Distribution of extended-spectrum β-lactamase types in a Brazilian tertiary hospital

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ABSTRACT

Introduction: Epidemiological data on the prevalence of extended-spectrum β-lactamases (ESBLs) are scarce in Brazil despite the fact that these data are essential for empirical treatment and control measures. The objective of this study was to evaluate the prevalence of different ESBLs by type and distribution in a tertiary hospital in southern Brazil.

Methods: We evaluated 1,827 enterobacterial isolates between August 2003 and March 2008 isolated from patients at a tertiary hospital. Samples were identified using a Vitek automated system and were confirmed by biochemical testing. The identified ESBL strains were characterized by phenotypic methods, polymerase chain reaction (PCR), and sequencing. Genetic similarities were evaluated by pulsed-field gel electrophoresis.

Results: It was 390 (21.3%) ESBL-producing strains, which expressed the ESBLs CTX-M (292), SHV (84), CTX and SHV (10), TEM (2), and PER (2).

Conclusions: The prevalence of ESBL-expressing strains was high, especially in \textit{Klebsiella pneumoniae} and \textit{Enterobacter} spp. CTX-M was the predominant type of ESBL observed, and its genetic variability indicates a polyclonal distribution.

Keywords: ESBL. CTX-M. \textit{Enterobacteriaceae}.

INTRODUCTION

Over three decades have passed since the identification of the first extended-spectrum β-lactamase (ESBL)-producing bacteria. Since then, the prevalence of ESBL-producing strains has increased, and new types and variants have been described. The first ESBLs were derived from TEM (temoniera) and SHV (sulfhydryl-variable) β-lactamases, which are mainly found in healthcare-associated infections. Cefotaxime (CTX-M) ESBLs have increased in importance due to the increased frequency of community-acquired infections caused by strains carrying this enzyme\textsuperscript{(1)(2)}.

The frequency and predominant types of ESBL vary from region to region and even between institutions within the same region. The study for monitoring antimicrobial resistance trends (SMART) surveyed ESBL-producing strains around the world and found that the CTX-M-15 variant predominates\textsuperscript{(3)}.

Extended-spectrum β-lactamases are mainly found in \textit{Escherichia coli}, \textit{Klebsiella pneumoniae}, and \textit{Klebsiella oxytoca}, but have also been described in many other species of \textit{Enterobacteriaceae} and non-fermenting Gram-negative rodshaped bacteria\textsuperscript{(1)}.

According to a study performed in the United States analyzing data from 2009 to 2011, North America has the lowest rates of ESBL-producers, with a prevalence of 11.4% to 16.1% in \textit{K. pneumoniae} and 8.1% in \textit{E. coli}. In this study, the SHV type of ESBL was most frequently observed, although the authors reported that CTX-M-14 and CTX-M-15 are increasing in prevalence and may soon replace SHV-12 as the most common ESBL types\textsuperscript{(4)}.

The prevalence of the various ESBL types in Europe varies from country to country. ESBL has been found in 20.1% of \textit{E. coli} (0.9%-89.7%) and 45.7% of \textit{Klebsiella} spp. (2.5-100%), with greater frequencies in Mediterranean countries, Belgium, and Poland. CTX-M is the predominant type of ESBL found in Europe, and it is frequently found in community-acquired infections. Moreover, in some regions, CTX-M is more commonly found in \textit{E. coli} than in \textit{K. pneumoniae}\textsuperscript{(4)(5)(6)}.

The prevalence of ESBL types in Asia also varies among Asian countries. The Antimicrobial Surveillance Program (SENTRY), conducted from 1998 to 2002, reported ESBL-producing \textit{K. pneumoniae} in Singapore (35.6%), China (30.7%), and Japan (10%), and ESBL-producing \textit{E. coli} in China (24.5%), Hong Kong (14.3%), and Singapore (11.3%). The most frequent ESBL types in Asia are CTX-M and SHV\textsuperscript{(6)(7)}.

