Plasmid Carrying $bla_{CTX\text{-}M\text{-}15}$, $bla_{PER\text{-}1}$, and $bla_{TEM\text{-}1}$ Genes in *Citrobacter spp.* From Regional Hospital in Mexico

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ABSTRACT

INTRODUCTION: *Citrobacter spp.* is an opportunistic bacteria that have been recognized as significant pathogens in patients with underlying diseases or immunocompromised status. The aim of this study was to identify extended-spectrum β-lactamases in clinical isolates of *Citrobacter spp.*

METHODS: This cross-sectional study was conducted at Hospital Central "Dr. Ignacio Morones Prieto" in San Luis Potosi, Mexico. Nineteen isolates of *Citrobacter spp.* were obtained from clinical specimens between April to December 2015. Four isolates were resistant to third-generation cephalosporins. The presence of genes encoding ESBL ($bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$, $bla_{VEB\text{-}1}$, bla_{SHV}, and $bla_{PER\text{-}1}$) was analyzed by PCR. For this purpose, plasmid DNA was extracted and horizontally transferred to recipient *E. coli* Top 10.

RESULTS: $bla_{CTX\text{-}M\text{-}15}$ and $bla_{VEB\text{-}1}$ genes were detected in *Citrobacter freundii* and *Citrobacter sedlakii*, whereas $bla_{PER\text{-}1}$ gene was identified in 1 isolate of *Citrobacter freundii*. In contrast, bla_{SHV} gene was not detected in any isolate. One strain carried $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$, $bla_{VEB\text{-}1}$, and $bla_{PER\text{-}1}$ genes, most in a 275-kb plasmid.

CONCLUSION: This study shows the presence of different types of ESBL in clinical isolates of *Citrobacter freundii* and *Citrobacter sedlakii*, which confer resistance to broad-spectrum β-lactams. The plasmid identified in this study harboring ESBL genes could play an important role in the dissemination of antibiotic resistance.

KEYWORDS: *Citrobacter*, ESBL, CTX-M-15, PER-1, VEB-1, TEM, plasmid

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Introduction

Bacteria of the genus *Citrobacter* belong to the family *Enterobacteriaceae* and comprise 13 species; *Citrobacter* are found in a variety of environmental sources, including soil and water, and occasionally are isolated from the gastrointestinal tract of animals and humans.[1,2] Despite being considered an unusual nosocomial pathogen, neonates and immunocompromised patients are a frequent target of infections caused by these microorganisms.[3] These conditions include sepsis, urinary tract infections, respiratory and intra-abdominal infections, and central nervous system infections.[4] According a large observational study, Citrobacter species account for 0.8% of all Gram-negative infections in a hospital setting, with a mortality rate in hospitalized patients that ranged from 6.8% to 56%.[3-5]

As expected, infections caused by multidrug-resistant *Citrobacter* strains are associated with a higher rate of in-hospital mortality compared to those caused by susceptible strains. These multidrug-resistant strains show high levels of molecular class C (Amp-C) and extended spectrum β-lactamases (ESBL) as well as plasmid-mediated quinolone and carbapenem resistance.[2]

ESBL confer resistance to penicillins, first to third-generation cephalosporins, and aztreonam, but not to cephamycins and carbapenems.[6] The production of ESBL has been recognized as a global problem, mainly in the case of *E. coli* and *K.*

pneumoniae.[7-9] However, this condition has been also described in recent years in other species, including the genus *Citrobacter*.[7-9]

Few studies have analyzed the presence and distribution of ESBL in clinical isolates of *Citrobacter spp.* Among them, an outbreak caused by 5 isolates of CTX-M-2 producing *C. koseri* in hematological patients was reported in 2006.[7] Furthermore, CTX-M-1/3 β-lactamases have been reported in Korea, France and Spain, CTX-M-9/30 in Canada, and CTX-M-14 in China.[7,8] In addition, CTX-M-14, TEM, SHV-4, and SHV-12 ESBL have been detected in Japan.[7,8]

The aim of this study was to detect ESBL in clinical isolates of *Citrobacter spp.* at the Hospital Central "Dr. Ignacio Morones Prieto" (HCIMP), in the State of San Luis Potosi, located at the center-north of Mexico.

