations and has highlighted gaps in this knowledge.[6] In humans it has been well established in numerous global studies that certain *E. coli* lineages (e.g. *bla*$_{\text{CTX-M}}$-encoding ST131) play a major role in the dissemination of ESBL genes, however such a depth of information does not exist for isolates from animal populations.[2] Human-associated pandemic lineages have been reported in animal populations albeit to a much lesser extent than in human populations.[7]

In humans, *bla*$_{\text{CTX-M}}$ variants are the globally dominant ESBL type with particular variants exhibiting geographical associations (e.g. *bla*$_{\text{CTX-M-15}}$ in Europe and North America and *bla*$_{\text{CTX-M-14}}$ in Asia).[2] Transmission of ESBLs is largely as a result of horizontal gene transfer, with conjugative IncF plasmids in particular being reported as the dominant vehicles for *bla*$_{\text{CTX-M}}$ genes.[8, 9] Previous studies using typing methodologies including WGS have suggested transmission of both strains and ESBL plasmids across animal and human populations .[10, 11] Epidemic plasmids have been reported across different host populations and in multiple countries.[12] For example, one particular epidemic plasmid type – pCT, encoding *bla*$_{\text{CTX-M-14}}$ – was identified in cattle and human *E. coli* isolates in England and found to exist in human isolates from several countries across 3 continents.[12]

Antimicrobial use in food animals may provide selective pressure for resistance genes/plasmids which could theoretically be spread to humans.[13] However, recent reports suggest that such transmission is very limited, at least in the UK.[14] In dairy farming, antibiotics are used both therapeutically in the treatment of common infections such as mastitis, and preventatively e.g. in so-called dry cow therapy, an antibacterial preparation inserted into a cow's udder between lactations to prevent against mastitis.[15] A survey of dairy farms in England and Wales in 2012 revealed that the fourth generation cephalosporin (4GC) cefquinome (another HP-CIA) was the most used dry cow therapy treatment[16]; in 2017, however, only 5.3% of total dry cow therapy active ingredients were HP-CIAs. Indeed, there has been a significant decline in the use of HP-CIAs on dairy farms in the UK.[17]

Given a history of 3GC/4GC usage on the dairy farms involved, this study sought to determine the prevalence and mechanisms of 3GC resistance – using cefotaxime resistance (CTX-R) as an indicator – in *E. coli* isolates from 53 dairy farms located in South West England. Furthermore, the study aimed to characterise the mechanisms of resistance in a subset of isolates using WGS.

Materials and Methods

Bacterial isolates, identification and susceptibility testing

Details of farm sample collection and microbiological analysis has recently been reported.[18] In brief, samples of faecally contaminated sites were collected using sterile overshoes on 53 dairy farms located in South West England between January 2017 and December 2018. Samples were plated onto TBX agar (Sigma-Aldrich, Poole, UK) containing 16 mg/L cephalexin. Up to 5 *E. coli* colonies per cefalexin plate were re-plated onto TBX agar containing 2 mg/L cefotaxime (CTX) in order to confirm resistance. Disc susceptibility testing was performed and interpreted according to EUCAST guidelines.[19]

Screening for β-lactamase genes by PCR

Two multiplex PCRs were performed to screen for β-lactamase genes. The first was to detect *bla*$_{CTX-M}$ groups as previously described [20] and the second was to detect the following additional β-lactamase genes (*bla*$_{CMY}$, *bla*$_{DHA}$, *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{OXA-1}$).[21]

Transconjugations

Transconjugations were performed using rifampicin-resistant (Rif-R) *E. coli* DH5α with both human and cattle *E. coli* isolates as the recipients (**Table 1**). Briefly, 1 mL each of overnight broth cultures of donor and recipient cells were mixed in a 3:1 ratio before centrifugation and resuspension in 50 µL of PBS. Five microlitre aliquots were spotted onto LB agar (Oxoid, Basingstoke, UK) plates and incubated at 37°C for 6 h. Growth was collected and resuspended in 100 µL of PBS before being plated on MacConkey agar (Oxoid) plates containing either 32 mg/L rifampicin (for Rif-R *E. coli* DH5α) or 0.5 mg/L ciprofloxacin (for strains HC4 and HG), and 2 mg/L cefotaxime. Transconjugant colonies were screened by PCR.

