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Corresponding Author: Dr. Francesco A. Luzzaro,

Corresponding Author's Institution:

First Author: Luigi Principe, MS

Order of Authors: Luigi Principe, MS; Valentina Viaggi, MS; Mathieu Clement, MS; Elisa Meroni, MS; Beatrice Pini, MD; Andrea Endimiani, MD; Francesco A. Luzzaro

To S. Stefani August 23, 2018
Editor in Chief
Journal of Global Antimicrobial Resistance

Enclosed please find the revised manuscript now entitled "*Emergence of CTX-M-1-producing Salmonella enterica serovar Napoli: a novel "enzyme-pathogen association" in the Italian ESBL endemic context"*, which I am resubmitting for possible publication as a *Letter to the Editor* in *Journal of Global Antimicrobial Resistance*.

In this manuscript, we describe the first isolation of CTX-M-1-producing *Salmonella enterica* serovar Napoli from a clinical sample. This finding is strictly related to the Italian ESBL endemic context, in which uncommon associations enzyme-pathogen can be discovered. This serovar, relatively uncommon in Europe, is among the most common serovars causing infection in Italy. Moreover, *Salmonella* Napoli, especially in children, is often associated to invasive infections. The acquisition of resistance determinants by a so-diffused pathogen is a major cause of concern, making difficult the treatment of related infections.

The manuscript has been revised according to reviewer's comments and suggestions. The final version has been seen and approved by all the authors. The material is original, unpublished, and has not been simultaneously submitted to another medical journal.

Yours sincerely,

Francesco Luzzaro

Please, address the correspondence concerning this manuscript to:
Francesco Luzzaro, M.D.
Mailing address: Microbiology and Virology Unit, A. Manzoni Hospital
Via dell'Eremo, 9/11 - 23900 - Lecco, Italy
Phone: +39 0341 489630
Fax: +39 0341 489601
E-mail: f.luzzaro@asst-lecco.it

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"First description of CTX-M-1-producing Salmonella enterica serovar Napoli: a new association enzyme-pathogen in the Italian ESBL endemic context"

Point-by-point response

Reviewer #1

The authors describe a case of salmonellosis with a CTX-M-1 producing strain of Salmonella Napoli in Italy. It is a pity that not more was done to determine the properties of the blaCTX-M-1 carrying plasmid.

R: Additional information about the plasmid carrying the *bla*$_{\text{CTX-M-1}}$ gene has been added in the text (see also response to point #4).

All comments are mentioned below.

1. Line 35: Please add the number of cases in this period of time in parenthesis.
R: The number of cases has been added as requested.

2. Line 34-38: Please shift this paragraph to the discussion below (e.g. line 60).
R: The paragraph has been moved to the discussion.

3. Line 50: Which method was used to determine the serovar?
R: The method used to determine the serovar has been specified as requested.

4. Line 59: How was the presence of only one plasmid confirmed without conjugation and plasmid isolation or plasmid number/size determination by S1 restriction and PFGE? PBRT just confirmed the presence of a replicon indicating the presence of at least one plasmid. Please rephrase.
R: The sentence has been rephrased adding new information as follows: "Conjugation experiments using *E. coli* J53 recipient (rifampicin-resistant) performed at 37°C, and selecting on MacConkey plates containing ampicillin and rifampicin (both 50 µg/ml), were successful (efficiency, 4 x 10^{-3}) and confirmed the presence of an IncI1 plasmid along with *bla*$_{\text{CTX-M-1}}$".

5. Line 69-71: An acquisition of the ESBL gene within the gut from other Enterobacteriaceae could be possible - where the stool samples tested for presence of other ESBL-producing Enterobacteriaceae?
R: We agree with this consideration. The acquisition of the ESBL gene within the gut from other *Enterobacteriaceae* could be possible. However, only selected stool pathogens (i.e., *Salmonella*, *Shigella*, and *Campylobacter* species) were searched for (according to our diagnostic routine methods).

6. Line 71-74: Please delete this sentence - it is redundant.
R: The sentence has been deleted as requested.

7. Line 78: Please discuss: Are 3rd generation cephalosporins used as treatment option for salmonellosis in children?
R: The possible role of third-generation cephalosporins for treating salmonellosis in children has been briefly discussed as requested.

8. Line 79-82: The role of IncI1 finding is not discussed - what is its relevance in the "Italian context"?
R: The role of IncI1 and the relevance in the Italian context have been briefly discussed as requested.

9. Table 1: Please always use the standard three-letter-code for antibiotic names.
R: The standard three-letter-code has been used in Table 1 as requested.

