

## BRIEF REPORT

CHARACTERIZATION OF *Escherichia coli* D7111 PRODUCING THE  $\beta$ -LACTAMASE TEM-176

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## ABSTRACT

The present report is the original description of *bla*<sub>TEM-176</sub>. The mechanisms of resistance to antimicrobial agents were determined in an enterotoxigenic *Escherichia coli*, determining the susceptibility to 22 antimicrobials classified in 15 different groups by agar diffusion and establishing the phylogenetic group, mechanisms of resistance and presence of Class 1 and 2 integrons. Integrons and  $\beta$ -lactam resistance genes were sequenced. The isolate, belonging to phylogenetic group A, showed the presence of resistance or diminished susceptibility to ampicillin, amoxicillin plus clavulanic acid, nalidixic acid, ciprofloxacin, streptomycin, kanamycin, tetracycline, trimethoprim, sulfisoxazole, cotrimoxazole, azithromycin and nitrofurantoin, carrying *bla*<sub>TEM</sub>, *aadA1/2*, *aphA1*, *sul3*, *tet(A)* and a Class 2 integron containing a *dfrA1* gene. Quinolone resistance was related to the substitution Ser<sub>83</sub>Ala. The TEM sequencing showed the presence of the new substitution Ala<sub>222</sub>Val, which led to the description of the new  $\beta$ -lactamase *bla*<sub>TEM-176</sub>.

**Keywords:** Ampicillin; Amoxicillin-Clavulanic Acid; beta-Lactamases; Antibacterial Drug Resistance; Enterotoxigenic *Escherichia coli*; Molecular Epidemiology; Integrons (source: MeSH NLM).

## INTRODUCTION

The  $\beta$ -lactam family is one of the most widely used antibiotic families worldwide, both in human and veterinary healthcare. However, the emergence of microorganisms resistant to these antibiotics compromises their usefulness. Thus, in some areas, including different regions of Peru, the levels of resistance to the oldest  $\beta$ -lactams, such as ampicillin, border or exceed 90%<sup>(1,2)</sup>. Meanwhile, resistance levels to more modern  $\beta$ -lactams, such as 3rd and 4th generation cephalosporins, are around 50%<sup>(3)</sup>.

In gram-negative bacteria, resistance to  $\beta$ -lactams is mainly associated with the production of  $\beta$ -lactamases. Among these, the TEM-type  $\beta$ -lactamases are the most frequent, and have been widely reported in Peru<sup>(2,3)</sup>, although in most cases only their presence has been determined, without developing sequencing studies to determine variants.

In this context, in 2007, during a study on childhood diarrhea and antimicrobial resistance carried out in Lima, a diarrheagenic (enterotoxigenic) strain of *Escherichia coli* D7111<sup>(1)</sup> was isolated, which was molecularly characterized by identifying the *bla*<sub>TEM</sub> gene variant. Therefore, this report aims to molecularly characterize the *E. coli* D7111 strain including the original description of the *bla*<sub>TEM-176</sub> gene.

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## THE STUDY

The *E. coli* D7111 strain was isolated during a passive prospective cohort study conducted in peri-urban areas of southern Lima that included 1,034 children between 2 and 12 months of age <sup>(1)</sup>.

Sensitivity to 22 antimicrobials categorized into 15 groups was obtained by agar diffusion <sup>(4)</sup>. Thus, we determined resistance to penicillins (ampicillin), penicillins +  $\beta$ -lactamase inhibitors (amoxicillin plus clavulanic acid [AMC]), cephamycins (cefotaxime), extended-spectrum cephalosporins (cefotaxime, ceftazidime, cefepime), monobactams (aztreonam), carbapenems (imipenem), aminoglycosides (amikacin, gentamicin, kanamycin), streptomycins (streptomycin), amphenicols (chloramphenicol), quinolones (nalidixic acid), fluoroquinolones (ciprofloxacin, norfloxacin), folate inhibitors (trimethoprim, cotrimoxazole), macrolides (azithromycin), tetracyclines (tetracycline), phosphonic acids (fosfomicin), nitrofurans (nitrofurantoin) and sulfonamides (sulfisoxazole). The results were interpreted according to the 2010 Clinical and Laboratory Standards Institute (CLSI) guidelines <sup>(4)</sup>.