Whole genome sequencing and analyses

One-hundred and eighty-four representative isolates were selected for WGS based on resistance phenotype, β-lactamase gene carriage and farm of isolation. WGS was performed by MicrobesNG (https://microbesng.uk/) on a HiSeq 2500 instrument (Illumina, San Diego, CA, USA) using 2x250 bp paired end reads. Reads were trimmed using Trimmomatic [22] and assembled into contigs using SPAdes 3.13.0 [23] (https://http://cab.spbu.ru/software/spades/). Resistance genes, plasmid replicon types and sequence types (according to the Achtman scheme[24]) were assigned using the ResFinder,[25] PlasmidFinder,[26] and MLST 2.0 on the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/) platform. Enhanced genome sequencing (combining Illumina and MinION reads) was performed on one transconjugant, also by MicrobesNG, and reads were assembled using Unicycler.[27] Contigs were annotated using Prokka 1.2.[28]

Reads and assembled contigs were aligned to reference sequences obtained from GenBank progressive Mauve alignment software [29] and CLC Genomics Workbench 12 (Qiagen, Manchester, UK). pMOO-32 was visualised using the CGView server [30] (http://stothard.afns.ualberta.ca/cgview_server/).

pMOO-32 PCR

A multiplex PCR, targeting five size-distinguishable regions of pMOO-32, was designed to indicate the presence of pMOO-32-like plasmids (**Table 2**).

Plasmid stability assay

Three representative pMOO-32 PCR-positive isolates, obtained from different farms in the study, and their transconjugant counterparts were subjected to 10 days of serial passaging on non-selective LB agar. After 10 days, colonies were screened for the presence of pMOO-32 by PCR.

Fitness cost assay

Fitness costs were assessed by a growth curve assay using M9 minimal medium (Sigma-Aldrich). Rif-R *E. coli* DH5α and the pMOO-32 transconjugant strain were grown with shaking at 37°C and OD_{600} measurements were taken at hourly intervals. Assays were performed on three biological replicates.

Results and Discussion

Detection of CTX-R genes of interest (GOIs) by PCR

We have previously reported our collection of 4581 samples from faecally contaminated sites from 53 dairy farms between January 2017 and December 2018. Of these, 4145 samples were positive for detectable levels of *E. coli* and 384 samples were positive for growth of CTX-R *E. coli*.[18] From these, 1226 colonies were taken forward for PCR testing for possible cephalosporinase genes of interest (GOIs): $bla_{CTX\text{-}M}$ (groups 1, 2, 8, 9 and 25),

146 *bla*$_{CMY}$, *bla*$_{DHA}$, and *bla*$_{SHV}$. Over half (648/1226; 52.7%) of all CTX-R isolates tested were
147 found to harbour *bla*$_{CTX-M}$ genes. Of these, 547/648 (84.4%) were of group 1, 99/648 (15.3%)
148 were of group 9, and in one case, both gene groups, were identified. Twelve isolates
149 harboured a *bla*$_{CMY}$ gene – one alongside *bla*$_{CTX-M}$ group 1 – and one isolate was *bla*$_{DHA-1}$-
150 positive. The remaining 566/1226 (46.2%) isolates were PCR-negative for all GOIs.

151 **Identification of acquired CTX-R genes by WGS**

152 One hundred and thirty-eight representative isolates, positive for at least one GOI and
153 chosen to give coverage of all positive farms, were subjected to WGS (**Table 3**). *bla*$_{CTX-M-32}$
154 was the most common GOI allele identified and was found in 79 isolates and 27 *E. coli* STs
155 from 25 farms. CTX-M-32 is a group 1 enzyme first described in a human clinical *E. coli*
156 isolate in 2004.[31] A number of other GOIs were identified: *bla*$_{CTX-M-14}$ (18 isolates, in 6 STs
157 and from 9 farms), *bla*$_{CTX-M-1}$ (16 isolates, 8 STs from 6 farms), *bla*$_{CTX-M-15}$ (16 isolates, 5 STs
158 from 10 farms), *bla*$_{CMY-2}$ (6 isolates, 3 STs from 3 farms) and *bla*$_{CTX-M-214}$ (3 isolates, 2 STs
159 from 3 farms) plus one isolate harbouring both *bla*$_{CTX-M-1}$ and *bla*$_{CTX-M-14}$. CTX-M-214
160 (GenBank Accession No. MH121688) is a novel CTX-M-9 variant, first identified in this
161 study, which differs from CTX-M-9 by a single amino acid, A112T. In all three isolates
162 encoding *bla*$_{CTX-M-214}$, the gene was identified on a contig which also encoded an IncI-ST26
163 plasmid replicon as well as *aadA2*, *sul1*, *dfrA16* in all three isolates, and additionally, *tetA*
164 was found in one isolate.

