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The role of horizontal gene transfer in the dissemination of extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates in an endemic setting

Yohei Doi, MD¹, Jennifer M. Adams-Haduch¹, Anton Y. Peleg, MD, PhD^{2,3,4}, and Erika MC D'Agata, MD, MPH^{2,*}

¹Division of Infectious Diseases, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

²Division of Infectious Diseases, Beth Israel Deaconess Medical Center, Boston, Massachusetts

³Department of Microbiology, Monash University, Clayton, Victoria, Australia

⁴Department of Infectious Diseases, The Alfred Hospital, Melbourne, Victoria, Australia

Abstract

The contribution of horizontal gene transmission (HGT) in the emergence and spread of extended-spectrum beta-lactamase (ESBL)-producing gram-negative bacteria during periods of endemicity is unclear. Over a 12-month period, rectal colonization with SHV-5 and SHV-12 producing-*Escherichia coli* and *Klebsiella pneumoniae* was quantified among a cohort of residents in a long-term care facility. Demographic and clinical data were collected on colonized residents. Transferability of SHV-encoding plasmids and pulsed-field gel electrophoresis was performed to quantify the contribution of HGT and cross-transmission, respectively. A total of 25 (12%) of 214 enrolled patients were colonized with 11 SHV-5- and 17 SHV-12-producing *E. coli* and *K. pneumoniae*. Clonally-related isolates were detected among multiple residents residing on the same and different wards. Among 12 clonally-distinct isolates, HGT of SHV-5- and SHV-12-encoding plasmids was identified among 6 (50%) isolates. HGT among clonally-distinct strains contributes to the transmission dynamics of these ESBL-producing gram-negative bacteria and should be considered when evaluating the spread of these pathogens.

Keywords

SHV-5; SHV-12; nursing homes; clonal; plasmid

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Corresponding author: Erika D'Agata, MD, MPH, Harvard Medical School, Beth Israel Deaconess Medical Center, Division of Infectious Diseases, Lowry Building Suite 6A, 110 Francis Street, Boston, MA 02215, Phone: (617)632-8407, edagata@bidmc.harvard.edu.

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1. Introduction

Extended-spectrum beta-lactamase (ESBL)-producing gram-negative bacilli are rapidly spreading throughout the world (Bradford 2001). ESBL genes are typically encoded on self-transmissible plasmids and can be mobilized between the same and different species of *Enterobacteriaceae*. As such, dissemination of ESBL-producing gram-negative bacilli is a complex process that involves both propagation of predominant clones between patients and horizontal gene transfer (HGT) of the responsible resistance genes between different clones ([Baraniak *et al.* 2002], [Fernandez *et al.* 2007], [Harris *et al.* 2007a], [Li *et al.* 2003], [Sidjabat *et al.* 2009], and [Velasco *et al.* 2007]).

Identification of the same or similar ESBL-encoding plasmids from different clones or species has been documented ([Fernandez *et al.* 2007], [Harris *et al.* 2007a], [Li *et al.* 2003], [Sidjabat *et al.* 2009], and [Velasco *et al.* 2007]). These studies typically investigated isolates that were collected from certain geographic areas in a cross-sectional fashion, or single patients over time. However, data regarding the actual contribution of HGT in non-outbreak, endemic settings remain rare. Our present study was thus carried out to define the relative role of plasmid transfer as opposed to clonal expansion in a longitudinal cohort of patients, using a long-term care facility (LTCF) as a model.

2. Materials and Methods

From October 2006 through September 2007, rectal swabs were collected every 1–3 months from 214 residents of a 600-bed long-term care facility in Boston, MA, residing in 4 of 12 wards. This institution-wide infection control initiative focused on the clinical and molecular epidemiology of multidrug-resistant gram-negative bacteria, the results of which have been reported elsewhere ([O’Fallon *et al.* 2009] and [O’Fallon *et al.* 2010]). This study specifically focuses on the molecular characterization of the subset of ESBL-producing *E. coli* and *K. pneumoniae* isolates. The Institutional Review Board at Hebrew Senior Life and the Clinical Committee of Investigation at Beth Israel Deaconess Medical Center approved the conduct of this study.

2.1. Resident data collection

All demographic and clinical data were collected from electronic medical and pharmacology records, and the Minimum Data Set (MDS). The MDS is a federally mandated, standardized assessment instrument required to be completed for all residents of nursing homes in the United States (Hawes *et al.* 1995). Clinical data included age, gender, co-morbidities and fecal and urinary continence. The Activities of Daily Living (ADL) score, obtained from the MDS, was used to characterize the ability of residents to conduct tasks independently (Katz *et al.* 1993). This metric ranges from 0, those residents completely dependent on healthcare workers for their ADLs, to 6, those fully independent. A composite score for co-morbidities was calculated using the Charlson score (Charlson *et al.* 1983). Antibiotic exposure and hospitalizations during the 12 months prior to enrollment and during the time period between the baseline culture obtained at enrollment and the follow-up culture was also collected.