Minor Comments

Title: Please modify: "Emergence of CTX-M-1-producing Salmonella enterica serovar Napoli: a novel "enzyme-pathogen association" in the Italian ESBL endemic context".
R: The title has been modified as requested.

Line 32: Please delete "first".
R: The word “first” has been deleted as requested.

Line 35: Please write "main cause".
R: The sentence has been modified as requested.

Line 45: Please delete "each month".
R: The sentence has been modified as requested.

Line 46: Please delete "having the same features”.
R: The sentence has been modified as requested.

Emergence of CTX-M-1-producing *Salmonella enterica* serovar Napoli: a novel "enzyme-pathogen association" in the Italian ESBL endemic context

Luigi Principe [1], Valentina Viaggi [1], Mathieu Clément [2], Elisa Meroni [1], Beatrice Pini [1], Andrea Endimiani [2], Francesco Luzzaro [1*]

[1] Clinical Microbiology and Virology Unit, A. Manzoni Hospital, Lecco, Italy; [2] Institute for Infectious Diseases, University of Bern, Bern, Switzerland

***Corresponding author:**

Francesco Luzzaro, M.D.

Mailing address: Microbiology and Virology Unit, A. Manzoni Hospital

Via dell'Eremo, 9/11 - 23900 - Lecco, Italy

Phone: +39 0341 489630

Fax: +39 0341 489601

E-mail: f.luzzaro@asst-lecco.it

Sir,

In a recent nationwide survey, Giani et al. reported on the spread of extended-spectrum beta-lactamase (ESBL) enzymes among *Enterobacteriaceae* isolated from both inpatients and outpatients, thus highlighting the Italian endemic context for these resistance determinants [1]. The CTX-M-1 group was the most common among ESBL enzymes (>80% of ESBL-producing enterobacterial isolates, including *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*). In this endemic scenario, ESBLs could be sometimes found in uncommon associations with other species. Here we describe the isolation of CTX-M-1-producing *Salmonella enterica* serovar Napoli from a clinical sample.

On June 2017, we recovered a *Salmonella* Napoli from the stool culture of a one-year-old Italian girl, presenting diarrhea, abdominal pain and vomiting. The infant showed leukocytosis (12,000/μl), without fever or other signs of invasive infection, hence no treatment was started. The child lived with the parents in Lombardy (Northern Italy, close to Milan) and did not attend the nursery school. Therefore, we speculated that the infection was attributable to the domestic environment or to family's habits, though no other cases of infection were observed at the family level. Follow-up stool cultures performed for three consecutive months, were also positive for *Salmonella* Napoli, thus showing a persistent colonization at the intestinal level without signs and symptoms of infection.

Bacterial identification at the genus level was conducted by MALDI-TOF mass spectrometry (Vitek MS, bioMérieux, Marcy l'Etoile, France), while serovar was determined by the regional Reference Laboratory for enterobacterial pathogens using the White-Kauffmann-Le Minor scheme (https://www.pasteur.fr/sites/default/files/veng_0.pdf). Antimicrobial susceptibility testing, carried out by broth microdilution using a dedicated TREK panel (DKMGN; Thermo Fisher Diagnostics, Milan, Italy) and interpreted according to the 7.1 EUCAST clinical breakpoints (www.eucast.org),

revealed that the isolate was non-susceptible to extended-spectrum cephalosporins, but susceptible to carbapenems, fluoroquinolones, colistin, tigecycline, and trimethoprim/sulfamethoxazole (Table 1). The presence of an ESBL determinant was phenotypically evaluated on the basis of synergistic activity between clavulanate and extended-spectrum cephalosporins using the double disk approximation method (www.eucast.org). Molecular analyses carried out by CT103XL microarray system (Check-Points, Wageningen, The Netherlands) and PCR/sequencing revealed the presence of $bla_{\text{CTX-M-1}}$ gene and a single IncI1 plasmid (PBRT kit; Diatheva, Cartoceto, Italy). Conjugation experiments using *E. coli* J53 recipient (rifampicin-resistant) performed at 37°C, and selecting on MacConkey plates containing ampicillin and rifampicin (both 50 µg/ml), were successful (efficiency, 4×10^{-3}) and confirmed the presence of an IncI1 plasmid along with $bla_{\text{CTX-M-1}}$.