165 In 46 isolates (from 33 farms) subjected to WGS that lacked any GOIs, AmpC hyper-
166 production was presumed to be the CTX-R mechanism because all carried the same *ampC*
167 promoter/attenuator mutation, previously reported to cause AmpC hyper-producton[21, 32]
168 Detailed analysis of these isolates is reported elsewhere.[33] Considering that all CTX-R *E.*
169 *coli* that tested negative for GOIs by PCR are likely AmpC-hyperproducers, this would mean
170 that AmpC-hyperproduction was the mechanism responsible in almost half (566/1226;
171 46.2%) of CTX-R isolates in this study, representing 186 samples and 38 farms.

172

173 **Identification and characterisation of pMOO-32**

174 Following observations of the high prevalence of *bla*$_{CTX-M-32}$, a search for common plasmid
175 replicon types was conducted which revealed an IncHI2-ST2 replicon in almost all the
176 sequenced *bla*$_{CTX-M-32}$-positive isolates. It was therefore hypothesised that there was a
177 dominant plasmid type occurring across the study. To test this hypothesis, transconjugations
178 were attempted into *E. coli* DH5α using *bla*$_{CTX-M-32}$-positive farm isolate DK (**Table 4**). One
179 successful transconjugant was sent for WGS employing both long and short read
180 methodologies to sequence the plasmid to closure. pMOO-32 is a 226,022-bp conjugative
181 plasmid belonging to the ST2-IncHI2 incompatibility group, harbouring *repHI2* and *repHI2A*
182 replication genes. It contains 245 putative ORFs and has a GC content of 45.5% (**Figure 1**).
183 pMOO-32 encodes the following antimicrobial resistance genes: *bla*$_{CTX-M-32}$, *strA*, *strB*,
184 *aph(6)-Ic*, *aph(3')-IIa* and *tetB* as well as genes encoding resistance to the heavy metal
185 compound, tellurite (*terABCDEFWXYZ*) and a HipAB type II toxin-antitoxin system along
186 with a second partial system (*higB* toxin gene). *bla*$_{CTX-M-32}$ is encoded downstream of an
187 IS*ECp1* element within which there is an IS*Kpn26* insertion encoded in the opposite
188 orientation (**Figure 2**). This same genetic environment was also observed in 4 *bla*$_{CTX-M-32}$-
189 positive but IncHI2 plasmid-negative ST10 isolates collected from 2 farms. There were 2
190 additional IncHI2 plasmid-negative ST765 isolates, both from the same farm, that encoded
191 *bla*$_{CTX-M-32}$ where the immediate genetic environment differed by a truncation in IS*Ecp1*.

192 Transconjugation attempts using the pMOO-32-positive farm *E. coli* isolate DK as donor into
193 a cefotaxime-susceptible (CTX-S) cattle ST88 *E. coli* (isolated from one of the study farms)
194 as well as into a CTX-S human urinary ST1193 *E. coli* isolate were both successful (**Table**
195 **4**). ST1193 is a recently described fluoroquinolone-resistant global clone, often implicated as
196 a cause of human infections,[34] whilst ST88 was selected as a particularly prevalent ST in
197 cattle isolates from both this and previous studies.[35] Antimicrobial disc testing showed that
198 the pMOO-32-carrying donor was, as expected from the genotype, resistant to ampicillin

(AMP), CTX, cefepime (FEP), aztreonam (AZT), streptomycin (STR), neomycin (NEO) and tetracycline (TET). The cattle ST88 and human ST1193 transconjugants were, additional to their starting wild-type resistance profile, resistant to CTX, FEP and AZT. These results (**Table 4**) are indicative of the functionality of the *bla*$_{CTX-M-32}$ gene harboured by pMOO-32.