2.2. Microbiological methods

Specimens were plated onto McConkey agar plates supplemented with ceftazidime (2mg/L) to minimize the recovery of gram-negative bacteria that do not produce ESBLs. Species and susceptibility testing using standard disk diffusion method was performed as per the Clinical and Laboratory Standards Institute (CLSI) methodology (CLSI, M02-A10 and M100-S20 (Clinical and Laboratory Standards Institute 2006). ESBL production among *E. coli* and *K.*

pneumoniae isolates that were resistant to ceftazidime was confirmed using the double disk method defined by the CLSI (M100-S20).

2.3. Detection of ESBL genes

The three common ESBL genes (TEM, SHV and CTX-M types) were detected by PCR experiments as described previously (Fernandez et al. 2007). In addition, bla_{CTX-M-15}-specific primers were used for the bla_{CTX-M}-positive *E. coli* isolate and all PCR products were sequenced to determine the exact ESBL gene (Fernandez et al. 2007).

2.4. Pulsed-field gel electrophoresis (PFGE)

All isolates producing SHV-5 or -12 ESBL were evaluated for genomic clonality by pulsed-field gel electrophoresis (PFGE). Fingerprints were generated by restriction endonuclease XbaI (New England Biolabs, Ipswich, MA) and subjected to electrophoresis using a CHEF-DR III system (Bio-Rad, Hercules, CA) as described previously (Fernandez et al. 2007). The relatedness of PFGE patterns was determined by the unweighted-pair group method using average linkages and cluster analysis with the Dice setting on Bionumerics software, version 6.01 (Applied Maths, Sint-Martens-Latem, Belgium).

2.5. Plasmid profiling

Plasmids from isolates producing SHV-5 or -12 ESBL genes were analyzed. The transferability of SHV-encoding plasmids was assayed by both transformation and conjugation as described previously (Fernandez et al. 2007). In brief, *E. coli* DH10B transformants or *E. coli* J53AziR transconjugants were selected by using 50 mg/L of ampicillin as the selective agent. The presence of the SHV gene was confirmed with PCR and supported by the ESBL phenotype in the transformants and transconjugants. The plasmids were then extracted using the standard alkaline lysis method (Sidjabat et al. 2009). The fingerprints of the SHV-encoding plasmids were generated by digesting plasmids with restriction enzyme HpaI or PstI (New England Biolabs). After an overnight electrophoresis, plasmid fingerprints were visualized by ethidium bromide staining and analyzed using the Bionumerics software as for PFGE.

3. Results

A total of 39 (18%) of 214 patients were colonized with ceftazidime-resistant isolates, of which 27 were *K. pneumoniae* and 12 were *E. coli*. Among these isolates, ESBL production was phenotypically confirmed among 23 *K. pneumoniae* and 7 *E. coli* isolates, recovered from 27 patients (3 patients were co-colonized with *K. pneumoniae* and *E. coli*). Antimicrobial susceptibility profiles are shown in Table 1.

3.1. ESBL genes

All 23 *K. pneumoniae* isolates had SHV-type ESBL genes. Of those, 5 were SHV-5, 17 were SHV-12 and 1 was SHV-31. Of the 7 *E. coli* isolates, 6 had SHV-5 and 1 had CTX-M-15. All *E. coli* and all but one *K. pneumoniae* isolates also had TEM-1, which is a non-ESBL that confers resistance to penicillins.

3.2. Clinical data among patients colonized with SHV-5 and SHV-12-producing isolates

A total of 25 (12%) patients were colonized with 28 isolates producing SHV-5 and SHV-12. The average age of residents was 94 years (range 81–96 years). Twenty-one residents (84%) were female and 22 (88%) were Caucasian. The average Charlson score was 2.6 (range 0–6) with the majority having a diagnosis of dementia (22 [88%]). The average ADL was 0 (range 0–2). Urinary or fecal incontinence was documented among 24 (96%) of residents. A

total of four (16%) and 6 (24%) residents had been hospitalized or received at least one course of antimicrobials during the year prior to enrollment, respectively. The average length of stay in the LTCF prior to the first culture was 8 years (range 5 months-14 years).

3.3. Clonality of SHV-5 and SHV-12-producing *E. coli* and *K. pneumoniae* isolates

The 28 isolates producing SHV-5 or SHV-12 were subjected to PFGE. Of the 6 *E. coli* isolates, all of which had SHV-5-encoding plasmids, 4 were closely related with >90% identity, whereas the other 2 isolates were unrelated (Fig. 1). The 5 SHV-5-producing *K. pneumoniae* isolates were grouped into 4 groups using a cutoff of 80% (Fig. 2). Thus, there were 7 groups of isolates producing SHV-5 for *E. coli* and *K. pneumoniae* combined. The 17 SHV-12-producing *K. pneumoniae* isolates were grouped into 5 groups using a cutoff of 80%, with the largest group containing 9 isolates (Fig. 2).

Ward location of residents harboring these isolates is shown in Figures 1 and 2. Clonally-related isolates were identified among residents residing on the same and different wards.

