Dissemination of a multidrug resistant CTX-M-65 producer *Salmonella enterica* serovar Infantis clone between marketed chicken meat and children.

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Running Title: *bla*$_{\text{CTX-M-65}}$-producing *S.* Infantis.

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Abstract

The objective of the present study was to characterize *Salmonella enterica* serovar Infantis isolated from chicken meat determining their clonal relationships with *S.* Infantis isolated from children with diarrhea. Fifteen meat-recovered *S.* Infantis were analyzed. Susceptibility levels to 14 antibacterial agents, the presence of ESBL and that of inducible plasmid-mediated AmpC (i-pAmpC) were determined by phenotypical methods. The presence of ESBL and pAmpC was confirmed by PCR, and detected ESBL-encoding genes were sequenced and their transferability tested by conjugation. The presence of *gyrA* mutations as well as Class 1 integrons was determined by PCR. Clonal relationships were established by REP-PCR and RAPD. In addition, 25 clinical isolates of *S.* Infantis were included in clonality studies. All meat-recovered *S.* Infantis were MDR, showing resistance to ampicillin, nitrofurans and quinolones, while none was resistant to azithromycin, ceftazidime or imipenem. ESBL ($bla_{\text{CTX-M-65}}$) and i-pAmpC (bla_{DHA}) were detected in 2 and 5 isolates respectively (in one case concomitantly), with $bla_{\text{CTX-M-65}}$ being transferable through conjugation. In addition, 1 isolate presented a bla_{SHV} gene. All isolates presented $D_{87}Y$ at GyrA, nalidixic acid active efflux pump and a Class 1 integron of ~1000 bp (*aadA1*). Clonal analysis showed that all isolates were related. Further they were identical to MDR $bla_{\text{CTX-M-65}}$-producing *S.* Infantis isolates causing children diarrhea in Lima. The dissemination of MDR $bla_{\text{CTX-M-65}}$-producing *S.* Infantis between marketed meat and children highlights a public health problem which needs be controlled at livestock level.

Keywords: ESBL; antibiotic resistance; foodborne diseases

1. Introduction

Non-Typhi *Salmonella* (NTS) are common inhabitants of the gut of poultry and other animals for food consumption, including cattle and pig (Threlfall, 2002), being a common contaminant of marketed meat (Arnedo-Pena *et al*., 2016; Khan *et al*., 2018; Ruiz-Roldán *et al*., 2018). NTS are also present in other sources such as eggs, fish or contaminated irrigation water (Martínez *et al*., 2017; Ruiz *et al*., 1999; Silva *et al*., 2014). These findings result in the food-chain being the most common route of acquisition of NTS infections (Threlfall, 2002).

Thus, NTS are a common cause of gastrointestinal disturbances such as diarrhea in both adult and infants (Cabrera *et al*., 2004; Granda *et al*., 2019). In addition, NTS have also been involved in extraintestinal infections such as local abscesses, bacteremia or meningitis, among others (Alonso *et al*., 2007; Mandomando *et al*., 2015; Molyneux *et al*., 2009; Sugimoto *et al*., 2017). While NTS diarrhea are described in all geographical areas and age-segments, extraintestinal NTS infections are of special relevance in low- and middle-income areas and in specific at-risk populations (e.g.: HIV or immunocompromised patients) (Chou *et al*., 2016; Mandomando *et al*., 2015; Molyneux *et al*., 2009).

NTS diarrhea is often self-limiting and does not require the use of antimicrobial agents, except in long-standing diarrhea, the presence of severe symptoms, young children (< 3 months), the presence of diverse comorbidities or in immunocompromised patients (Lübbert, 2016; Threlfall, 2002). However, extraintestinal cases are life-threating and may result in fatal outcomes or severe sequelae (Alonso *et al*., 2007; Ao *et al*., 2015; Chou *et al*., 2016; Mandomando *et al*., 2015; Molyneux *et al*., 2009).

Classically, NTS had been treated with antibiotics such as ampicillin, chloramphenicol or cotrimoxazole (van Duijkeren and Houwers, 2000). Nonetheless, since the 1990's, a rise

in the levels of NTS resistance to these antibiotics has been observed (Cabrera *et al*., 2004; Gallardo *et al*., 1999; Mandomando *et al*., 2015; Ruiz *et al*., 1999), and their role as first-line treatment has declined (Ao *et al*., 2015; Mandomando *et al*., 2015). Currently, when treatment of NTS causing diarrhea is needed, the antimicrobial agents most frequently used are fluoroquinolones or azithromycin (Cabrera *et al*., 2004; Lübbert, 2016), while NTS extraintestinal infections are often treated with fluoroquinolones or cephalosporins (Crump *et al*., 2015). Nonetheless, in the present century the spread of NTS exhibiting multidrug resistance (MDR) patterns, including resistance to fluoroquinolones, macrolides, 3rd and 4rd generation cephalosporins, carbapenems, fosfomycin, or even to polymyxins has been described (Carattoli *et al*., 2017; Granda *et al*., 2019; Hawkey *et al*., 2019; Quino *et al*., 2019).