Materials and Methods

Bacterial isolates

This study was conducted after approval (July 23, 2015) by the Research Committee [COFEPRIS 14 CI 24 028 083] and the Research Ethics Committee of the Hospital Central "Dr. Ignacio Morones Prieto" [CONBIOETICA-24-CEI-001-20160427]. The registration number was 48-15. A written informed consent was obtained from all participants or legal guardians/parents for those under the age of 16 years.

The HCIMP has 250 beds and 32 beds in the intensive care unit and provides medical services to mid-and low-income populations from all over the State of San Luis Potosi.

Between July and December 2015, the Microbiology Laboratory of HCIMP identified 19 consecutive and non-repeated isolates of *Citrobacter spp.* The clinical isolates were identified using Vitek 2C (bioMérieux, Marcy l'Étoile, France) and were transferred to the Section of Medical Genomics from the Research Center of Health Sciences and Biomedicine, UASLP for molecular characterization.

ESBL phenotypic confirmatory test

The phenotypic confirmatory test of ESBL was performed by using the combined disk method on Mueller Hinton Agar, according to the Clinical and Laboratory Standards Institute (CLSI) recommendations, with Cefotaxime (CTX, 30 mg) and Ceftazidime (CAZ, 30 mg) disks, alone and in combination with Clavulanic Acid (CA, 10 mg) (Becton, Dickinson, Sparks, MD). Results were considered positive when the growth-inhibitory zone around either the CTX/CL or CAZ/CL disk was 5 mm or greater.[10]

Molecular identification of β-lactamases

DNA amplification of β-lactamase genes was carried out with specific primers (Table S1). In brief, 3 colonies of an overnight culture were suspended in 100 µL of DNase free water and incubated at 94°C for 5 min and at −70°C for additional 5 min. Then, tubes were centrifuged at 13 000 rpm for 5 min and the supernatant was used as DNA template.[11] The PCR reaction mixture contained 1X buffer, 2.0 mM of each dNTP, 5.0 µM of oligonucleotides for bla_{TEM}, bla_{CTX-M}, bla_{SHV}, bla_{VEB-1}, and bla_{PER-1} genes, 1.0 U of Taq DNA polymerase, bacterial genomic DNA, and 1.5-3.0 mM $MgCl_2$. PCR conditions were performed at 94°C for 5 min for initial denaturation, followed by 30-40 cycles of 30 s at 94°C, 1 min at 50-60°C, and 1 min at 72°C, followed by a final extension of 5 min at 72°C, using a Multigene thermo-cycler (Labnet International Inc, New Jersey, United States). The amplified products were analyzed by electrophoresis on 2% agarose gels. PCR products were purified using a Wizard DNA Clean-Up system (Promega), according to the manufacturer's instructions and were sequenced by using the dideoxynucleotide method, in a 3130 Genetic Analyzer device (Applied Biosystems, Foster City, California). BLAST analysis was performed in the NCBI http://www.ncbi.nlm.nih.gov/page.

Plasmid isolation

Plasmid DNA extraction to *Citrobacter spp.* (R-135 and R-086) was made by using the Plasmid Mini Kit (QIAGEN), which was performed through the following 3 steps: (1) Isolates grown in Luria Bertani medium for 12-16 h to a cell density of approximately $3.0–4.0 \times 10^9$ cells/mL were centrifuged at 6000*g* for 15 min at 4°C. Then, bacterial pellet was resuspended in 0.3 mL of Buffer P1, followed by the sequential addition of P2 and P3 buffers. After an additional centrifugation, the supernatant containing the plasmid was obtained. (2) Supernatant was loaded onto an anion-exchange Genomic tip (QIAGEN 20/G) and incubated for 20 min. (3) Plasmid DNA was eluted from the tip with 0.8 mL of QF buffer. Then, DNA was precipitated with isopropanol, centrifuged at 15 000 rpm for 30 min. Finally, the DNA pellet was washed with 1 mL of 70% ethanol, centrifuged and dissolved in a 10 mM Tris-HCl, pH 8.5. Extracted DNA was visualized on a 1% agarose gel.

Bacterial transformation

In brief, *E. coli* Top 10 bacteria was permeabilized with 75.0 mM calcium chloride and incubated with plasmid DNA (50-100 ng/µL) and heat-shocked at 42°C for 45 s/ice for 5 min. Afterward, bacteria were resuspended in 250 µL of Luria Bertani (LB) broth and incubated at 37°C under continuous shaking for 1 h. Finally, transformed bacteria were selected by incubating on LB/ampicillin (100 µg/mL) agar plates at 37°C for 24 h. ESBL genes in these bacteria were detected as described above.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was carried out by using the broth microdilution method, according to CLSI recommendations. Briefly, antibiotics were serially diluted 2-fold in

Table 1. Type of bacterial isolates from different clinical samples.

TYPES OF THE SPECIMENS	*C. FREUNDII*	*C. BRAAKII*	*C. KOSERI*	*C. SEDLAKII*
	N = 14	N = 2	N = 2	N = 1
Skin and soft tissue	11	1	0	1
Urine	2	0	2	0
Blood	1	1	0	0

Table 2. Phenotypic and genotypic detection of ESBL for strains studied.

STRAIN	PHENOTYPIC CONFIRMATORY TEST OF ESBL			*BLA* GENES				
	CAZ/CAZ-CL (MM)	CTX/CTX-CL (MM)	RESULT	CTX-M	TEM	SHV	VEB-1	PER-1
C. sedlakii R-099	18/24	11/24	Positive	–	–	–	+	–
C. freundii R-086	19/25	13/25	Positive	+	+	–	–	–
C. freundii R-134	10/13	10/13	Negative	–	–	–	–	–
C. freundii R-135	20/22	16/21	Positive	+	+	–	+	+

Abbreviations: CAZ, Ceftazidime; CAZ/CL, Ceftazidime/Clavulanic acid; CTX, Cefotaxime; CTX/CL, Cefotaxime/Clavulanic acid.

50 µL of Mueller-Hinton broth, mixed with 50 µL of bacteria at a density of 10^6 colony-forming units/mL and incubated for 18 h at 37°C. Quality control was performed using the reference *E. coli* Top 10 and results were expressed as the minimum inhibitory concentration (MIC).

Results

Bacterial isolates

The Microbiology Laboratory identified 14 isolates of *C. freundii*, 2 isolates of *C. koseri*, 2 isolates of *C. braakii*, and 1 isolate of *C. sedlakii* in the different samples studied (Table 1).

ESBL phenotypic confirmatory test

Three isolates resistant to third-generation cephalosporins showed an ESBL phenotype: *C. freundii* R-086, R-135, and *C. sedlakii* R-099. Moreover, the *C. freundii* R-134 isolate, which was also resistant to third-generation cephalosporins, did not exhibit a resistance phenotype indicative of the presence of ESBL (Table 2).

Molecular identification of β-lactamases

As shown in Figure 1, bla_{TEM} and bla_{CTX-M} genes were identified in *C. freundii* R-086 and R-135. In 2 additional isolates (*C. freundii* R-135 and *C. sedlakii* R-099), bla_{VEB-1} genes were detected Figure 1c, whereas the bla_{PER-1} gene was identified in *C. freundii* R-135 Figure 1d. In contrast, the bla_{SHV} gene was not identified in any isolate (data not shown). Moreover, bla_{TEM}, $_{CTX-M}$, $_{VEB-1}$, and $_{PER-1}$ genes were identified in the *C. freundii* R-135 isolate (Table 2). Finally, DNA sequence analysis showed that the TEM β-lactamase detected in *C. freundii* R-086 had 100% homology to TEM-1 (GenBank accession number: ALW82937.1.), whereas the predicted amino acid sequence of the CTX-M β-lactamase showed 99% homology to CTX-M-15 (GenBank accession number: AKO22374.1).

Plasmid isolation and bacterial transformation

A 275-kb plasmid DNA was isolated from *C. freundii* R-086 (R-086p) and R-135 (R-135p) strains (data not shown). On the other hand, In the Top 10-R-135 *E. coli* transformant we were able to amplify the $bla_{CTX-M-15}$, $_{PER-1}$, and $_{TEM-1}$, genes Figure 2. In contrast, the bla_{VEB-1} gene was not amplified in R-135p or Top-10-R-135.

Antimicrobial susceptibility testing

The MIC of antimicrobial agents for *C. freundii* R-135, *E. coli* Top 10-R-135, and *E. coli* Top 10 are shown in Table 3.

Discussion

In recent years, infections caused by *Citrobacter spp*. have become important because they cause nosocomial outbreaks, mainly affecting neonates and immunocompromised patients.[12] Although *C. koseri* has been most frequently identified in sporadic cases and hospital outbreaks,[6] in our study, the predominant species was *C. freundii*.

Since its first description in the 1990s, ESBL of the CTX-M family have showed an increased frequency in *Enterobacteriaceae*, followed by SHV and TEM as well as, to a lesser extent, by the OXA, Tla, GES, VEB, and PER types. However, the frequency of these types is variable in different geographical regions.[6] In our study, we detected that the *C. freundii* R-086 and R-135

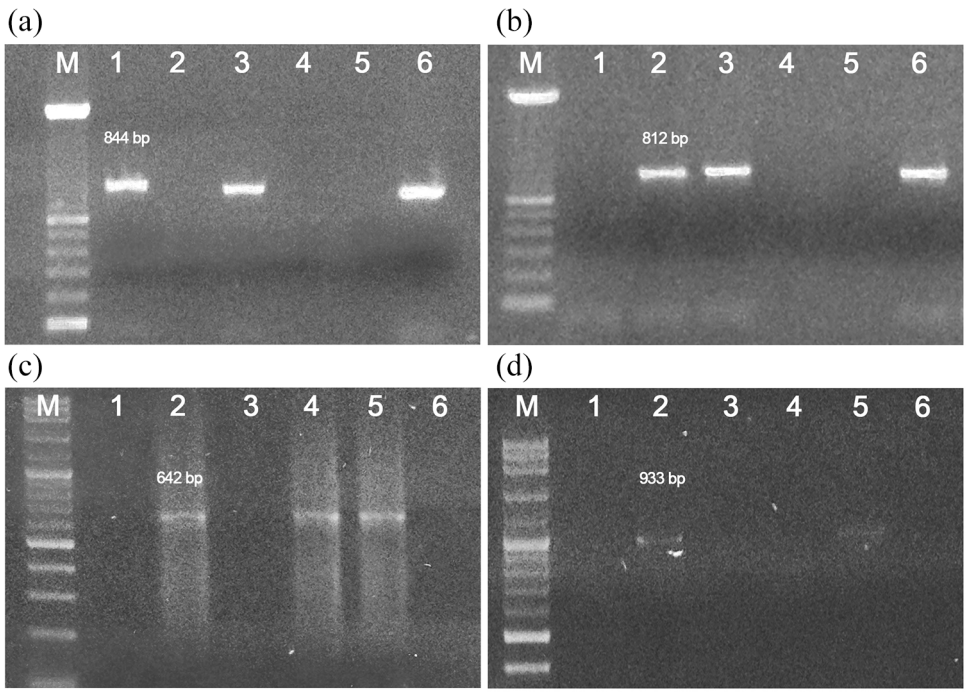

Figure 1. Agarose gel electrophoresis for the PCR products of ESBL: (a) the bla_{TEM} gene. Line M: 100 bp ladder, line 1: positive control, line 2: negative control, lines 3 and 6: R-086 and R-135 strains (bla_{TEM} positive), lines 4 and 5 bla_{TEM} negative strains, (b) the bla_{CTX-M} gene. Line M: 100 bp ladder, line 1: negative control, line 2: positive control, lines 3 and 6: R-086 and R-135 strains (bla_{CTX-M} positive), lines 4 and 5: R-099 and R-134 strains (bla_{CTX-M} negative), (c) the bla_{VEB-1} gene. Line M: 100 bp ladder, line 1: negative control, line 2: positive control, lines 3 and 6: R-086 and R-134 strains (bla_{VEB-1} negative), lines 4 and 5: R-099 and R-135 strains (bla_{VEB-1} positive), and (d) the bla_{PER-1} gene. Line M: 100 bp ladder, line 1: negative control, line 2: positive control, line 5: R-135 strain (bla_{PER-1} positive), lines 3, 4, and 6: bla_{PER-1} negative strains.