The presence of extended-spectrum  $\beta$ -lactamases (ESBL) was analyzed with the double-disk synergy test <sup>(3)</sup>, while presence of plasmid AmpC (pAmpC) was determined by comparison of cefoxitin disk inhibition diameters in the presence or absence of 200  $\mu$ g cloxacillin <sup>(5)</sup>.

The presence of  $\beta$ -lactamases CARB type, OXA-1-like, OXA-2-like, OXA-5-like, SHV and TEM was determined by PCR (Polymerase Chain Reaction) (Table 1). Positive products were recovered, purified and sent to Macrogen (Seoul, South Korea) for two-way sequencing.

Phylogenetic groups were established by PCR (simultaneous amplification of *chuA*, *yjaA* and *tspE4C2*). We also established the presence of *aadA1/2*, *aphA1* (*aph(3')-Ia*) and *aphA2* (*aph(3')-IIa*) genes involved in resistance to streptomycins and aminoglycosides; *tet(A)* and *tet(B)* involved in resistance to tetracyclines; *dfrA1*, *dfrA5*, *dfrA6*, *dfrA7*, *dfrA12*, *dfrA13*, *dfrA14*, *dfrA15*, *dfrA15b*, *dfrA16*, *dfrA16b*, *dfrA17* related to the development of resistance to trimethoprim and *sul1*, *sul2* and *sul3* which cause resistance to sulfonamides. Additionally, the presence of Class 1 and Class 2 integrons was determined by PCR, and the quinolone resistance determinant region of the *gyrA* and *parC* genes was amplified (Table 1). In the case of the *dfr* genes, the variant was identified by RFLP (Restriction Fragment Length Polymorphism) of the amplicons obtained according

## KEY MESSAGES

**Motivation for the study:** Although several studies in Peru have established the presence of *bla*<sub>TEM</sub>, there is a lack of data on the circulating variants.

**Main findings:** An *Escherichia coli bla*<sub>TEM</sub> gene was sequenced, presenting a single variation, leading to the Ala<sub>222</sub>Val change, with respect to TEM-1. At the time of the study (2010) the sequence obtained had not been described, and was given the name of *bla*<sub>TEM-176</sub>.

**Implications:** The detection of a new  $\beta$ -lactamase, shows the presence of resistance genes not described in the area, suggesting that whole genes should be sequenced when possible.

to the methodology previously described <sup>(6,7)</sup>. Mutations in *gyrA* and *parC* as well as integron-positive products were characterized by sequencing.

Information on the reference sequences (DNA/ Amino Acids) of the resistance genes considered in this study (as well as others) can be found at <https://www.ncbi.nlm.nih.gov/refseq/>.

The original study was approved by the Ethics Committee of the Institute for Nutritional Research under code 246-2006/CEI-IIN.

## FINDINGS

Strain *E. coli* D7111, identified as belonging to phylogroup A, showed resistance to ampicillin, AMC, azithromycin, cotrimoxazole, kanamycin, sulfisoxazole, tetracycline, trimethoprim and nalidixic acid, as well as intermediate sensitivity to streptomycin, nitrofurantoin and ciprofloxacin (Table 2). In the case of azithromycin, the inhibition halo was <6 mm, so that even in the absence of the cut-off point established by CLSI, we classified it as resistant.

The phenotypic study of  $\beta$ -lactamases was negative for both ESBL and pAmpC, with *bla*<sub>TEM</sub> being the only  $\beta$ -lactamase coding gene amplified. The sequencing result showed the presence of a nucleotide-level difference (A665T) regarding the *bla*<sub>TEM-1</sub> sequence, leading to the amino acid change Ala<sub>222</sub>Val. After comparing the amino acid sequence with those described to date, it was verified as novel and was submitted to the corresponding repository ([www.lahey.com](http://www.lahey.com)), currently transferred to the National Center for Biotechnology

**Table 1.** Primers used in this study.

Gene	Primer 1	Primer 2	bp	°C	Ref
<i>bla</i> <sub>TEM</sub>	ATCTTGAAGACGAAAGGGC	ACGCTCAGTGAACGAAAAC	1,150	60	3
<i>bla</i> <sub>SHV</sub>	ATGCGTTATATTCGCCTGTG	TTAGCGTTGCCAGTGCTCG	841	55	3
<i>bla</i> <sub>CARB</sub>	AATGGCAATCAGCGCTTC	GGGGCTTGATGCTCACT	586	56	3
<i>bla</i> <sub>OXA-1-LIKE</sub>	ACCAGATTCAACTTTCAA	TCTTGGCTTTTATGCTTG	598	55	3
<i>bla</i> <sub>OXA-2-LIKE</sub>	CGATAGTTGTGGCAGACGAA	CCACTCAACCCATCCTACCC	550	55	3
<i>bla</i> <sub>OXA-5-LIKE</sub>	TATATTCCAGCATCAACATT	ATGATGCCCTCACTTGCCAT	605	55	3
<i>gyrA</i>	AAATCTGCCCGTGTCTGGT	GCCATACCTACGGCGATAACC	343	55	3
<i>parC</i>	AAACCTGTTCAGCGCCGATT	GTGGTGCCGTTAAGCAAA	395	59	3
<i>aphA1</i>	ATGGGCTCGCGATAATGTC	CTCACCGAGGCAGTTCAT	600	50	6
<i>aphA2</i>	GAACAAGATGGATTGCACGC	GCTCTTCAGCAATATCACGG	680	50	6
<i>aadA1, aadA2</i>	GCAGCGCAATGACATTCITG	ATCCTTCGGCGCGATTTTG	282	60	6
<i>tet(A)</i>	GTAATTCAGCACTGTCCG	CTGCCGACAACATTGCTT	937	62	6
<i>tet(B)</i>	CTCAGTATTCCAAGCCTTTG	CTAAGCACTGTCTCCTGTT	416	57	6
<i>dfrA1, dfr5, dfr15, dfr15b, dfr16, dfr16b</i>	GTGAAACTATCACTAATGG	TAAACCCCTTTTGCCAGATTT	474	55	6
<i>dfrA6, dfr14</i>	GAGCAGCTICTTTTAAAGC	TTAGCCCTTTIICCAATTTT	393	60	6
<i>dfrA7, dfr17</i>	TTGAAAATTTCAITGATT	TTAGCCCTTTTTCAAAATCT	474	55	6
<i>dfrA12, dfr13</i>	GGTSGCAGAAAGATTTTTCGC	TGGGAAGAAGCGGTCACCCCTC	319	60	6
<i>sul1</i>	TGGTGACGGTGTTCGGCATT	GCGAGGGTTTCCGAGAAGGTG	789	63	6
<i>sul2</i>	CGGCATCGTCAACATAACC	GTGTGCGGATGAAGTCAG	722	50	6
<i>sul3</i>	CATTCTAGAAAACAGTCGTAGTTCC	CATCTGCAGCTAACCTAGGGCTTTGGA	990	51	6
VR1	GGCATCCAAGCAGCAAG	AAGCAGACTTGACCTGA	Var.	55	6
VR2	CGGGATCCCGACGGCATGCAGATTGTA	GATGCCATCGCAAGTACGAG	Var.	60	6
<i>chuA</i>	GACGAACCAACGGTCAGGAT	TGCCGCCAGTACCAAAGACA	279	59	7
<i>yjaA</i>	TGAAGTGTGAGGAGACGCTG	ATGGAGAATGCGTTCCCTCAAC-	211	59	7
<i>tspE4C2</i>	GAGTAATGTCCGGGCATTCA	CGCGCCAACAAAGTATTACG	152	59	7

bp: amplicon size in base pairs; °C: banding temperature; Ref: Reference; VR: Variable Region (1 - of Class 1 integrons; 2 - of Class 2 integrons); Var: Variable

Information - NCBI - <https://www.ncbi.nlm.nih.gov/pathogens/submit-beta-lactamase/>), being confirmed as a new TEM variant, and received the name TEM-176 (Figure 1). Consequently, the sequence was reported to GenBank, and is accessible under the codes: GU550123 - Original DNA Sequence; NG\_050215 - Reference DNA Sequence; Reference Protein Sequence WP\_038976851.