The number of described NTS serotypes is higher than 2500, with a few being frequently described worldwide as a cause of human illness, and a high number being sporadically described as infecting patients. In this scenario, *Salmonella enterica* serovar Infantis ranks worldwide among the common NTS serotypes isolated as a cause of human disease, after those NTS belonging to serovars Typhimurium and Enteritidis (Lamas *et al*., 2018).

Since 2010, in Peru a series of diarrhea cases related to MDR *S.* Infantis have been detected both in Lima and other regions of the country (Gonzales-Escalante, 2015; Granda *et al*., 2019; Quino *et al*., 2019). Further, an increase of ESBL-carrying *S. enterica* isolates emerged in 2011 (Gonzales-Escalante, 2015).

In the present study a series of MDR *S.* Infantis isolated from meat samples were characterized and clonal relationships with *S.* Infantis isolated from children with diarrhea were established.

2. Material and Methods

2.1 Microorganisms

Fifteen non-duplicate *S.* Infantis isolated in August 2012 from fresh meat samples were included in the present study; Of these, 13 were from chicken meat, and the other 2 isolates were from beef and pork samples (Martínez-Puchol *et al*., 2020; Ruiz-Roldán *et al*., 2018).

Briefly, the samples included in the above-mentioned study were randomly bought in 5 traditional markets of 3 different areas, South, Center and North, of Lima (Peru), in a study designed to determine the burden of *Enterobacteriaceae* in meat samples (Ruiz-Roldán *et al*., 2018). Regarding samples containing *S.* Infantis, these were bought in 4 out of 5 sampling markets representing the 3 above-mentioned areas (Martínez-Puchol *et al*., 2020). In the previously mentioned study *Salmonella* strains were ~3% of the total microorganisms isolated (N=830) (Ruiz-Roldán *et al*., 2018).

In all cases the isolates were identified by biochemical tools and confirmed by *invA* gene amplification and sequencing (Barletta *et al*., 2013; Ruiz-Roldán *et al*., 2018). Furthermore, after recovery from frozen stock and prior to use, the microorganisms were reconfirmed as *S. enterica* by PCR amplification of the *16S rRNA* gene (Salazar de Vegas *et al*., 2006).

In addition, 27 *S.* Infantis from other sources were included in the analysis of clonal relationships (see section 2.9 - Clonal determinations); when available, antibiotic resistance levels and molecular data of these isolates were also compared with the results obtained analyzing food-related *S.* Infantis.

2.2 Antibiotic susceptibility

The antibiotic susceptibility to ampicillin (β-lactam - βL), amoxicillin plus clavulanic acid (β-lactam plus β-lactamase inhibitor - βL-I), cefotaxime, ceftazidime (cephalosporins - CPH), chloramphenicol (phenicol - CHL), cotrimoxazole (Antifolate - AF), gentamicin (aminoglycoside - AMG), imipenem (carbapenem - CBP), nalidixic acid, ciprofloxacin (quinolones - Q), azithromycin (macrolides - MC) and tetracycline (tetracyclines - TC) was established by disk diffusion in accordance with CLSI guidelines (CLSI, 2018).

The nalidixic acid breakpoint considered was that of *Enterobacteriaceae*. Regarding azithromycin, as no generic *S. enterica* clinical susceptibility cut-off has been established, the disk diameters were interpreted following the CLSI criteria for *Salmonella enterica* serovar Typhi (CLSI, 2018). Meanwhile, data on furazolidone and nitrofurantoin (nitrofurans - NF) resistance have previously been reported (Martínez-Puchol *et al*., 2020).

Multidrug resistance (MDR) was defined as a resistance to at least 1 antibacterial agent from 3 unrelated families. In all cases, the *Escherichia coli* ATCC 25922 was used as Minimal Inhibitory Concentration (MIC) quality control strain.

2.3 Phenotypic detection of ESBL

In the food-recovered isolates the presence of ESBL was phenotypically established in all isolates, irrespective of the pattern of susceptibility/resistance to CPH, by double disk methodology (Palma *et al*., 2017; Ruiz-Roldán *et al*., 2018). Briefly, cefotaxime and ceftazidime disks were placed at 20 mm (center-center distance) of an amoxicillin plus clavulanic acid disk. For a representative image see Lezameta *et al*. (2010).

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148 **2.4 Phenotypic detection of inducible plasmidic AmpC**

149 The presence of inducible plasmidic AmpC (i-pAmpC) was determined in all isolates,
150 irrespective of the susceptibility/resistance to CPH, using the double disk test induction
151 with imipenem and ceftazidime as described previously (Ruiz-Roldán *et al*., 2018).
152 Briefly, ceftazidime and amoxicillin plus clavulanic acid disks were placed at 20 mm
153 (center-center distance). For a representative image see Del Valle Martinez Rojas (2009).