Epidemiological data on the frequencies of ESBL-expressing strains in Africa and Australia are rare. One study...
conducted in the Pacific region reported that 10% and 28.1% of
*K. pneumoniae* in Australia and South Africa, respectively,
are ESBL-producers. Additionally, a local study conducted in a
Tunisian hospital found *E. cloacae* ESBL isolates that produce
ESBL types CTX-M and SHV (17).

The highest prevalence of ESBL-producing strains was
recorded in South America, although the frequencies vary
among countries; in Guatemala, Mexico, and Peru, ESBLs
are prevalent in *E. coli*, whereas in Brazil, Cuba, Ecuador,
and Venezuela, ESBL prevalence is greater in *Klebsiella* spp.
(i.e., ≥50%). The most common type of ESBL is CTX-M,
although a few isolates harbor the TEM and SHV ESBL types.
The PER-2 type, which is exclusive to South America, is the
second most ESBL isolated frequently type in Argentina and has
been frequently found in Uruguay, Chile, and Bolivia (9, 10, 11).

A Brazilian survey performed as part of the SENTRY
program revealed ESBL frequencies of 10% in *E. coli* and 50%
in *K. pneumoniae*. Epidemiological data on the prevalence of
ESBL types in Brazil are scarce and suggest that CTX-M is the
predominant ESBL type. Two epidemiological studies conducted
in the State of Rio de Janeiro found that the ESBL
CTX-M predominates. One study found a predominance of
In the second study, CTX-M-15 was predominant in more than 50% of
*K. pneumoniae*, *E. coli*, and *E. cloacae* isolates (13). A study in
Sao Paulo found 20 CTX-M-2, 14 CTX-M-59, 12 CTX-M-15, and 13 SHV genes in ESBL-expressing strains (14). GES (Guiana
Extended Spectrum β-Lactamases), BES (Brazilian Extended
Spectrum β-Lactamases), and PER (Pseudomonas Extended
Resistance) types have also been reported in Brazil (10).

Most studies suggest that ESBL-producing strains are
polyclonal and that the ESBL features are spread by plasmid
transfer; however, during outbreaks, clonal dissemination may
occur (14, 15, 16).

The aim of this study was to investigate the prevalence of
ESBL types in patients and the genetic similarities between
ESBL strains in a tertiary southern Brazilian hospital.

**METHODS**

**Sample and control strains**

Our survey included 1,827 enterobacterial samples obtained
between August 2003 and March 2008 from inpatients at the
Clinical Hospital at the Federal University of Paraná. Only one
isolate per patient was included in our analysis. The samples
obtained were from urine (856), blood (391), liquor (18), other
sterile liquids (138), respiratory samples (336), and abscesses
and wounds (88). Identification was performed using a Vitek
automated system (bioMérieux, Marcy-l’Etoile, France) and
was confirmed by biochemical testing. Control strains included
*E. coli* RJ-15317 (*bla*<sub>CTX-M-2</sub> positive control), *E. coli* RJ-694
(*bla*<sub>CTX-M-9</sub> positive control), *E. aerogenes* RJ-159 (*bla*<sub>CTX-M-8</sub>
positive control), *K. pneumoniae* TEM-3 SP (*bla*<sub>TEM</sub> positive
control), *K. pneumoniae* SHV-5 SP (*bla*<sub>SHV</sub> positive control),
*E. cloacae* 532 PR (*bla*<sub>PER-2</sub> positive control), *E. coli* ATCC
25922 (control for susceptibility testing), and *K. pneumoniae*
ATCC 700603 (positive ESBL phenotype control).

