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Presence of Extended-Spectrum β-lactamase, CTX-M-65 in *Salmonella enterica* serovar Infantis Isolated from Children with Diarrhea in Lima, Peru

Ana Granda[1] Maribel Riveros[1] Sandra Martínez-Puchol[2] Karen Ocampo[1] Laura Laureano-Adame[3] Alfredo Corujo[3] Isabel Reyes[4] Joaquim Ruiz[2] Theresa J. Ochoa[1,5]

[1] Department of Pediatrics, Instituto de Medicina Tropical "Alexander von Humboldt," Universidad Peruana Cayetano Heredia, Lima, Perú
[2] ISGlobal, Hospital Clinic, Universitat de Barcelona, Barcelona, Spain
[3] Food Research Center, Nutreco, Toledo, Spain
[4] Unidad de investigación pediátrica, Hospital de Emergencias Pediátricas, Lima, Perú
[5] Center for Infectious Diseases, University of Texas Health Science Center at Houston, Houston, TX, United States

Address for correspondence Theresa J. Ochoa, MD, Department of Pediatrics, Instituto de Medicina Tropical "Alexander von Humboldt," Universidad Peruana Cayetano Heredia, Av. Honorio Delgado 430, San Martin de Porras, Lima 33, Perú (e-mail: Theresa.J.Ochoa@uth.tmc.edu; Theresa.Ochoa@upch.pe).

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Abstract

Gastroenteritis in children is a serious condition in many parts of the world. *Salmonella enterica* is one of the causes of the disease. In this study, 280 fecal samples from children with diarrhea in four hospitals in Lima, Peru, were collected between September 2012 and March 2013. *Salmonella* was detected in 26 of the samples. Serotyping demonstrated that 25 of the isolates were *S. enterica* Infantis, and one isolate was *S. enterica* Typhimurium. Repetitive extragenic palindromic–polymerase chain reaction analysis suggests that all *S.* Infantis belong to the same clone. All but one of the *S.* Infantis isolates exhibited an extended-spectrum β-lactamase phenotype as they harbored $bla_{\text{CTX-M 65}}$. Two strains also carried $bla_{\text{TEM-1}}$. Nine of the isolates were resistant to azithromycin and two to ciprofloxacin. This study demonstrates that a multidrug-resistant *S.* Infantis clone carrying $bla_{\text{CTX-M 65}}$ was circulating among children in Lima, Peru. The development of molecular epidemiology studies in *Salmonella*-causing diarrhea or other pathologies in Lima and in other areas will be useful to determine the permanence, geographical spread, and clinical implications of this clone.

Keywords
- extended-spectrum β-lactamases
- *Salmonella enterica* serovar Infantis
- diarrhea

Introduction

Worldwide, gastroenteritis is one of the major causes of morbidity and mortality in children under 5 years of age; it was estimated that the annual incidence of diarrhea is 2.8 million cases,[1] leading to around 500,000 deaths each year.[2]

Salmonella is a common cause of gastroenteritis.[1] This bacterium has more than 2500 serovars, but *Salmonella enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis are isolated most frequently worldwide.[3] *Salmonella enterica* serovar Infantis has increasingly been detected in several parts of the world.[4] In the recent years, it has been associated with outbreaks in Latin American countries like Brazil, Ecuador,[5,6] and Peru, where it has been reported as the third most common serotype.[7]

In Peru, *Salmonella sp.* resistant to β-lactam antibiotics is a problem, and extended-spectrum β-lactamases (ESBLs) are frequently present. In 2010, Lima and its vicinity reported a considerable increase in the number of isolates resistant to ceftriaxone (CRO), with the presence of β-lactamases belonging to the CTX-M family.[8] Previously, in 2006, a nosocomial outbreak of *S.* Typhimurium harboring a $bla_{\text{SHV-5}}$ gene was also reported.[9] These findings are relevant because although antibiotic treatment is not usually indicated in acute diarrhea,

Joaquim Ruiz's ORCID is https://orcid.org/0000-0002-4431-2036.

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third-generation cephalosporins, together with azithromycin (AZM) and ciprofloxacin (CIP), can be used as a treatment in high-risk groups such as infants, the elderly, and immunocompromised patients.[10]

The main genes encoding ESBLs are bla_{TEM}, bla_{SHV}, bla_{OXA}, and bla_{CTX-M}. The latter, which confers high levels of resistance to cefotaxime (CTX), encodes enzymes that are subdivided into five subclasses (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25).[11] Currently, the CTX-M family is the most commonly disseminated in the world and different microbial species.[12]

The objectives of this study were to determine the presence of *Salmonella* in fecal samples of children admitted to four hospitals in Lima, Peru, and to determine whether the *Salmonella* isolates were related and expressed an ESBL phenotype. The genetic basis of the ESBL phenotype was also determined.