154

155 **2.5 Molecular detection of β-Lactamases**

156 The presence of *bla*$_{\text{CARB}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{OXA-1}}$-like, *bla*$_{\text{OXA-2}}$-like and *bla*$_{\text{OXA-5}}$-like
157 was determined by Polymerase Chain Reaction (PCR) in all the isolates. Meanwhile, the
158 presence of *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{DHA}}$ was determined by PCR in those isolates displaying a
159 phenotype compatible with the presence of ESBL and i-pAmpC, respectively.

160 The strategy to amplify *bla*$_{\text{CTX-M}}$ was a preliminary screening with *bla*$_{\text{CTX-M}}$ universal
161 primers followed, in positive isolates, by the use of 5 pairs of primers able to amplify
162 different *bla*$_{\text{CTX-M}}$ groups (*bla*$_{\text{CTX-M-1, 2, 8, 9, 10}}$ - like) as described elsewhere (Palma *et al*.,
163 2017). In all cases the primers and amplification conditions have been described
164 previously (Palma *et al*., 2017) (Table 1).

165 All PCR products were resolved in a 2% agarose gel which was stained with Sybr Safe
166 (Invitrogen, Carlsbad, USA). The amplified products were purified (Gel Extraction Kit,
167 Omega Bio-tek Norcross, GA, United States) and sequenced (Beckman Coulter; Takeley,
168 United Kingdom).

169

2.6 ESBL transferability

The transferability of detected ESBL was determined by conjugation in Luria-Bertani broth (Conda, Madrid, Spain) using the azide-resistant *E. coli* J53 and Mueller-Hinton supplemented with sodium azide (150 µg/mL) and cefotaxime (10 µg/mL) following the methodology previously described (Pons *et al*., 2015).

2.7 Mechanisms of quinolone resistance

The presence of mutations in the *gyrA* gene was determined by PCR and sequencing as previously described (Vila *et al*., 1995) (Table 1). In addition, the MIC of nalidixic acid and ciprofloxacin was performed in the presence and absence of 20 µg/mL of Phenyl-Arginyl-β-Naphtylamide (PAβN). The PAβN effect on the MIC levels was considered when $MIC_I/MIC_{PA\beta N} > 2$ (Palma *et al*., 2017).

2.8 Class 1 Integron

The presence of Class 1 integrons was established by PCR following the procedures previously described (Levesque and Roy, 1993) (Table 1). The amplified products were recovered and sequenced as above.

2.9 Clonal determinations

The clonal relationships between the *S.* Infantis isolates was established by two different typing-PCR: Repetitive Extragenic Palindromic (REP)-PCR and Random Amplified Polymorphic DNA (RAPD). In both cases the techniques were performed following methodologies described previously (Granda *et al*., 2019; Lin *et al*., 1996).

As indicated above, in addition to the isolates recovered from food samples, 25 *S.* Infantis previously reported as a cause of children's diarrhea in Lima, from which data on antibiotic susceptibility and presence of extended-spectrum β-Lactamases (ESBL) were available, were included in the analysis. Two other *S.* Infantis, kindly provided by Dr. Mandomando (CISM, Manhiça, Mozambique) and by Dr. Garcia (UPCH, Lima Peru), isolated as a cause of infantile diarrhea in Mozambique and from a bacteremia in Peru respectively, were also included in the clonal analysis. In addition, *Salmonella enterica* belonging to other serotypes were also used as controls.

RAPD and REP-PCR profiles were analyzed using the fingerprinting software GelJ (Heras *et al.*, 2015). Phylogenetic trees were constructed using the Dice coefficient with clustering by the unweighted pair-group method with arithmetic mean (UPGMA) with a 1% tolerance in band position differences. The isolates were considered to belong to the same epidemiological group when the profiles showed ≥80% of homology.

2.10 Ethical issues

The study was approved by the Ethical Review Board of the Universidad Peruana Cayetano Heredia (UPCH).

3. Results

Food-recovered *Salmonella* were identified through phenotypic and genotypic methods, with a full concordance.

All food-recovered *S.* Infantis displayed high levels of antibiotic resistance irrespective of the origin of the sample (chicken, pork or beef). Furthermore, all isolates were MDR displaying resistance to at least 4 different antibacterial agent families. Eight different antibiotic resistance patterns were observed, with the most common antibacterial agent resistance profile being Q, NF, TC, βL (4 isolates) followed by Q, AF, NF, TC, βL (3 isolates) and Q, CHL, AF, NF, TC, βL and Q, NF, βL, βL-I (2 isolates each), with all the remaining antibiotic-resistant profiles accounting for 1 isolate each (Table 2). All 8 antibiotic resistance patterns were classified as MDR, showing resistance to 4 to 8 different antibiotic families.

The highest levels of resistance were those to ampicillin, furazolidone, nitrofurantoin, nalidixic acid and ciprofloxacin with 100% of the isolates exhibiting a resistance phenotype, followed by tetracycline (13 isolates, 86.7%). Meanwhile, no isolate was resistant to azithromycin, ceftazidime, or imipenem and only 2 showed gentamicin or cefotaxime resistance (Table 3).