**Detection of ESBL producers**

The susceptibility disk diffusion test was performed
according to Clinical and Laboratory Standards Institute (CLSI)
guidelines (17) with the following β-lactam antimicrobials:
ceftazidime, cefotaxime, cefpodoxime, cefepime, aztreonam,
cefotaxime, imipenem, meropenem, and ertapenem (Oxoid,
Cambridge, UK). *Enterobacteriaceae* with reduced sensitivity
to one or more β-lactams (18) were tested for the presence of
ESBL and the following genes: *bla*<sub>CTX-M</sub>, *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>PER</sub>,
*bla*<sub>OXA</sub>, *bla*<sub>GES</sub>, and *bla*<sub>GES</sub> (19). Template deoxyribonucleic acid
(DNA) was obtained using post-boiling extraction of a heavy
suspension of colonies on PCR water. Primers specific for
the 16S region were included in all reactions as an internal
control. When the internal control was negative, the DNA
was extracted again using PureLink® Spin Column-based
DNA extraction kits (Invitrogen, New York, USA). PCR
products were purified using the GFX-TM PCR purification kit
(Amersham Biosciences, Uppsala, Sweden) and amplified
using Dye Terminator GT (Amersham Biosciences). The
product was precipitated with 7.5M ammonium acetate and
95% ethanol, after which the product was sequenced using
MegaBACE (Amersham Biosciences) with the following
primers: *bla*<sub>CTX-M</sub> (5′-F TGT TAG TGA GTG TGC CGC
TG-3′; 5′-R GAC GGC TTT CTG CCT TAG GTT G-3′), *bla*<sub>TEM</sub>
(5′-F T-A GTG ATI ATT CAA CAT TTC CG3′; 5′-R
CCA ATG CCT TCC ACA TCA CTG AC3′), *bla*<sub>SHV</sub> (5′-F
TCA GCC AAA AAC ACC TTG; 5′-R
TCC CGA AGA TAA ATC ACC ACC A-3′), and *bla*<sub>PER</sub>
(5′-F CGTCTTCGCTGCTGATG3′;
5′-R-GGCAGCTTCTTAAAACG3′). The sequences were
compared using basic local alignment search tool (BLAST)
gov/BLAST).

**Determination of minimum inhibitory concentration**

All ESBL-producing samples were tested for ceftazidime,
cefotaxime, cefpodoxime, cefepime, aztreonam, and ceftoxitin
(Sigma-Aldrich, St Louis, MO, USA; imipenem and ertapenem
(Merk Sharp & Dohme Farmacêutica Ltd., Campinas, Brazil);
meropenem (AstraZeneca, London, UK); and tigecycline
(Wyeth, Philadelphia, USA). Cation-adjusted Mueller Hinton
agar (Difco, Franklin Lakes, USA); Cation-adjusted Mueller-Hinton
agar (Difco, Franklin Lakes, USA) were tested for the presence of
*P. aeruginosa* strain ATCC 25922 and *P. aerugi nosa* strain ATCC 27853 were used
as control strains. The ranges tested were 0.03-256µg/mL for
ceftazidime, cefotaxime, cefpodoxime, cefepime, aztreonam,
and cefoxitin, 0.03-16µg/mL for imipenem, ertapenem, and
ceftazidime, cefotaxime, cefpodoxime, cefepime, aztreonam,
and cefoxitin, 0.03-16µg/mL for meropenem, and 0.12-16µg/mL for tigecycline (20).

**Analysis of genetic similarity**

Macrorestriction [XbaI (10)] analysis by pulsed-field gel
electrophoresis (PFGE) [CHEF-DRIII; Bio-Rad Laboratories,
USA] was performed for 262 CTX-M-producing *K. pneumoniae*
(120), *E. cloacae* (59), *E. aerogenes* (50), and *E. coli* (33)

![www.rsbmt.org.br – www.scielo.br/rsbmt](http://www.rsbmt.org.br)
The survey of 1,827 samples yielded 483 (26.4%) strains with reduced susceptibility to third-generation cephalosporins and/or aztreonam, according to CLSI criteria. Of these strains, 390 (21.3%) were ESBL-producers, including CTX-M-292, SHV-84, CTX and SHV (10), TEM (2), and PER (2). Table 1 shows the results of the screening and genotype testing for ESBL expression by species. Species with 12 or fewer isolates were grouped as other and included Citrobacter amalonatus (two non-ESBL), Citrobacter diversus (one non-ESBL), Cronobacter sakazakii (two non-ESBL), Enterobacter gergoviae (three ESBL and seven non-ESBL), Klebsiella ozaenae (six non-ESBL), Pantoea agglomerans (one ESBL and one non-ESBL), Providencia rettgeri (one ESBL), Providencia stuartii (two ESBL and two non-ESBL), Proteus vulgaris (one ESBL and 11 non-ESBL), and Serratia liquefaciens (six non-ESBL). The antimicrobial susceptibility testing of the ESBL strains is presented in Table 2.