Salmonella Napoli is an emerging public health concern. During 2000–2006, a 140% increase in *Salmonella* Napoli cases was reported in Europe, mostly in France, Italy and Switzerland (87% of total cases) [2]. This serovar is one of the most common causing *Salmonella* infections in Italy, being on the rise since 2000 [3]. In Lombardy, *Salmonella* Napoli was the main cause of invasive salmonellosis among nontyphoidal serotypes (40 out of 687) during 2010-2014 [4]. Outbreaks of *Salmonella* Napoli infection reemerged in Italy during 2011–2015, with the proportion of related cases increased from 4.3% in 2011 to 5.8% in 2015 [5]. *Salmonella* Napoli is an important concern, especially in children, being often associated to invasive infections [4]. In a recent Italian study, compared to other serovars, the risk of *Salmonella* Napoli infection was higher in the age group <1-year-old, and lower in the other age groups [3]. Its diffusion is mostly associated to waterborne and foodborne sources, although environmental and zoonotic sources have been suggested [3,5]. In particular, vegetables irrigated with contaminated water are the main food vehicle for *Salmonella* Napoli [5]. Of note, ESBL enzymes belonging to CTX-M-1 group are widely spread in *E. coli* strains isolated from animals and in the environment, probably explaining this uncommon

association with *Salmonella* Napoli. In our case, $bla_{\text{CTX-M-1}}$ was carried by the IncI1 plasmid, that is considered a global epidemic resistance plasmid, being worldwide detected in *Enterobacteriaceae* of different origin. In particular, the IncI1 plasmid is widely diffused in Italy in *E. coli* strains of animal and human origin, being responsible for the dissemination of beta-lactamase genes, especially $bla_{\text{CTX-M-1}}$.

To the best of our knowledge, this is the first isolation of an ESBL-producing *Salmonella* Napoli from a clinical sample. In particular, the Italian ESBL endemic context could make real the acquisition of CTX-M-1 resistance determinant by this pathogen, representing a very worrisome occurrence, especially in the case of invasive infection in children. Furthermore, as a major concern, some outbreaks caused by *Salmonella* Napoli have been formerly described [5]. As widely diffused in *E. coli*, the association of ESBLs with other resistance determinants (i.e., those for fluoroquinolones and trimethoprim/sulfamethoxazole) could represent a possible occurrence also in *Salmonella* Napoli, making difficult the treatment of related infections. Of note, use of third-generation cephalosporins is recommended for children at high risk for invasive disease. In this context, the prompt detection at the laboratory level of ESBL-producing *Salmonella* isolates is essential. Moreover, molecular and epidemiological investigations together with whole genome sequencing approaches conducted on clinical and environmental isolates could provide meaningful data to understand the exchange of resistance traits and the real sources of diffusion of this potentially problematic pathogen.

Funding

This work was partially supported by NRP-72, "National Research Programme, Antimicrobial Resistance" (Swiss National Science Foundation; grant No. 177378 due to AE). MC is a PhD student (2018-2021) supported by NRP-72.

Competing interests

None declared.

Ethical approval

Not required.

References

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***Corresponding author:**

Francesco Luzzaro, M.D.

Mailing address: Microbiology and Virology Unit, A. Manzoni Hospital

Via dell'Eremo, 9/11 - 23900 - Lecco, Italy

Phone: +39 0341 489630

Fax: +39 0341 489601

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Table

Table I. Characteristics and susceptibility profile of the CTX-M-1-producing *Salmonella enterica* serovar Napoli

Code	Source	Resistance determinant	MIC values (mg/L) of antimicrobial agents and clinical interpretation (S/I/R)*															
			AMC	TZP	ATM	CAZ	CTX	FEP	ETP	IPM	MEM	AMK	GEN	CIP	TGC	CST	SXT	CZA	CTT
LC0541/17	Stools	CTX-M-1	8/2 (S)	4/4 (S)	32 (R)	4 (I)	>8 (R)	4 (I)	≤0.12 (S)	≤0.5 (S)	≤0.12 (S)	≤4 (S)	≤0.5 (S)	≤0.06 (S)	≤0.25 (S)	0.5 (S)	≤1/19 (S)	≤0.5/4 (S)	1/4 (S)

*MIC values indicate minimal inhibitory concentrations, as obtained by standard broth microdilution method. Interpretation based on EUCAST criteria (version 7.1, 2017; http://www.eucast.org).

Abbreviations: AMC, amoxicillin-clavulanate; TZP, piperacillin-tazobactam; ATM, aztreonam; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; ETP, ertapenem; IPM, imipenem; MEM, meropenem; AMK, amikacin; GEN, gentamicin; CIP, ciprofloxacin; TGC, tigecycline; CST, colistin; SXT, trimethoprim-sulfamethoxazole; CZA, ceftazidime-avibactam; CTT, ceftolozane-tazobactam; S, susceptible; I, intermediate; R, resistant.