Epidemiology of pMOO-32-like plasmids

The complete nucleotide sequence of pMOO-32 was submitted to GenBank under the accession number MK169211. Subsequently a multiplex PCR was designed, based on the pMOO-32 sequence, to screen all group 1 *bla*$_{CTX-M}$-positive isolates for the presence of pMOO-32-like plasmids. 26/53 (49.1%) farms within this study, all located within a 40 km radius of each other, tested positive for the presence of pMOO-32-like plasmids using this test. WGS performed on 73 isolates from 24 of these farms identified the pMOO-32-like plasmids in 27 STs, suggesting that its dominance is largely a result of horizontal rather than clonal transmission. Ten farms harboured pMOO-32-like plasmids in isolates of more than one ST. The most frequently identified STs were ST69 and ST10, found in 18 isolates from 7 farms, and 6 isolates from 4 farms, respectively. Using the closed sequence of pMOO-32 as a reference, sequencing reads from all 73 isolates were mapped; this indicated that the plasmids exhibited 94-100% identity to the reference sequence. The differences between the reference plasmid, pMOO-32 and the 73 isolates could be attributed to a loss or gain of mobile genetic elements, but no rearrangements were observed to the plasmid backbone or changes to resistance gene content.

The presence of pMOO-32-like plasmids in 27 STs indicates that plasmid-mediated transmission plays a significant role in the dissemination of *bla*$_{CTX-M-32}$ in the farms in this study. The origins and geographical reach of pMOO-32 remain to be established, however it was shown that none of the 10 farms located in a geographically separated sub-region of this study area were found to harbour this plasmid, or *bla*$_{CTX-M-32}$, suggesting the plasmid may be specific to a particular geographic area; further investigations would be necessary to prove this.

The high-level prevalence and stability of pMOO-32-like plasmids could be a consequence of the HipAB-type II toxin-antitoxin system, making it likely that this plasmid can persist in the absence of antimicrobial selection pressure. Growth curve assays indicated a 12-40% fitness cost (reduction in OD_{600}) of pMOO-32 carriage in *E. coli* DH5α at the start and end of the exponential growth phase in M9 minimal medium (data not shown). However, despite this cost in growth terms, pMOO-32 was stably maintained over 10 days of passaging in the absence of antibiotic pressure in the farm isolates, their respective transconjugants, as well as the human and cattle transconjugants tested. The ability of pMOO-32 to readily transfer into the human isolates and be maintained despite the lack of antibiotic pressure indicates the zoonotic potential of this plasmid. Despite this, a recent study looking into CTX-R urinary *E. coli* from primary care in the same geographical region did not identify any $bla_{CTX\text{-}M\text{-}32}$ genes or pMOO-32-like plasmids.[21]

Dominance of CTX-M-32

In this study $bla_{CTX\text{-}M\text{-}32}$ was clearly shown to be the dominant mechanism of CTX resistance due to the very high prevalence of pMOO-32-like plasmids across 26 farms. A previous study where $bla_{CTX\text{-}M\text{-}32}$ was first described found that it confers increased hydrolytic activity towards the 3GC ceftazidime compared with $bla_{CTX\text{-}M\text{-}1}$, from which it differs by a single amino acid substitution - Asp240-Gly.[31] It could be hypothesised therefore that CTX-M-32 gives some advantage in an environment of 3/4GC use on dairy farms over other CTX-M variants, but this requires further investigation.

Conclusions

E. coli harbouring acquired β-lactamases capable of conferring resistance to 3GCs were evident in most farms within this study. The carriage of $bla_{CTX\text{-}M}$ genes was the dominant acquired mechanism of CTX-R identified; bla_{CMY} carriage was evident, but to a much lesser extent. The prevalence of $bla_{CTX\text{-}M}$ genes, and particularly the $bla_{CTX\text{-}M\text{-}32}$ allele, could be largely attributed to the dissemination of a single plasmid type, pMOO-32. pMOO-32 is

252 capable of conjugating and remaining stable in both animal-associated and human clinical *E.*
253 *coli* isolates, so the possibility exists that pMOO-32-like plasmids will disseminate further,
254 perhaps into other animal populations and even into humans.