A genetic similarity analysis of CTX-M-producing strains revealed two forms of dissemination: the spread of resistant clones persisting for several years in hospitals and the transmission of resistance-encoding plasmids. This hypothesis is supported by the observation of identical clones, different types of CTX-M, and unrelated bacterial strains harboring identical ESBL genes. CTX-M-59 was an exception that was found in a single clone of E. aerogenes. ESBL clones were not restricted to certain hospital areas but were found in intensive care units (ICU) and other hospital units. They were also present as causative agents of urinary tract infections, respiratory tract infections, and bacteremia, among other diseases. Genetic similarities between CTX-M ESBL-producing K. pneumoniae, E. coli, E. cloacae, and E. aerogenes are presented in Figure 1.

The observed prevalence of ESBL-producing strains (21.3%) was similar to the prevalence reported in another Brazilian study (24.8%) based on phenotype testing. Notably, previous reports have not been conducted on the prevalence of various ESBL genotypes. E. aerogenes, E. cloacae, and K. pneumoniae were the most frequently ESBL-positive species isolated. Epidemiological analysis revealed an unexpectedly high frequency of Enterobacter spp. (i.e., E. aerogenes and E. cloacae) when compared with K. pneumoniae, which is probably due to the spread of a CTX-M-59-producing E. aerogenes clone throughout the hospital. In a SENTRY study that included strains from Brazil, the authors found ESBL rates of 12.8% in Escherichia coli and 49.9% in Klebsiella spp.

The CTX-M ESBL type was present in 74.8% (292/390) of all ESBL-positive strains. CTX-M was first identified in South America and is prevalent in Brazil. It is also the predominant ESBL type found in other regions of the world and is increasing in frequency, particularly in the context of community-acquired infections. The CTX-M-2 variant was predominant in our study and was identified in 229 (58.7%) samples. CTX-M-2 was first described in a strain of Salmonella isolated in Argentina and was soon reported in Brazil and other South American countries. CTX-M-2 is the predominant ESBL type in Brazil and has been found in several species of Enterobacteriaceae. More recent studies have reported an increasing number of CTX-M-15-producing isolates.

The CTX-M-59 variant found in isolates of E. aerogenes is an H89 L derivative of CTX-M-2 and was first described in ESBL-producing K. pneumoniae in Brazil. CTX-M-59 has not been reported outside Brazil or in different species. The bla<sub>CTX-M-2</sub> and bla<sub>CTX-M-59</sub> genes were identified in IncA/C plasmids, reinforcing the possibility of the intraplasmid evolution of bla<sub>CTX-M-59</sub> from bla<sub>CTX-M-2</sub> and the implication of an effect of IncA/C on bla<sub>CTX-M</sub> dissemination.

CTX-M-15 is predominant in E. coli and K. pneumoniae worldwide but was found in only four of the samples in this study: E. coli (1), E. cloacae (1), E. aerogenes (1), and Serratia marcescens (1). In some countries, outbreaks of E. coli have been driven by a single ESBL-expressing clone that can be found throughout the world. CTX-M-15 was reported in Brazil for the first time in 2010. Moreover, an epidemiological study from Rio de Janeiro identified CTX-M-15 in more than 50% of ESBL-positive samples.

The CTX-M-8 variant was found in five strains of E. coli and one strain of K. pneumonia in our study. CTX-M-8 was first described in Brazil in Citrobacter amalonaticus and E. cloacae, the only other report of this ESBL type was in Spain in 2009. The CTX-M-9 variant was found in nine strains, and CTX-M-14 was found in one strain. CTX-M-9 has been reported in several Brazilian studies and is considered pandemic in Europe similarly to CTX-M-15. CTX-M-14 was first reported in China and was reported in Brazil in 2010.