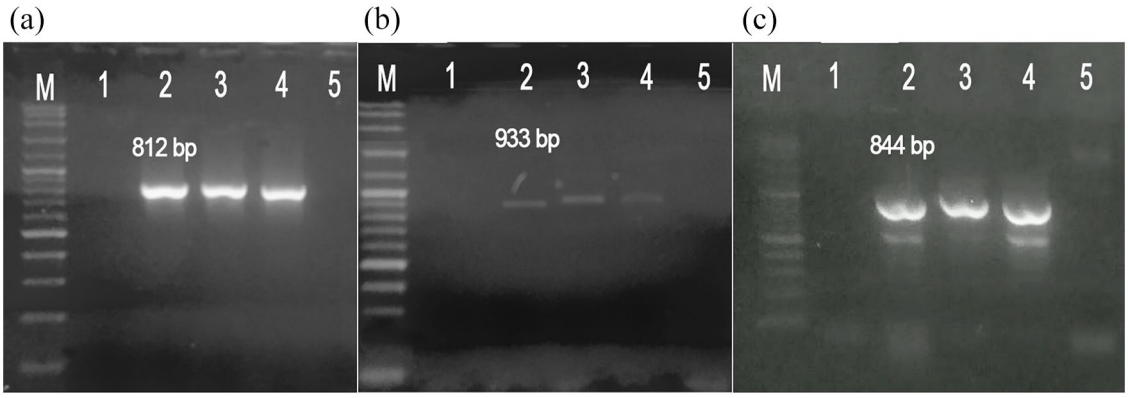

Figure 2. Agarose gel electrophoresis for the PCR products of Top10R-135 strain: (a) the $bla_{CTX-M-15}$ gene. Line M: 100 bp ladder, line 1: negative control, line 2: R-135 strain ($bla_{CTX-M-15}$ positive), line 3: R-135p, line 4: Top10R-135 (transformant strain), and 5: *E. coli* Top-10, (b) the bla_{PER-1} gene. Line M: 100 bp ladder, line 1: negative control, line 2: R-135 strain (bla_{PER-1} positive), line 3: R-135p, line 4: Top10R-135 (transformant strain), and 5: *E. coli* Top-10, and (c) the bla_{TEM-1} gene. Line M: 100 bp ladder, line 1: negative control, line 2: R-135 strain (bla_{TEM} positive), line 3: R-135p, line 4: Top10R-135 (transformant strain), and 5: *E. coli* Top-10.

strains synthesized CTX-M-15, an ESBL worldwide distributed and characterized by its efficient ceftazidime hydrolyzing activity.[13,14] The first report of CTX-M-15 in Mexico was in 2011, in *E. coli* isolate,[11] when 2 different groups reported strains of *E. coli*, *Enterobacter cloacae*, and *K. pneumoniae* as carriers of CTX-M-15 ESBL.[9,15] Two years later, 58 isolates of *E. coli* and 16 isolates of *K. pneumoniae* producers of CTX-M-15 ESBL were detected in a tertiary care hospital in the city of Guadalajara, State of Jalisco, México.[11] The present study is the first report of *Citrobacter spp*. able to synthesize CTX-M-15 ESBL in Mexico. In the same strain the TEM-1 β-lactamase was identified, which efficiently hydrolyze penicillins and low spectrum cephalosporins and that is not considered an ESBL.

The presence of VEB-1 ESBL in the genus *Citrobacter* had not been described so far and in the case of PER ESBL, there is only a previous report of *C. koseri* PER-2 producer.[16] This

Table 3. Minimum inhibitory concentration (μg/mL).

ANTIMICROBIAL	*C. FREUNDII* R-135	*E. COLI* TOP10-R-135	*E. COLI* TOP 10	BREAKPOINTS (CLSI)
Ampicillin	⩾1024	⩾1024	8	⩾32
Cephalotine	1024	⩾1024	8	⩾32
Ceftriaxone	1024	⩾2048	⩽4	⩾4
Ceftazidime	32	32	⩽4	⩾16
Meropenem	⩽0.25	⩽0.25	⩽0.25	⩾4

ESBL has emerged in clinical isolates of *E. coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella spp.*, and *Proteus mirabilis*.[6]

The first report on the resistance to β-lactams in *C. sedlakii* was an isolate resistant to aminopenicillins, carboxypenicillins, low spectrum cephalosporins (but sensitive to broad-spectrum cephalosporins, and carbapenems), which was mediated by a β-lactamase called Sed-1.[12] In our study, the *C. sedlakii* R-099 isolate was resistant to aminopenicillins, carboxypenicillins, broad-spectrum cephalosporins including cefepime. In this case, the PCR analysis showed the presence of VEB-1 but not of *bla* TEM, SHV, CTX-M or PER-1. At our best knowledge, this is the first report of a *C. sedlakii* strain able to produce VEB-1 ESBL.

We identified the $bla_{\text{TEM-1}}$, and $bla_{\text{CTX-M-15}}$ genes in *C. freundii* R-086, an isolated that also showed resistance to quinolones, aminoglycosides, and inhibitors of the folate pathway (data not shown). Moreover, in the *C. freundii* R-135 strain, $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{VEB-1}}$, and $bla_{\text{PER-1}}$ genes were identified. This phenomenon may be related to the presence of genetic mobile structures such as integrons, transposons, and plasmids that have been previously described in other species of Gram-negative bacilli; In this regard, an analysis of 40 isolates of *Citrobacter spp*. performed in 2010, the simultaneous presence of $bla_{\text{CTX-M}}$, $_{\text{ampC, SHV}}$, and $_{\text{TEM}}$ genes was observed, and in 32.5% of these isolates class 1 integron was identified. In addition, in 48% of CTX-M-15 positive isolates the insertion sequence IS26 was detected.[17] In the same year, a class 1 integron and ISCR1 insertion sequence were identified in a multiresistant *C. freundii* isolate, which showed resistance to quinolones and β-lactams by presence of CTX-M-15 ESBL.[18]

Previous reports suggest that different plasmids are responsible for the global spread of CTX-M-15 ESBL[19] since $bla_{\text{CTX-M-15}}$ has been identified in plasmids ranging from 40 to more than 200-Kb[20] and belonging to IncFII and IncI1 groups.[20,21] In these mobile elements, more resistance determinants have been identified, as $bla_{\text{TEM-1}}$ and $bla_{\text{SHV-12}}$ genes. However, there is only 1 previous report on a plasmid containing the $bla_{\text{CTX-M-15}}$ and $bla_{\text{PER-1}}$ genes, which was detected an *Aeromonas caviae* strain, which was isolated from a wild-growing Mediterranean mussel.[22] Thus, this is the first report of a plasmid R-135p carrying the $bla_{\text{CTX-M-15}}$, $_{\text{TEM-1}}$, and $_{\text{PER-1}}$ genes in *C. freundii*. The genetic context of $bla_{\text{CTX-M-15}}$ includes an ISEcp1 sequence, upstream of the gene, which can be an efficient factor for the mobilization and expression of $bla_{\text{CTX-M-15}}$, as it has been observed in previous reports.[19,23]

We consider that it would be important to investigate the genetic context of β-lactamase genes in both, plasmids and chromosome as well as to determine the structure of the plasmid identified in *C. freundii* R-135.

In conclusion, our study shows the presence of different ESBL types in clinical isolates of *C. freundii* and *C. sedlakii*, which mediate the resistance to broad-spectrum β-lactams. The simultaneous presence of several antibiotic resistance genes seems to be related to genetic mobile elements that may favor their dissemination.

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Author Contributions

CNG acquired clinical data and samples, interpreted results, and drafted the manuscript. CNG and MBM performed the experiments. ETM and PNM co-designed and supervised the study and interpreted the results of experiments. DEN analyzed and interpreted data. RGA and LPG critically revised and edited the manuscript. All authors have read and approved the manuscript.

Ethical Statement

This study was conducted at Hospital Central Dr. Ignacio Morones Prieto (HCIMP) in San Luis Potosi, Mexico after approval by the Research Committee [COFEPRIS 14 CI 24 028 083] and the Research Ethics Committee of the HCIMP [CONBIOETICA-24-CEI-001-20160427]. The registration number was 48-15.

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Supplemental Material

Supplemental material for this article is available online.

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