255

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266 The authors declare no conflict of interests. Farming and veterinary businesses who
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Isolate	Source/Host	ST	Resistance Genes	Use
DK	Cattle	155	*strA*, *strB*, *aph(6)-Ic*, *aph(3')-IIa*, *tet(B)*, $bla_{CTX\text{-}M\text{-}32}$	Donor
HC4	Human	1193	*aadA5*, *dfrA17*, *mdf(A)*, *sul1*	Recipient
HG	Cattle	88	*aph(6)-Id*, *ant(2'')-Ia*, *aph(3')-Ia*, *aadA24*, *aph(3'')-Ib*, *sul1*, *sul2*, *tet(A)*, *tet(B)*, $bla_{TEM\text{-}1}$, $bla_{OXA\text{-}1}$, *catA1*, *floR*	Recipient

Table 1. Characteristics of *E. coli* strains used in transconjugation experiments.

Primer	Sequence (5'-3')	Product Size (bp)	Target
aph(3')-IIa_F	TGGCTACCCGTGATATTGCT	642	*aph(3')-IIa*/*aph(6)-Ic* junction
aph(6)-Ic_R	CTGGCGGACGGGAAGTATC		
HI2A_F	AGCCTTTCTCACGGTAGCAT	526	HI2 *repA*
HI2A_R	TTCAATTGTCGGTGAGCGTC		
TraI_F	CGGGAAAAGCTGCACTCAAT	396	*traI*
TraI_R	AAGACTTTGTGAGCTTGGCG		
TetB_F	TTCAGCGCAATTGATAGGCC	285	*tetB*
TetB_R	ATCCCACCACCAGCCAATAA		
CTX-M-32_F	TTAGGAAGTGTGCCGCTGTA	180	$bla_{CTX\text{-}M\text{-}32}$
CTX-M-32_R	CACGGCCATCACTTTACTGG		

Table 2. The primers used for the pMOO-32 multiplex PCR.

	CTX-R mechanisms identified and no. of STs/farms																
). of olates/farms	*bla*$_{CTX-M-32}$	STs/farms	*bla*$_{CTX-M-1}$	STs/farms	*bla*$_{CTX-M-14}$	STs/farms	*bla*$_{CTX-M-15}$	STs/farms	*bla*$_{CTX-M-214}$	STs/farms	*bla*$_{DHA-1}$	STs/farms	*bla*$_{CMY-2}$	STs/farms	AmpC-hyp	STs/farms
4/46	79	27/25	16[a]	8/6	18[a]	6/9	16	5/10	3	2/3	1	1/1	6	3/3	46	13/33

Table 3. Characteristics of 184 isolates subjected to whole genome sequencing.

[a]One isolate harboured both *bla*$_{CTX-M-1}$ and *bla*$_{CTX-M-14}$.

Antimicrobial Agent	Zone Diameters (mm)												
	E. coli DK	S/I/R	*E. coli* DH5α	S/I/R	*E. coli* DH5α TR	S/I/R	*E. coli* HG	S/I/R	*E. coli* HG TR	S/I/R	*E. coli* HC4	S/I/R	*E. coli* HC4 TR	S/I/R
AMP	<6	R	30	S	<6	R	<6	R	<6	R	20	S	<6	R
CTX	10	R	45	S	18	I	35	S	10	R	34	S	13	R
CAZ	20	I	45	S	30	S	34	S	20	I	32	S	23	S
FEP	19	R	45	S	28	S	28	S	18	R	35	S	24	I
ETP	30	S	45	S	44	S	34	S	33	S	36	S	36	S
ATM	15	R	45	S	24	I	34	S	15	R	35	S	19	R
STR[a]	<6	R	25	S	10	R	<6	R	<6	R	13	R	<6	R
TOB	17	S	28	S	26	S	<6	R	<6	R	18	S	19	S
NEO[a]	8	R	20	S	12	R	<6	R	<6	R	14	I	8	R
TET[a]	<6	R	36	S	<6	R	<6	R	<6	R	30	S	<6	R

Table 4. Disc susceptibility testing of *E. coli* DK and pMOO-32 transconjugants of various *E. coli* strains.

AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; ETP, ertapenem; ATM, aztreonam; STR, streptomycin; TOB, tobramycin; NEO, neomycin; TET, tetracycline.

[a]Streptomycin and neomycin sensitivities were determined using tobramycin EUCAST interpretation guidelines, and tetracycline according to guidelines for *Yersinia enterocolitica*.