The SHV type was the second most common group of ESBLs observed in this study, as SHV was found in 94 (24.2%) samples. The SHV-12 variant was found in 90 (23.1%) strains. The SHV enzymes, and SHV-12 in particular, are commonly observed and have been previously reported in Brazil. The SHV type is the second most common ESBL group in Brazil and other countries. SHV-2 and SHV-27 were each found in a single (0.3%) isolate, and SHV-38 was found in two (0.6%) isolates.

The TEM type (TEM-136 variant) was observed in two (0.6%) samples. TEM ESBLs are not common in Brazil, as the only TEM type previously reported in Brazil was TEM-116. The TEM type is also less frequently observed than the CTX-M and SHV types worldwide.

Klebsiella pneumoniae

Enterobacter cloacae

Escherichia coli

Enterobacter aerogenes
<table>
<thead>
<tr>
<th>Species</th>
<th>Isolates</th>
<th>Screening (+) n (%)</th>
<th>ESBL (+) n (%)</th>
<th>ESBL variants</th>
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<td>10 (17.5)</td>
<td>7 (12.3)</td>
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<td>Morganella morganii</td>
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<td>5 (14.7)</td>
<td>2 (5.9)</td>
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<td>2</td>
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<td>Proteus mirabilis</td>
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<td>10 (20.0)</td>
<td>6 (12.0)</td>
<td>CTX-M-2</td>
<td>6</td>
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<td>Serratia marcescens</td>
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<td>18 (38.3)</td>
<td>7 (14.9)</td>
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<td>6</td>
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<td>CTX-M-15</td>
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<tr>
<td>Other*</td>
<td>46</td>
<td>12 (26.1)</td>
<td>8 (17.4)</td>
<td>CTX-M-2</td>
<td>5</td>
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<td>1</td>
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</table>

wards Enterobacter aerogenes did not disseminate and was expressed by a single clone of clones with different types of CTX-M ESBLs as well as by dissemination is also supported by the incidence of identical previously reported by our group the PER-2 type is infrequent and was observed in two isolates were also described during an outbreak in Uruguay. In Brazil, clinical isolates, suggesting that Clone A Klebsiella pneumoniae isolates consisted of two clonal groups subdivided into groups A1-A15 (related) and group B. Clonal group A contains CTX-M-2 and CTX-M-9. Clone A K. pneumoniae was found throughout the study period in several clinical isolates, suggesting that Clone A Klebsiella pneumoniae is an endemic microorganism in the hospital. Clone B was present from 2004 to 2008 in different departments, primarily in intensive care units. The other K. pneumoniae isolates exhibited similarities below the level of highly related or related samples.

The dissemination of ESBLs in E. coli was mostly polyclonal, with small highly related clonal groups designated A, B, C, and D. Clone A was found in urine samples of three hematology patients in 2008, with one isolate producing CTX-M-15 and two isolates producing CTX-M-2. Clone B appeared in samples from 2007 and 2008 and included CTX-M-2 and CTX-M-8. Clone C was present in samples from 2004 to 2008; four ICU isolates expressed CTX-M-2, and two ESBL subclones, CTX-M-2 and CTX-M-8, were isolated in other wards. Clone D consisted of three representative isolates from different wards and different years.

E. aerogenes clones producing CTX-M-59 were isolated from 2004 to 2008, mainly from blood and urine samples obtained from patients in the ICU. Only two isolates showed no clonal relationship with the other isolates.

The spread of E. cloacae was mostly polyclonal, with five main groups, each including more than three similar strains. Clonal groups were designated A, B, C, D, and E, all of which expressed the ESBL CTX-M-2. Except for clone E, which was mainly isolated from blood samples, the remaining clones were predominantly isolated from urine. Clone A was isolated in 2004-2007, clone B was isolated in 2005-2006, C and E were isolated in 2005-2007, and D was isolated in 2004-2006. All clones were found in different clinics suggesting that they were spread across various hospital departments.