Additionally, *E. coli* strain D7111 presented the genes *aadA1/2*, *aphA1* (coding for the aminoglycoside-modifying enzyme, APH(3')-Ia), *tet(A)*, *dfrA1* and *sul3* as well as the amino acidic change Ser<sub>83</sub>Ala in GyrA. The integron study showed the presence of a class 2 integron, of approximately 800 bp, containing the *dfrA1* gene cassette (Table 2).

## DISCUSSION

This study reports the presence of a TEM variant that, at the time of the study, had not been identified and, after being submitted to the entity who oversees nomenclature, was given the name TEM-176.

The  $\beta$ -lactamase family of TEM-type  $\beta$ -lactamases was initially described in 1965<sup>(8)</sup>, currently counting 190 variants sequenced and present at [https://www.ncbi.nlm.nih.gov/pathogens/refgene/#gene\\_family:\(blaTEM\)](https://www.ncbi.nlm.nih.gov/pathogens/refgene/#gene_family:(blaTEM)) ranging from TEM-1 to TEM-242 (note the absence in the NCBI database of TEM-type  $\beta$ -lactamases with assigned numbering).

TEM-176 differs by only one amino acid from TEM-1. Although apparently insignificant, differences of one amino acid may imply differences in the spectrum of activity (both on existing  $\beta$ -lactams and on  $\beta$ -lactams to be designed at later times after their description). Likewise, these differences may have evolutionary implications, since the acquisition of a point mutation could make TEM-176 evolve more easily to other TEM variants, including ESBL, as would be the case of TEM-147 (Reference Protein Sequence: WP\_063864859), which has only one amino acid difference with respect to TEM-176, Arg<sub>162</sub>His, in addition to the presence of Val<sub>222</sub>.

After its detection in strain D7111 and its inclusion in the nomenclature, TEM-176 has been described on several occasions. Thus, GenBank (<https://www.ncbi.nlm.nih.gov/>)

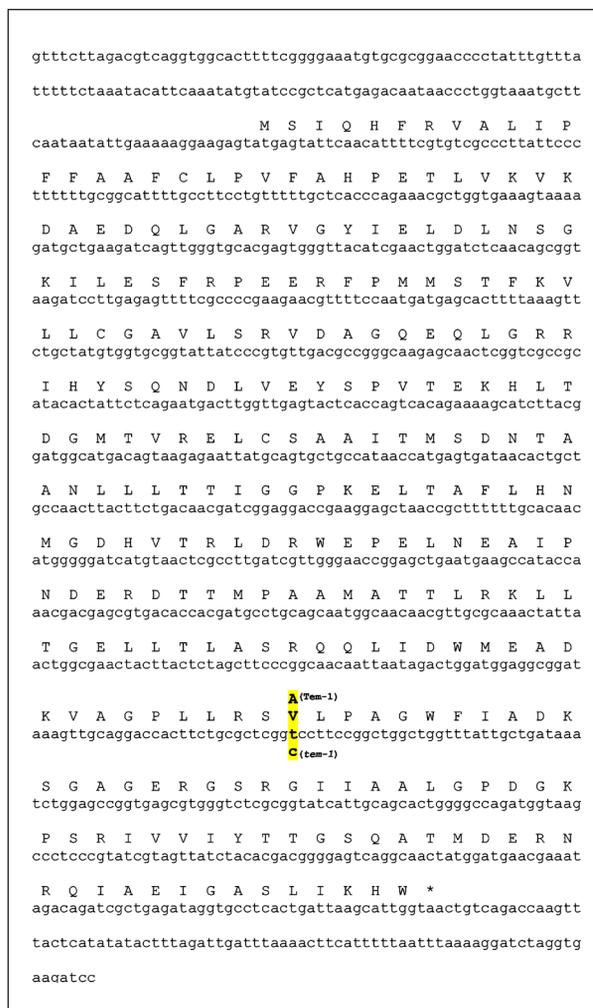
**Table 2.** Resistance profile and underlying mechanisms.