The MIC to quinolones showed that all isolates displayed nalidixic acid MIC levels of >256 µg/mL with a ciprofloxacin MIC of 1 µg/mL. In all cases, resistance to nalidixic acid was associated with the presence of a $D_{87}Y$ amino acid change in GyrA plus an active efflux pump. In 3 and 11 nalidixic acid-resistant isolates the use of PAβN resulted in decreases from >256 µg/mL to 64 µg/mL and to 32 µg/mL respectively ($MIC_I/MIC_{PA\beta N}$ > 4) remaining classified as nalidixic acid-resistant, while in the last isolate the nalidixic acid MIC decreased to 16 µg/mL ($MIC_I/MIC_{PA\beta N}$ > 16), just under the CLSI resistance breakpoint.

The search for ESBL and i-pAmpC showed the presence of 1 isolate carrying an ESBL, 4 carrying a i-pAmpC and 1 carrying both ESBL and i-pAmpC. In addition, in 1 case (concomitantly with a i-pAmpC) the presence of a *bla*$_{SHV}$ was detected. No βL resistance mechanism was detected in 9 βL-resistant isolates. When the ESBL and i-pAmpC were determined the results showed the presence of *bla*$_{CTX\text{-}M\text{-}65}$ (ESBL) and *bla*$_{DHA}$ (i-pAmpC) (Table 3). Of note, the presence of a positive result for a specific *bla*$_{CTX\text{-}M}$ group does not preclude the absence of another; the presence of all 5 *bla*$_{CTX\text{-}M}$ groups sought was performed in all isolates in which the presence of a *bla*$_{CTX\text{-}M}$ was detected irrespective of the detection of the presence of a group. Of note, the 2 *bla*$_{CTX\text{-}M\text{-}65}$-carrying isolates were those exhibiting resistance to cefotaxime. Regarding horizontal transferability, the conjugation assays showed the transferability of *bla*$_{CTX\text{-}M\text{-}65}$.

Finally, all isolates presented a Class 1 integron of c. 1000 bp, which contained an *aadA1* gene.

Both the REP-PCR and the RAPD analyses showed visual identical band-pattern in all food-related *S.* Infantis, which was confirmed by dendrogram construction (minimal RAPD identity degree: 100%, minimal REP-PCR identity degree >94%) being therefore considered as belonging to the same clone (data not shown). Furthermore, the incorporation of diarrhea-related *S.* Infantis to the analysis highlighted that these isolates belonged to the same clone. The bacteremic isolate incorporated in the analysis showed a distinctive band pattern, being then considered as belonging to another clone. In all cases the external controls showed different band-patterns, irrespective of the *Salmonella* serotype (data not shown).

4. Discussion

Antibiotic resistance is an emerging threat which affects most clinically relevant microorganisms. This problem is of special concern when food-transmissible pathogens are affected, because this may easily result in large difficult to treat outbreaks (Plumb *et al*., 2019). The presence of antibiotic-resistant microorganisms in marketed meat may be related to different factors, but the prophylactic, therapeutic or grown-promoter use of antibiotics during animal production is by far the most relevant cause acting as a powerful evolutionary force that selects antibiotic-resistant microorganisms (Angulo *et al*., 2000). In this context, a series of *S*. Infantis were recovered from meat samples in the area of Lima, mostly from chicken samples, but also from other meat sources.

Identification was done by both phenotypic and genotypic tools. Phenotypic approaches, such as biochemical tools, have the advantage to become accessible on low-income areas, providing of useful results. Nevertheless, these techniques need for an additional culture, and then final results are delayed. Genotypic tools, such as *invA* detection, while unaffordable in a series of low-income areas, provide of rapid and reliable results (Barletta *et al*., 2013). Of note, the amplification and sequence of the *16S rRNA* gene, while time consuming, may provide of data to resolve discrepancies between identification approaches (Wang *et al*., 2006), providing an accurate identification.

In *S. enterica*, the presence of the phenotype of nalidixic acid resistance of high level, and ciprofloxacin resistance of low level is a frequent event, being concordant with the presence of a single amino acid substitution in GyrA and a basal PAβN-inhibitible efflux pump activity (Kim *et al*., 2011; Merino *et al*., 2007). Furthermore, our results fully agree with the previously described more efficient extrusion of nalidixic acid than that of ciprofloxacin by *Enterobacteriales* PAβN-inhibitible efflux pump (Sáenz *et al*., 2004). None of the present isolates showed resistance of high level to ciprofloxacin.

Despite the description of successful *S. enterica* clones expressing resistance of high level to fluoroquinolones such as ciprofloxacin (Le Hello *et al.*, 2013), it has been suggested that as a general rule the presence of additional quinolone-target alterations, be able to result in high MIC levels of fluoroquinolones, also have a strong impact on *Salmonella* fitness (Fàbrega *et al*., 2014). Regarding the amino acid substitution $D_{87}Y$, its presence in *Salmonella enterica* has been previously reported (Cabrera *et al*., 2004), including the detection in *S.* Infantis recovered in Peru from both human and meat samples (Quino *et al*., 2019; Vallejos-Sánchez *et al*., 2019). In fact, while in other *Enterobacteriales* as well as other Gram-negative bacteria the first point mutation usually arises on position 83 (*E. coli* or *Salmonella* numeration) (Ruiz, 2003; Vila *et al*., 1995), in *Salmonella* the presence of isolates presenting position 87 as first point mutation seems to be especially frequent (Cabrera *et al*., 2004; Quino *et al*., 2019; Ruiz *et al*., 1999). Of note, the use of the *S. enterica* CLSI MIC breakpoints for ciprofloxacin before 2012 would have resulted in discordant nalidixic acid and ciprofloxacin susceptibility patterns, as reported in a series of studies developed previous to this change (Bertrand *et al*., 2006; Cabrera *et al*., 2004; Girish *et al*., 2013; Pérez-Moreno *et al*., 2013; Ruiz *et al*., 1999).

The presence of isolates with and without $bla_{\text{CTX-M-65}}$ and/or bla_{DHA} may be interpreted as the presence of different plasmid profiles either related to the acquisition or the loss of plasmid content. In this line, previous studies have shown the presence of $bla_{\text{CTX-M-65}}$ within megaplasmids (125 - >300 Kb) (Riccobono *et al*., 2015; Silva *et al*., 2017; Tate *et al*., 2017; Vallejos-Sánchez *et al*., 2019). Further, an in-silico comparison of plasmids p-F219 (~321 Kb; GenBank access CP038508) and pFSIS1502916 (~322 Kb; Genbank access: CP016409), from a Peruvian and USA *S.* Infantis isolates respectively, showed ~99% of identity, despite being reported as belonging to different Inc groups (IncI - p-F219; IncFI1 - pFSIS1502916) (Tate *et al*., 2017; Vallejos-Sánchez *et al*., 2019).

310 Interestingly, while in the studies of Granda *et al* (2019) as well as Quino *et al* (2019)
311 almost all the isolates analyzed possessed the *bla*$_{\text{CTX-M-65}}$ gene, in the present meat
312 samples this gene was present in the 2/15 (13.3%) of the isolates.

313 Previous data by Riccobono *et al*. (2015) showed that when the *bla*$_{\text{CTX-M-65}}$ plasmid
314 spreading in Bolivia is transferred to *Salmonella enterica*, it tends to be lost in the absence
315 of selective pressure. While the analyzed Bolivian plasmid is different to p-F219, reported
316 in Peru, both plasmids have ~40% of identity (Riccobono *et al*., 2015; Vallejos-Sánchez
317 *et al*., 2019). In the absence of further data, it can be proposed that a similar *Salmonella*
318 instability may take place in *bla*$_{\text{CTX-M-65}}$-carrying plasmids spreading in Peru; the heavy
319 level of use of CPH in both hospital and community settings likely underlie the
320 differences in the prevalence of *bla*$_{\text{CTX-M-65}}$ in meat and humans. These findings
321 suggesting the presence of a widely spreading conjugative *bla*$_{\text{CTX-M-65}}$-borne
322 megaplasmid among present isolates

323 Although the presence of the most common β-lactamases was sought, the βL resistance
324 mechanisms remained to be established in 9 out of 15 *S.* Infantis. This finding suggests
325 the presence of an uncommon mechanism of βL resistance (either β-lactamase or not),
326 and has also been observed in a βL-resistant *S.* Infantis isolated as a cause of children's
327 diarrhea in the area of Lima in which no specific βL resistance mechanism was identified
328 (Granda *et al*., 2019). Meanwhile the resistance to cefotaxime observed in the 2 CTX-M-
329 65-producer isolates is in agreement with the high cefotaxime hydrolysis efficiency of
330 CTX-M (Rossolini *et al*., 2008).

331 The presence of *bla*$_{\text{DHA}}$ has been scarcely reported in *S. enterica* (Pérez-Moreno *et al*.,
332 2013). While very few data on the prevalence of i-pAmpC in Peru are available, with
333 descriptions in *E. coli* recovered from chicken and beef samples (Ruiz-Roldán *et al*.,
334 2018), a high rate of pAmpC producing *Enterobacteriaceae* acquisition has been

observed in travelers returning from Peru (Lorme *et al*., 2018). Meanwhile, *bla*$_{\text{CTX-M-65}}$ has been previously reported as being frequent in the area, including its detection in *S.* Infantis, being described in Peru and in neighboring countries, such as Bolivia, Chile or Ecuador (Cartelle Gestal *et al*., 2016; Fuentes-Castillo *et al*., 2019; Riccobono *et al*., 2015; Sánchez-Salazar *et al*., 2020). Regarding Peru, the *bla*$_{\text{CTX-M-65}}$ gene has been previously described in bacteremic *E. coli*, and also in *S.* Infantis recovered from human and meat samples (Granda *et al*., 2019; Palma *et al*., 2017; Quino *et al*., 2019; Tate *et al*., 2017; Vallejos-Sánchez *et al*., 2019). In fact, during the last years there has been continuous isolation of MDR and ESBL-producer *S.* Infantis in Lima, as well as in other Peruvian areas as a cause of diarrhea or other human infections, mostly affecting children (Gonzales-Escalante, 2015; Granda *et al*., 2019; Quino *et al*., 2019).

In this scenario the isolation of food-carried *S.* Infantis also displaying an MDR phenotype and some carrying *bla*$_{\text{CTX-M-65}}$ was suggestive of the presence of a possible clonal relationship. Thus, 25 *S.* Infantis clinical isolates from Granda *et al* (2019) study were compared with present meat recovered *S.* Infantis. Twenty-four out of 25 clinical isolates presented the *bla*$_{\text{CTX-M-65}}$ gene, showing a resistance pattern (Q, CHL, AF, NF, TC, βL, CPH) compatible with present pattern VI, with 10 isolates also presenting resistance to azithromycin and without data of resistance to AMG (Granda *et al*. 2019). REP-PCR and RAPD analysis confirmed clonal relationship, highlighting the transference of *S.* Infantis between food samples and humans resulting in the development of infectious diarrhea. Furthermore, in addition to the above-mentioned frequent description of diarrhea cases related to *S.* Infantis in other Peruvian region, the present data open the door to the possible presence of at least a nationwide disseminated highly resistant clone. Furthermore, the presence of MDR ESBL-producer *S.* Infantis in marketed bushmeat in Peruvian Amazonia has just been highlighted (Maguiña in press).

In the absence of molecular comparisons, this finding strongly suggesting the dissemination of this *S.* Infantis clone out of human and poultry borders. In this sense, the presence of reports showing the presence of *bla*$_{\text{CTX-M-65}}$-producer *S.* Infantis in neighboring countries such as Chile or Ecuador is of note. Thus, in Chile a *bla*$_{\text{CTX-M-65}}$-producer *S.* Infantis was recovered in a wild bird admitted to a wildlife rescue and rehabilitation center in Chile (Fuentes-Castillo *et al.*, 2019), while in Ecuador *bla*$_{\text{CTX-M-65}}$-producer *S.* Infantis have been isolated from patients, farm-environment, farm animals or animal-feed (Cartelle Gestal *et al.*, 2016; Sánchez-Salazar *et al.*, 2020). In the absence of molecular determinations, it cannot be excluded that these isolates, together with those recovered in Peru, belong to a common *S.* Infantis clone disseminated throughout different countries.

The transference of this *S.* Infantis clone between food and humans may be related to different situations, including poor cooking, or indirect transmission related to food manipulation and/or poor hygienic practices. In this sense, the presence of *S.* Infantis from the same clone in beef and pork samples may be related to either the true presence of the microorganism in the animal environment, or cross-contamination from the slaughterhouse to retail stalls.

While most *Salmonella* infections are self-limiting and do not require the use of antibacterial agents, the presence of concomitant comorbidities, the severity, long duration, the presence of the infection in newborns or the elderly or some other specific circumstances may require the use of antibacterial agents (Lübbert, 2016; Threlfall, 2002). The present meat-recovered isolates displayed high levels of resistance to almost all antibiotics, usually administered in the treatment of gastrointestinal *Salmonella* infections, with the exception of azithromycin, highlighting the need to preserve the activity of this antibacterial agent, and strongly alerting about the need to restrict the use

385 of macrolides outside of human health. Of concern, 40% of clinical isolates were resistant
386 to azithromycin (Granda *et al*., 2019), with this finding probably being related to the
387 antibiotic pressure exerted on clinical settings and community.

388 In the present study no data about the slaughterhouse origin of samples was recovered,
389 and therefore being unknown whether the chickens were grown on the same or on
390 different farms, thereby limiting the knowledge about the real dissemination of this *S.*
391 Infantis clone in the farm environment. Furthermore, this lack of knowledge does not
392 allow the risk of cross-contamination in the slaughterhouse to be established.
393 Nonetheless, the wide distribution of samples analyzed in Lima, strongly suggests the
394 presence of chicken meat from different origins and slaughterhouses, and subsequently
395 the relevant presence of this *S.* Infantis clone on chicken farms. Of note, Sanchez-Salazar
396 *et al*, (2020) described the presence of *S.* Infantis carrying the $bla_{\text{CTX-M-65}}$ gene in poultry
397 feed from Ecuadorian farms. This possibility remains to be analyzed in Peruvian farms,
398 but suggests the possible transmission of ESBL-producer *S.* Infantis from poultry feed
399 through marketed chicken meat to humans.

400 The widespreading of CTX-M-65-producer *S.* Infantis in Peru, which has been isolated
401 in distant areas, from a variety of sources, including marketed chicken meat and human
402 infections, and suggested in bushmeat, shows the need to develop further studies to
403 expand present clonal studies, determine the relationships between these isolates and
404 establish measures to break dissemination pathways.