Methods

Bacterial Isolates

A total of 280 stool specimens from children under 6 years of age hospitalized with gastroenteritis and persistent diarrhea were collected from September 2012 to April 2013 from four hospitals in Lima: Hospital de Emergencias Pediátricas (HEP) ($n = 212$), Hospital Nacional Cayetano Heredia (HNCH) ($n = 17$), Hospital Nacional Docente Madre Niño San Bartolomé (HSB) ($n = 2$), and Centro de Salud Materno Infantil Tahuantinsuyo Bajo (CST) ($n = 49$) (►**Fig. 1**). *Salmonella* sp. were isolated on differential culture media such as xylose lysine deoxycholate agar and Salmonella–Shigella agar. Presumptive *Salmonella* colonies were identified through biochemical[13] and serological (detection of somatic antigen O, [Probac do Brasil, Sao Paulo, SP, Brazil]) tests and then confirmed by polymerase chain reaction (PCR)[14] in the Laboratory of Enteric Diseases and Nutrition (LEEN), Tropical Medicine Institute Alexander von Humboldt from Universidad Peruana Cayetano Heredia (UPCH). *Salmonella* isolates were sent to the Nutreco Food Research Center (Toledo, Spain), where serotyping was performed using a commercial DNA microarray system, Check & Trace Salmonella (Check-Points, the Netherlands, Holland).

Antibiotic Susceptibility

The antibiotic susceptibility was determined by Kirby–Bauer tests according to the Clinical & Laboratory Standards Institute (CLSI) guidelines.[15,16] Antibiotics included in this study were chloramphenicol (C), aztreonam (ATM), trimethoprim–sulfamethoxazole (TMP/SMX), cefotaxime (CTX), ampicillin (AMP), furazolidone (F), nalidixic acid (NA), CRO, CIP,

Fig. 1 Geographical distribution of point locations of four hospitals in Lima: Hospital de Emergencias Pediátricas (HEP), Hospital Nacional Cayetano Heredia (HNCH), Hospital Nacional Docente Madre Niño San Bartolomé (HSB), and Centro de Salud Materno Infantil Tahuantinsuyo Bajo (CST).

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tetracycline (TE), AZM, erythromycin (E), amoxicillin plus clavulanic acid (AMC) and ceftazidime (CAZ). In the case of AZM, an isolate was considered resistant if the halo was <12 mm as proposed for other enterobacteria.[15]

Phenotypic Methods for Detection of ESBL

The presence of ESBLs was verified by two tests: double-disc synergy test with AMC (30 µg), CAZ (30 µg), CTX (30 µg), CRO (30 µg), and ATM (30 µg)[17]; and the CLSI confirmatory test using both CTX (30 mg) and CAZ (30 mg) disks alone and in combination with clavulanic acid (10 mg). The second test was considered positive when an increase in the growth inhibitory zone diameter around a disk containing CTX or CAZ with the addition of clavulanic acid was 5 mm or greater than the diameter around the disk containing CTX or CAZ alone.[15]

Molecular Detection of ESBL Genes

The presence of bla_{TEM},[18] bla_{SHV},[19] and bla_{CTX-M}[20] was determined by PCR in isolates presenting an ESBL phenotype. The CTX-M variants, CTX-M 1, CTX-M 2, CTX-M 8, and CTX-M 9,[21,22] were also confirmed by PCR (►**Table 1**). The amplification products were purified (Gel Extraction Kit from Omega Bio-tek, Norcross, Georgia, United States) and sequenced (Beckman Coulter; Takeley, Great Britain).

Repetitive Extragenic Palindromic–Polymerase Chain Reaction

Clonal relationships between strains were determined by repetitive extragenic palindromic–PCR (REP-PCR) using the primer GCG CCG ICA TGC GGC ATT[23] with the following amplification conditions: 95°C for 5 minutes followed by 30 cycles of 95°C for 1 minute, 40°C for 1 minute, and 65°C for 1 minute, and final extension at 65°C for 16 minutes. A phylogenetic tree was constructed by the unweighted pair group method with arithmetic mean analysis using the Phoretix 1D Pro software.

Results

Bacterial Isolates

Of 280 clinical fecal samples from children with diarrhea, 26 (9%) were identified as *Salmonella sp*., 25 were serotyped as *S*. Infantis, and 1 as *S*. Typhimurium. When analyzed for Hospital settings, it was observed that *Salmonella* sp. isolates were only recovered from HEP (21/212; 10%) and HNCH (5/17; 29%).