High rates of resistance to cephalosporins and aztreonam were observed; however, a few samples were susceptible according to CLSI criteria18). Thus, the clinical efficacy of cephalosporin and aztreonam against ESBL-producing bacteria remains uncertain. Cefepime susceptible-dose dependence (SDD) was found in 12.8% of the ESBL isolates; in cases such as these, higher doses are recommended for infected patients18).

Carbapenems are considered the drug of choice for treating infections by ESBL-producing microorganisms. This study showed good sensitivity of isolates to carbapenems (99.3% for imipenem, 98.2% for meropenem, and 86.4% for ertapenem), but resistant isolates were also observed (mainly ertapenem). ESBLs do not degrade carbapenems efficiently but can confer resistance when associated with reduced permeability of the membrane to the drug. Carbapenems displayed good potency and activity, reinforcing the fact that they are an important therapeutic option against these microorganisms. Aminoglycosides have also been associated with high sensitivity rates but are also associated with high toxicity and limited indications. The carbapenems are the most widely used antimicrobial agents to treat infections caused by ESBL-producing strains9(10). However, the indications for their use should be clearly defined because the indiscriminate use of carbapenems has led to the emergence of resistant strains10).

The clinical isolates analyzed in our study were susceptible to tigecycline but to a lesser degree than they were to carbapenems. Tigecycline use was approved by the Food and Drug Administration for intra-abdominal infections, skin and soft tissue infections, and community-acquired pneumonia.

**TABLE 2 - Antimicrobial susceptibility of ESBL-producing strains.**

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; (µg/ml)</th>
<th>Susceptibility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceftazidime</td>
<td>32</td>
<td>&gt; 256</td>
<td>19.6</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>256</td>
<td>&gt; 256</td>
<td>4.3</td>
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<tr>
<td>Aztreonam</td>
<td>128</td>
<td>&gt; 256</td>
<td>10.4</td>
</tr>
<tr>
<td>Cefepime</td>
<td>32</td>
<td>&gt; 256</td>
<td>12.1</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.06</td>
<td>0.12</td>
<td>99.3</td>
</tr>
<tr>
<td>Meropenem</td>
<td>0.03</td>
<td>0.25</td>
<td>98.2</td>
</tr>
<tr>
<td>Ertapenem</td>
<td>0.06</td>
<td>0.5</td>
<td>86.4</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>0.5</td>
<td>2</td>
<td>90.3</td>
</tr>
</tbody>
</table>

ESBL: extended spectrum β-lactamase; MIC<sub>50</sub>: minimum inhibitory concentration 50%; MIC<sub>90</sub>: minimum inhibitory concentration 90%.
Tigecycline has not been approved for the treatment of bacteremia and urinary tract infections; additional studies are needed to demonstrate its efficacy.  

ESBLs are increasing in frequency among several species of enterobacteria. This information is of paramount importance for nosocomial infection control. CTX-M was the predominant type observed in our study, which is consistent with other reports from South America. The ESBL CTX-M has been found in hospitals and in community, animal, and environmental samples, which illustrates the challenge associated with containing bacteria expressing this ESBL type. The diversity of species in which CTX-M was found and the great genetic variability observed among *E. coli*, *K. pneumoniae*, and *E. cloacae* ESBL-producing bacteria indicate a predominantly polyclonal spread and high transfer efficiency of ESBL genes between bacteria in the hospital environment. Moreover, the spread of resistance can occur through the transmission of resistant strains, and resistant clones can be established and remain in hospitals for several years, eventually spreading to other hospitals and causing various types of infections. One example is the CTX-M-59 gene expressed in an *E. aerogenes* strain, which has not been found in other species and has exhibited monoclonal spread. Epidemiological evidence and local studies are important to elucidate the spread of EBSL types, which present a worldwide public health problem.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES