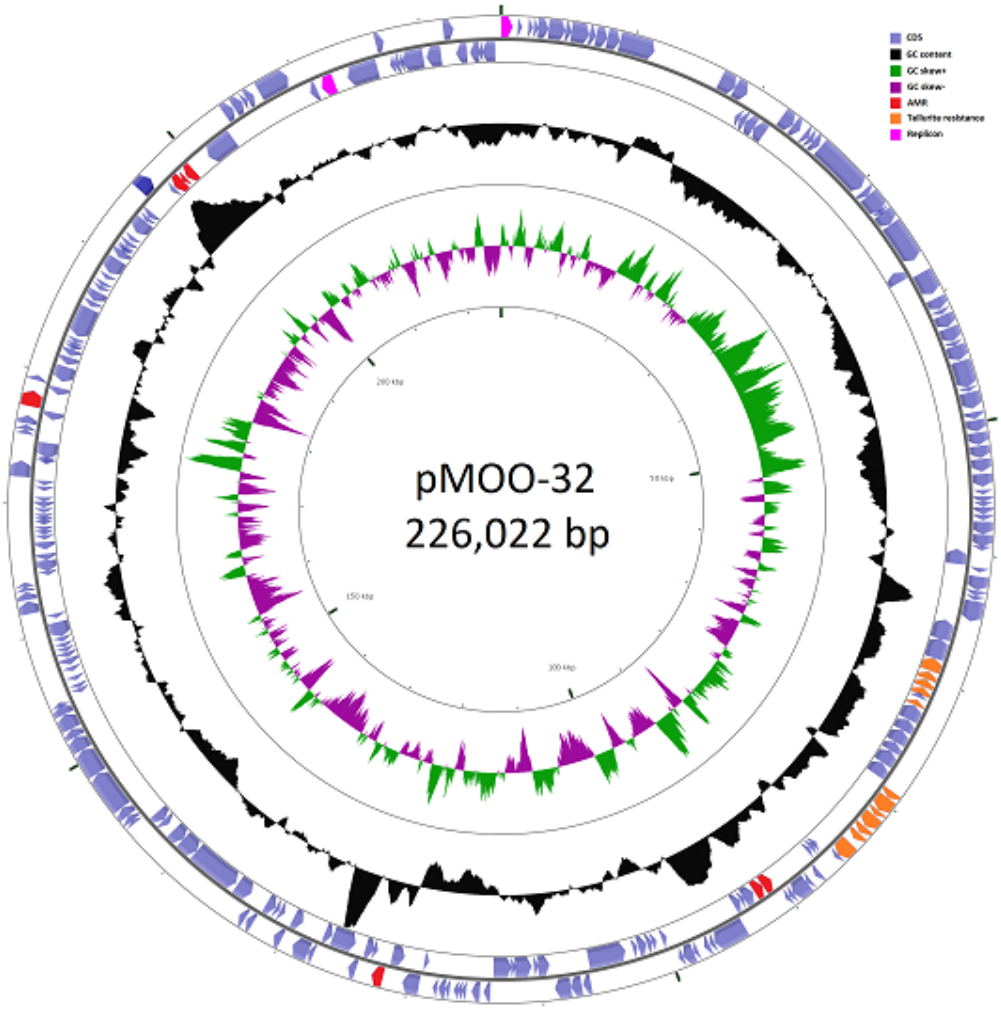

Figure 1. Plasmid pMOO-32 created using CGView.[30]

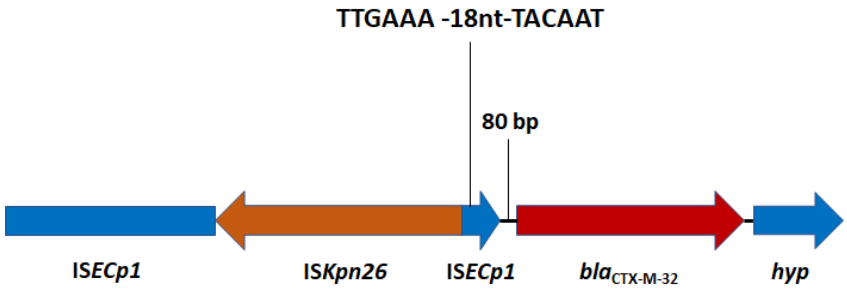

Figure 2. The genetic environment of *bla*$_{CTX-M-32}$ in pMOO-32 and other IncHI2 positive, *bla*$_{CTX-M-32}$-harbouring isolates.