Antibiotic Susceptibility

All *S*. Infantis were resistant to AMP, E, F, NA, SXT, and TE, 24/25 isolates were also resistant to ATM, CRO, CTX, and C, and 10/25 (40%) were resistant to AZM. Two out of the 25 isolates were resistant to CIP, and the remaining 92% (23/25) exhibited intermediate resistance. Four isolates were resistant to CAZ and 18 presented intermediate resistance. *Salmonella* Typhimurium only presented resistance to E and AZM. The CLSI confirmatory test results were concordant with the result of the double-disc synergy test.

Molecular Detection of Extended-Spectrum β-Lactamase

All strains with an ESBL phenotype harbored bla_{CTX-M}. Two isolates, E-25 (from HNCH) and 2-094 (from HEP), also presented with bla_{TEM} (►**Table 2**). Subsequent analysis showed the presence of a $bla_{CTX-M\ 65}$ gene belonging to CTX-M group 9. The bla_{TEM} gene was identified as encoding TEM-1 and therefore not classified as ESBL. No bla_{SHV} gene was detected.

Table 1 Primers used for the molecular detection of ESBL and variants

Primer	Nucleotide sequence 5̀—3̀	Amplicon size (bp)	Annealing temperature used for PCR (°C)	Reference
TEM-F	ATTCTTGAAGACGAAAGGGC	1,150	60	18
TEM-R	ACGCTCAGTGGAACGAAAAC			
SHV-F	CACTCAAGGATGTATTGTG	885	52	19
SHV-R	TTAGCGTTGCCAGTTATTGTG			
CTXM- Univ- F	CGATGTGCAGTACCAGTAA	585	52	20
CTXM- Univ- R	TTAGTGACCAGAATCAGCGG			
CTXM-3G (group 1)-F	GTTACAATGTGTGAGAAGCAG	1,017	60	21
CTXM-3G (group 1)-R	CCGTTTCCGCTATTACAAAC			
CTXM-9 (group 9)-F	TGACCGTATTGGGAGTTTCAG	917	55	22
CTXM-9 (group 9)-R	GATTTATTCAACAAAACCAG			
CTXM-8 (group 8)-F	TGATGAGACATCGCGTTAAG	873	60	21
CTXM-8 (group 8)-R	TAACCGTCGGTGACGATTTT			
CTXM-10-F	CCGCGCTACACTTTGTGGC	944	60	21
CTXM-10-R	TTACAAACCGTTGGTGACG			

Abbreviation: ESBL, extended-spectrum β-lactamase.

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Table 2 Characteristics of *Salmonella* Infantis strains

Isolate	Date	Hospital	Specie/Serovar	ESBL type
E-25	September 12, 2012	HNCH	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$, $bla_{\text{TEM-1}}$
E-28	November 12, 2012	HNCH	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$
2-015	January 7, 2013	EP	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$
2-016	January 7, 2013	EP	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$
2-017	January 8, 2013	EP	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$
2-023	January 14, 2013	EP	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$
2-024	January 14, 2013	EP	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$
2-026	January 15, 2013	EP	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$
2-029	January 17, 2013	EP	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$
2a-002	January 18, 2013	HNCH	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$
2-031	January 21, 2013	EP	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$
2-032	January 22, 2013	EP	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$
2-040	January 24, 2013	EP	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$
2-047	January 28, 2013	EP	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$
2-050	29 January, 2013	EP	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$
2-065	February 1, 2013	EP	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$
2-068	February 4, 2013	EP	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$
2-094	February 14, 2013	EP	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$, $bla_{\text{TEM-1}}$
2-125	February 27, 2013	EP	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$
2-144	March 14, 2013	EP	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$
2-161	March 20, 2013	EP	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$
DEC 1-009	March 25, 2013	HNCH	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$
1-013	April 4, 2013	HNCH	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$
2-204	April 17, 2013	EP	*Salmonella* Infantis	$bla_{\text{CTX-M 65}}$
2-013	January 3, 2013	EP	*Salmonella* Infantis	–
2-145	March 18, 2013	EP	*Salmonella* Typhimurium	–

Abbreviations: EP, Hospital de Emergencias Pediátricas; ESBL, extended-spectrum β-lactamase; HNCN, Hospital Nacional Cayetano Heredia.