405 In summary, the present study demonstrates the food-origin of the diarrhea-related *S.*
406 Infantis clone which is spreading in Lima. On the other hand, the description of $bla_{\text{CTX-M-65}}$-possessing *S.* Infantis in both other Peruvian regions and neighboring countries
408 suggests extensive spreading of this clone. Present data strongly suggest the *in vivo* farm-
409 selection of MDR microorganisms and warms about on the imperious necessity to

implement effective non-antibiotic human/animal-pathogen control pathways in order to reduce the use of antimicrobial agents in food-production.

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7. Declarations of interest

None

8. Author Contributions

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Joaquim Ruiz: Conceptualization, Formal analysis, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Funding acquisition.

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686

687

688 **Table 1: Primers used in this study**

689

Gene	Sequence	Size (bp)	ºC [1]	Cycles	Reference
bla$_{CTX-M}$	CGA TGT GCA GTA CCA GTA A TTA GTG ACC AGA ATC AGC GG	585	53	35	Palma *et al*., 2017
bla$_{CTX-M-1G}$	GTT ACA ATG TGT GAG AAG CAG CCG TTT CCG CTA TTA CAA	1041	50	35	Palma *et al*., 2017
bla$_{CTX-M-2G}$	ATG ATG ACT CAG AGC ATT CG TCA GAA ACC GTG GGT TAC	876	52	35	Palma *et al*., 2017
bla$_{CTX-M-8G}$	TGA TGA GAC ATC GCG TTA AG TAA CCG TCG GTG ACG ATT TT	875	52	35	Palma *et al*., 2017
bla$_{CTX-M-10G}$	CCG CGC TAC ACT TTG TGG C TTA CAA ACC GTT GGT GAC G	944	55	35	Palma *et al*., 2017
bla$_{CTX-9/14G}$	TGA CCG TAT TGG GAG TTT CAG GAT TTA TTC AAC AAA ACC AG	917	56	35	Palma *et al*., 2017
bla$_{CARB}$-like	AAT GGC AAT CAG CGC TTC CC GGG GCT TGA TGC TCA CTC CA	586	56	30	Palma *et al*., 2017
bla$_{OXA-1}$-like	ACC AGA TTC AAC TTT CAA TCT TGG CTT TTA TGC TTG	598	55	30	Palma *et al*., 2017
bla$_{SHV}$-like	ATG CGT TAT ATT CGC CTG TG TTA GCG TTG CCA GTG CTC G	841	55	30	Palma *et al*., 2017
bla $_{OXA-5/7}$-like	TAT ATT CCA GCA TCA ACA TT ATG ATG CCC TCA CTT GCC AT	605	55	30	Palma *et al*., 2017
bla $_{OXA-2/3}$-like	CGA TAG TTG TGG CAG ACG AA CCA CTC AAC CCA TCC TAC CC	550	55	30	Palma *et al*., 2017
bla$_{DHA}$	AAC TTT CAC AGG TGT GCT GGG T CCG TAC GCA TAC TGG CTT TGC	405	64	30	Palma *et al*., 2017
bla$_{TEM}$-like	ATT CTT GAA GAC GAA AGG GC ACG CTC AGT GGA ACG AAA AC	1150	55	30	Palma *et al*., 2017
gyrA	AAA TCT GCC CGT GTC GTT GGT GCC ATA CCT ACG GCG ATA CC	343	55	30	Vila *et al*., 1995
RAPD [2]	CCG AAG CTG C	---	35	39 [3]	Lin *et al*., 1996
REP-PCR [2]	GCG CCG ICA TGC GGC ATT	---	40	30	Granda *et al*., 2019
Class 1 integron	GGC ATC CAA GCA GCA AG AAG CAG ACT TGA CCT GA	Variable	59	30	Levesque and Roy, 1993

690 bp: base pair

691

692 [1] Annealing Temperature

693 [2] Both RAPD and REP-PCR are typing PCR, therefore do not amplify any specific gene
694 and are expected to obtain a variable number of bands with different sizes

695 [3] The program used was: 4 cycles of 94ºC for 4 min, 35ºC for 4 min, and 72ºC for 4 min;
696 30 cycles of 94ºC for 30 s, 35ºC for 1 min, and 72ºC for 2 min; and final cycle of 72°C
697 for 5 min was added for final extension (Lin *et al*., 1996).

698

699 **Table 2: Antibiotic resistance profiles of meat-recovered *S.* Infantis**

700

		Antibiotic resistance [1]											
Profile	**N**	**βL**	**βL-I**	**CPH**	**NF**	**Q**	**AF**	**CHL**	**AMG**	**TC**	**MDR**	**ESBL**	**i-pAmpC**
I	**4**	■			■	■				■	**Yes**	**-**	**-**
II	**3**	■			■	■	■			■	**Yes**	**-**	**-**
III	**2**	■			■	■	■	■		■	**Yes**	**-**	**-**
IV	**2**	■	■		■	■					**Yes**	**-**	**Yes**
V	**1**	■	■	■	■	■	■	■	■	■	**Yes**	**Yes**	**Yes**
VI	**1**	■		■	■	■	■	■	■	■	**Yes**	**Yes**	**-**
VII	**1**	■	■		■	■				■	**Yes**	**-**	**Yes**
VIII	**1**	■	■		■	■	■			■	**Yes**	**-**	**Yes**

701

702 N: Number of isolates; MDR: Multidrug resistance; ESBL: Extended-spectrum β-
703 lactamase; i-pAmpC: inducible plasmid mediated AmpC.

704 βL: β-lactams; βL-I; β-lactams plus β-lactamase inhibitor; CPH: Cephalosporins; NF:
705 Nitrofurans; Q: Quinolones; CHL: Phenicols; AF: Antifolate; AMG: Aminoglycosides;
706 TC: Tetracyclines.

707 [1] At least to one antibiotic of the antibiotic-families considered. No isolate was resistant
708 to carbapenems or macrolides.

709

Table 3: Antibiotic susceptibility and mechanisms of antibiotic resistance of meat-recovered *S.* Infantis

																		β-lactamases [1]					Class 1 integron	
Strain	**Source**	**Area**	NAL	CIP	C	SXT	FX	NIT	GM	AZM	IMP	TE	AMP	AMC	CAZ	CTX	***gyrA***	***bla*$_{SHV}$**	**ESBL**	***bla*$_{CTX-M}$**	**i-pAmpC**	***bla*$_{DHA}$**	**size (bp)**	**cassette**
S1	Chicken	South	R	R	R	R	R	R	S	S	S	R	R	S	S	S	$D_{87}Y$	-	-	-	-	-	~1000	aadA1
S2	Chicken	North	R	R	R	R	R	R	R	S	S	R	R	R	S	R	$D_{87}Y$	-	+	***bla*$_{CTX-M-65}$**	+	***bla*$_{DHA}$**	~1000	aadA1
S3	Chicken	Center	R	R	S	S	R	R	S	S	S	R	R	S	S	S	$D_{87}Y$	-	-	-	-	-	~1000	aadA1
S4	Chicken	Center	R	R	R	R	R	R	S	S	S	R	R	S	S	S	$D_{87}Y$	-	-	-	-	-	~1000	aadA1
S5	Chicken	North	R	R	S	R	R	R	S	S	S	R	R	S	S	R	$D_{87}Y$	-	-	-	-	-	~1000	aadA1
S6	Chicken	North	R	R	S	S	R	R	S	S	S	R	R	S	S	S	$D_{87}Y$	-	-	-	-	-	~1000	aadA1
S7	Chicken	North	R	R	R	R	R	R	R	S	S	R	R	S	S	S	$D_{87}Y$	-	+	***bla*$_{CTX-M-65}$**	-	-	~1000	aadA1
S8	Chicken	North	R	R	S	S	R	R	S	S	S	R	R	S	S	S	$D_{87}Y$	-	-	-		-	~1000	aadA1
S9	Pork	South	R	R	S	S	R	R	S	S	S	R	R	S	S	S	$D_{87}Y$	-	-	-		-	~1000	aadA1
S11	Chicken	Center	R	R	S	S	R	R	S	S	S	S	R	R	S	S	$D_{87}Y$	-	-	-	+	***bla*$_{DHA}$**	~1000	aadA1
S12	Beef	Center	R	R	S	S	R	R	S	S	S	R	R	R	S	S	$D_{87}Y$	-	-	-	+	***bla*$_{DHA}$**	~1000	aadA1
S16	Chicken	South	R	R	S	R	R	R	S	S	S	R	R	R	S	S	$D_{87}Y$	+	-	-	+	***bla*$_{DHA}$**	~1000	aadA1
S17	Chicken	Center	R	R	S	R	R	R	S	S	S	R	R	S	S	S	$D_{87}Y$	-	-	-	-	-	~1000	aadA1
S18	Chicken	Center	R	R	S	R	R	R	S	S	S	R	R	S	S	S	$D_{87}Y$	-	-	-	-	-	~1000	aadA1
S19	Chicken	Center	R	R	S	S	R	R	S	S	S	S	R	R	S	S	$D_{87}Y$	-	-	-	+	***bla*$_{DHA}$**	~1000	aadA1

NAL: Nalidixic acid; CIP: Ciprofloxacin; C: Chloramphenicol; SXT: Cotrimoxazol; FX: Furazolidone; NIT: Nitrofurantoin; GM: Gentamicin; AZM: Azithromycin; IMP: Imipenem; TE: Tetracycline; AMP: Ampicillin; AMC: Amoxicillin plus clavulanic acid; CAZ: Ceftazidime; CTX: Cefotaxime; ESBL: Extended-spectrum β-lactamase; i-pAmpC: inducible plasmid mediated AmpC. bp: base pair.

[1] No *bla*$_{CARB}$, *bla*$_{TEM}$, *bla*$_{OXA-1-like}$, *bla*$_{OXA2/3-like}$ or *bla*$_{OXA5/7-like}$ was detected.

717