Antimicrobial	Sensitivity	Mechanism
Ampicillin	R	TEM-176
AMC	R	TEM-176?
Cefoxitin	S	---
Cefotaxime	S	---
Ceftazidime	S	---
Cefepime	S	---
Aztreonam	S	---
Imipenem	S	---
Nalidixic Acid	R	Ser <sub>83</sub> Ala (GyrA)
Ciprofloxacin	I	Ser <sub>83</sub> Ala (GyrA)
Norfloxacin	S	Ser <sub>83</sub> Ala (GyrA)
Amikacin	S	---
Gentamicin	S	---
Kanamycin	R	APH(3')-Ia
Streptomycin	I	ANT(3'')-I
Azithromycin	R	ND
Chloramphenicol	S	---
Trimethoprim	R	DfrA1
Sulfisoxazole	R	Sul3
Cotrimoxazole	R	DfrA1 + Sul3
Tetracycline	R	Tet(A)
Nitrofurantoin	I	ND
Fosfomycin	S	---

AMC: Amoxicillin plus clavulanic acid; S: Susceptible; I: Intermediate; R: Resistant; ND: Not determined; ?: Not established

ipg/WP\_038976851.1) reports its presence in *E. coli* (29 strains), *Salmonella enterica* (61 strains, belonging to frequent serotypes such as Typhimurium, Enteritidis or Hadar, or infrequent ones such as Brancaster or Schwarzengrund), *Klebsiella pneumoniae* (2 strains), *Citrobacter amalonaticus* (2 strains), *Citrobacter portucalensis* (1 strain) and *Vibrio parahaemolyticus* (2 strains), being in most cases detected during genomic sequencing studies.

The presence of this (or other) *bla*<sub>TEM</sub> variants has probably gone unnoticed in numerous studies, given the need to sequence the *bla*<sub>TEM</sub> amplicon in order to make the correct identification. The fact that it does not affect 3rd and 4th generation cephalosporins may also have contributed to this. Nevertheless, since its description and introduction in GenBank on January 31, 2010, *bla*<sub>TEM-176</sub> has been reported in microorganisms isolated from samples of different origins (e.g. human, animal or food), and from several geographic locations, such as Austria, Australia, Ecuador, Singapore or Tanzania, thus showing worldwide dissemination (9-13).



In lowercase letters is the sequence of a 1043 bp DNA fragment containing the *bla*<sub>TEM-176</sub> gene (GenBank: GU550123), above it is annotated in uppercase letters the amino acid sequence of TEM-176. In bold and highlighted in yellow are the differences with respect to TEM-1 (GenBank: NG\_050145), amino acid at the top, nucleotide at the bottom.

**Figure 1.** *bla*<sub>TEM-176</sub> gene sequence.

Although there is scarce data on its genomic location, in some cases its presence has been proven in plasmids of the IncX group (10,11), such as pSGMCR103 (a plasmid of the IncX1 group), which, among other resistance determinants, in addition to *bla*<sub>TEM-176</sub>, carries the *mcr-5.1* gene (11).

In addition, its presence has been detected in metagenomic studies carried out in Peru (14). This fact is interesting, since it would appear that despite the scarcity of studies aimed at sequencing β-lactamase type TEM in Peru, the presence of TEM-176 has been described by different authors independently in the country, suggesting that it could be relatively frequent in the area.