Molecular Typing by Repetitive Extragenic Palindromic–Polymerase Chain Reaction

REP-PCR data suggested a clonal relationship among 24 of the 25 isolates of *S.* Infantis. All ESBL-producing strains have identical REP-PCR profiles. The isolate, *S.* infantis 2-013, that did not have the ESBL phenotype showed a different REP-PCR profile (►**Fig. 2**).

Discussion

In this study, 92% of 25 *S.* Infantis strains were ESBL. Since 2010, an unusual increase in *Salmonella* cases has been reported in pediatric patients from various hospitals in Lima, including the National Institute for Child Health,[8] and in food samples.[24] *S.* Infantis is the third most frequent serovar identified in Peru and is associated with the consumption of contaminated eggs and meat products.[23] Some findings show that this serotype can persist and proliferate in the environment, as well as in hospitals, for a long period of time,[7,25,26] as observed in studies conducted in Brazil,[5] Ecuador,[6] and Argentina.[27]

Most cases of gastroenteritis caused by *Salmonella* do not require treatment with antibiotics unless patients are young infants, are malnourished, have systemic disease, or are immunocompromised. In these cases, cephalosporins or quinolones are commonly used.[28] Nonetheless, severe cases related to multidrug-resistant *S.* Infantis have been described in Peru, including cases of bacteremia.[29]

A previous report analyzing quinolone resistance in *Salmonella* sp. from the area of Lima, Peru, showed that 33 and 14% of *Salmonella* Typhi and non-Typhi, respectively, were resistant to NA, whereas 24% and 13% presented diminished susceptibility to CIP.[30] In our study, 100% of the isolates were NA resistant and 8% were resistant to CIP.

Usually, a single mutation in *gyrA* leads to resistance to NA but only decreased susceptibility to fluoroquinolones.[31] In fact, this is the most common scenario in *Salmonella* sp.[32,33] as the presence of additional mutations in quinolone-targets leading to high levels of CIP resistance seems to have a deleterious effect on bacterial fitness.[34] Despite this impaired fitness, isolates exhibiting resistance to CIP likely

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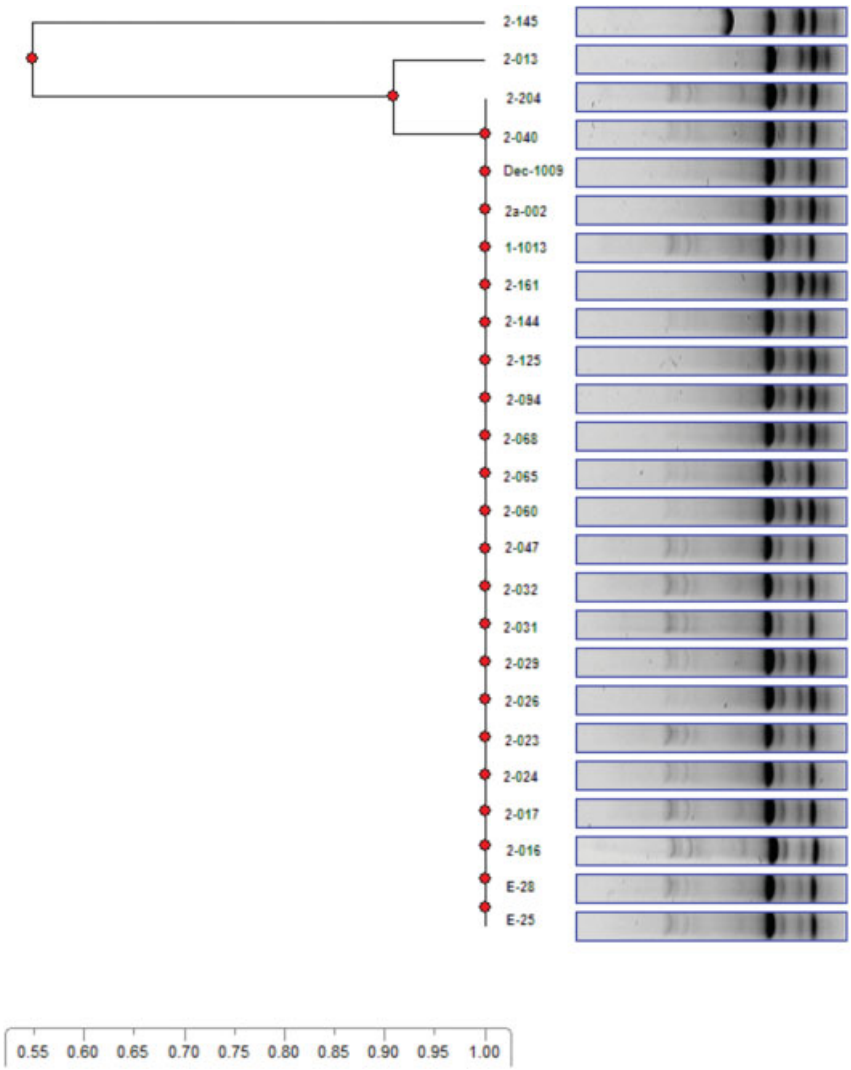

Fig. 2 *Salmonella sp.* similarity dendrogram: analysis of repetitive extragenic palindromic–polymerase chain reaction profiles using the unweighted pair group method with arithmetic mean analysis method shows that all S. Infantis carrying the bla$_{CTX\text{-}M\ 65}$ belong to the same clone, whereas isolate 2-103 does not possess the bla$_{CTX\text{-}M\ 65}$ presented an identity level of >90%. The *S.* Typhimurium 2-145 showed a fully unrelated pattern.

harbor additional target mutation, as was observed for highly fluoroquinolone-resistant S. Kentucky ST198-X1-SGI1 that has been disseminated worldwide.[35] Regarding other antimicrobial agents, studies in our geographical area have shown the presence of *S.* Typhimurium exhibiting resistance to CRO, CAZ, and amikacin, producing the bla$_{SHV\text{-}5}$.[9]

CTX-M group 9 (*bla*$_{CTX\text{-}M\ 65}$) was found in 24 of our *S.* infantis isolates with resistance to CTX and CRO and 4 out of 24 (17%) with decreased susceptibility to CAZ, characteristic of an isolate carrying the *bla*$_{CTX\text{-}M}$ gene.[36] Although *bla*$_{CTX\text{-}M\ 65}$ has been frequently found in neighboring countries[37] and has been observed in strains of *Escherichia coli* causing bacteremia in children[22] in Peru, to our knowledge, this is the first report in Peru of the *bla*$_{CTX\text{-}M\ 65}$ gene in *Salmonella* strains.

In Peru, the prevalence of *bla*$_{CTX\text{-}M\ 14}$, *bla*$_{CTX\text{-}M\ 24}$, *bla*$_{CTX\text{-}M\ 15}$, and *bla*$_{CTX\text{-}M\ 2}$ in *E. coli* has been reported as 45, 22, 22, and 11%, respectively.[38] Between July 2012 and January 2013, *bla*$_{CTX\text{-}M}$ was detected in four strains of *Salmonella* at the National Institute of Child Health in Lima, Peru.[39] These strains are contemporaneous with *Salmonella* ESBL strains found in this study, which suggests the presence of an outbreak in the city of Lima. This hypothesis is supported by the REP-PCR results, which showed the presence of an *S.* Infantis clone disseminated between two of the Hospitals included in the study. Furthermore, it is necessary to consider the distance of around 6 km between HEP and HNCH, which likely precludes the presence of common environments such as schools.

Between 2014 and 2015, 5 of 28 *Salmonella* strains isolated in Ecuador were clonally related to S. Infantis harboring a *bla*$_{CTX\text{-}M\ 65}$ gene.[6] Also, in 2005, strains of S. Infantis with an ESBL phenotype belonging to an outbreak were found in Argentina.[27] All these findings suggest that an epidemic clone of S. Infantis has spread to different countries in Latin America. Riccobono et al[40] studied the spread of the *bla*$_{CTX\text{-}M\ 65}$ gene, and the authors concluded it was because of a polyclonal spread of the Inc11 ST71 epidemic plasmid transferable to other enterobacteria by conjugation. Therefore, the presence of this epidemic plasmid in *S.* Infantis clone is highly probable.

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The S. Infantis 2-013 isolate, the only S. Infantis without ESBL pattern, showed a different REP-PCR profile. Nonetheless, the pattern was closely similar to exhibiting more than 90% identity and therefore could possibly be an "ancestral" non-ESBL isolate or it could have lost the resistance gene at some point.

In conclusion, this is the first report of an epidemic strain of *S. enterica* serovar Infantis carrying $bla_{\text{CTX-M 65}}$ in Peru. The data suggest that this strain is widely distributed in the area of Lima. Furthermore, indirect evidence (e.g. similarity of antibiotic resistance patterns or the presence of CTX-M 65) suggests the presence of multidrug-resistant ESBL carrying S. Infantis spreading in Latin America. Further studies on *S.* Infantis from nonhuman origin are needed in order to determine the possible acquisition of this pathogen. Moreover, new research should include samples from different Latin American countries to determine cross-border dissemination of a multidrug-resistant *S.* Infantis.

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Conflict of Interest
None declared.

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