The fact that strain D7111 is resistant to AMC, together with the non-detection of other β-lactamases, opens the door to the possibility that this β-lactamase inhibitor does

not completely inhibit TEM-176, which would allow this β-lactamase to be categorized as IRT (Inhibitor Resistant TEM). However, this aspect should be specifically and unequivocally verified, since unidentified β-lactamases could coexist, just as it has been found that overexpression of β-lactamases of the TEM type, such as TEM-1, can lead to decreased sensitivity or resistance to AMC<sup>(15)</sup>.

Strain D7111 also presented other resistance determinants, such as the *aphA1* gene, underlying the detection of resistance to kanamycin<sup>(16)</sup>. APH(3')-Ia has been previously described in numerous microorganisms, including all gram-negative bacteria of the ESKAPE group (*Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) as well as other microorganisms causing different diseases such as *Escherichia coli* or *Salmonella* spp. (<https://card.mcmaster.ca/ontology/39041>). However, to our knowledge, this is the first description of this enzyme in Peru. The ubiquity of APH(3')-Ia together with the high levels of kanamycin resistance detected in clinical isolates in Peru makes this finding not surprising. Another aminoglycoside-modifying enzyme-encoding gene, an *aadA*-type gene, was also detected. The *aadA* genes encode nucleotidyltransferases inactivating streptomycin and spectinomycin<sup>(16)</sup>, and are widely disseminated worldwide, although unlike in this case, they are often associated with integrons, both Class 1 and Class 2 (<http://integrral.bio.ua.pt/>).

Consistent with what has been described in the literature, the Ala<sub>83</sub> substitution in GyrA was considered to be the cause of resistance to nalidixic acid<sup>(17,18)</sup>. This amino acid change is associated with less alteration of the hydrophobicity patterns of GyrA than other amino acid changes at the same position such as Ser<sub>83</sub>Leu or Ser<sub>83</sub>Val, and therefore is usually associated with lower levels of resistance<sup>(17,18)</sup>.

In addition to tetracycline, Tet(A) has been associated with slight increases in resistance to tigecycline, moreover, the development during treatments of new allelic variants (amino acidic change Ser<sub>251</sub>Ala) capable of conferring minimum inhibitory concentration levels of 32 mg/L has been observed<sup>(19)</sup>.

Integrons are mobilizable genetic structures, although not mobile *per se*, which are characterized by having a 5'

end consisting of a gene coding for an integrase, a series of gene cassettes coding for genes of various kinds and a 3' end that varies among the different types of integrons, but that is highly conserved<sup>(20)</sup>. Integrons are classified according to the integrase gene, with classes 1 and 2 being the most common, usually carrying resistance genes<sup>(20)</sup>, and they receive a specific numbering based on their content (type and order) of gene cassettes (<http://integrral.bio.ua.pt/>). The integron detected in this strain is a class 2 integron containing the *dfrA1* gene, concordant with the In2-2 integron (<http://integrral.bio.ua.pt/>). This *dfrA1* gene underlies resistance to trimethoprim, and together with the *sul3* gene, responsible for resistance to sulfisoxazole, underlies resistance to co-trimoxazole.

The complete study of this strain and the sequencing of *bla*<sub>TEM-176</sub> was developed in 2010, so the elapsed time is the major limitation for this study, creating a paradox in which there are publications where the presence of *bla*<sub>TEM-176</sub> is reported prior to the original report of this β-lactamase. Likewise, it remains to evaluate the real effect of AMC and the potential as IRT of TEM-176, which should be done by cloning this gene in a cloning vector that does not contain another β-lactamase, in order to avoid erroneous interpretations due to the added action of both enzymes.

In conclusion, the mechanisms of antimicrobial resistance present in strain D7111 are characterized, and reporting the original description of TEM-176. Likewise, the detection of TEM-176 in subsequent studies developed in Peru is emphasized, suggesting the stable presence of this resistance determinant in the area.

**Author contributions:** All authors participated in the conception and design of the article. MJP and SM participated in experimental development; data analysis and interpretation. JR and MJP participated in the drafting of the article. All authors critically reviewed the article, approved the final version and assume responsibility for the contents of the manuscript.

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