3.4. SHV-5 and SHV-12 plasmid analysis

Isolates representing each group as defined by a cutoff of 80% on PFGE were included for plasmid profiling. This included 7 SHV-5-producing isolates (3 *E. coli* and 4 *K. pneumoniae*) and 5 SHV-12-producing isolates (all *K. pneumoniae*). *E. coli* DH10B transformants were used for all isolates except one (*K. pneumoniae* R63RC), for which only *E. coli* J53 transconjugant was available. Transconjugants could be obtained for 10 isolates except *K. pneumoniae* R36RA and R215NA. As can be seen in the Fig. 3 and 4, 4 of the 7 SHV-5-encoding plasmids (2 from *E. coli*, 2 from *K. pneumoniae*) shared nearly identical restriction patterns (87 to 93% identity). Of the other 3 SHV-5-encoding plasmids, 2 of them (1 from *E. coli* and 1 from *K. pneumoniae*) shared 67% identity with each other using PstI but not with HpaI. The sizes of the SHV-5-encoding plasmids ranged between 50 and 80kb.

Of the 5 SHV-12-encoding plasmids, 2 were nearly identical with each other using both enzymes (84.0 to 93.7% identity), with another pair sharing the majority of restriction bands with this pair.

Therefore, in our assessment of 12 clonally-distinct isolates producing SHV-5 or SHV-12, HGT of ESBL-encoding plasmids was identified among 6 (50%) isolates.

3.5. Spatial and temporal analysis among patients colonized with identical or nearly identical plasmids

A total of 6 residents were colonized with clonally-distinct *K. pneumoniae* and *E. coli* carrying similar plasmids. The average age of these residents was 91 years old (range 88–96 years) and all were female and Caucasian. The average Charlson score was 2 (range 1–6) and all six had an ADL score of 0. Five had a diagnosis of dementia. Only one (17%) and 2 (33%) residents were previously hospitalized or received at least one course of antimicrobials, respectively, in the 12 months prior to study enrollment.

A total of 4 residents were colonized with *K. pneumoniae* and *E. coli* carrying similar SHV-5 encoding plasmids. All four residents resided on the same ward (Ward A) an average of 6.4 years (range 5 months to 13.5 years) prior to enrollment. The ESBL-producing isolates were identified from the baseline rectal culture obtained at study enrollment among 3 of these residents. None of these residents received antimicrobials during the 12-months prior to the baseline culture. For the fourth resident, ESBL-producing *K. pneumoniae* was first identified on the third follow-up culture obtained 162 days after the baseline culture. The only antimicrobial exposure in this resident was a 7-day course of oral trimethoprim-

sulfamethoxazole received during the interval between the baseline and third follow-up culture.

The two residents colonized with *K. pneumoniae* carrying the similar SHV-12 encoding plasmid resided on different wards (Ward A and B). Their average length of stay in these wards prior to obtaining the baseline culture was 8 years (2 and 14 years). Neither resident received antimicrobials or was hospitalized in the 12 months prior to enrollment. Colonization was identified from the baseline culture among both residents.

4. DISCUSSION

This study characterized the spread of SHV-5 and SVH-12-producing *E. coli* and *K. pneumoniae* isolates during a 12-month period of endemicity. At least 12% of residents in this LTCF were colonized with these ESBL-producing gram-negative bacteria. Although cross-transmission of clonally-related strains between patients was documented, transfer of ESBL-carrying plasmids also contributed to the spread of these multidrug-resistant organisms. In this study, 50% of clonally-unrelated strains harbored identical or nearly identical SHV-5 and SVH-12-carrying plasmids. This HGT occurred between the same *Enterobacteriaceae* species as well as between *E. coli* and *K. pneumoniae*.

In the outbreak setting, clonal dissemination is the predominant mechanisms of acquiring ESBL-producing gram-negative bacteria (Filozov *et al.* 2009). However, during periods of endemicity, several studies have documented that cross-transmission of these resistant bacteria only explains 4–50% of acquisition events ([Harris *et al.* 2007a], [Harris *et al.* 2007b], and [Kola *et al.* 2007]). One study showed that endogenous acquisition of multidrug-resistant gram-negative bacteria, defined as recovery of a non-MDR-gram-negative bacteria at baseline with subsequent recovery of an MDR-gram-negative bacteria of the same species and PFGE pattern during follow-up cultures, occurred among 69% of patients in an out-patient chronic hemodialysis unit over a 4-month period (Pop-Vicas *et al.* 2008). The findings of our study corroborate these data and imply that during periods of endemicity, both exogenous and endogenous acquisition of these ESBL-producing gram-negative bacteria contribute to their dissemination.

HGT occurs predominantly in niches with high bacterial densities, where the likelihood of cell-to-cell contact, and therefore plasmid conjugation is increased (Sorenson *et al.* 2005). After gene transfer, the newly formed resistant strain requires amplification via antimicrobial pressure ([Faure *et al.* 2010] and [Tenover *et al.* 2006]. In this study, only one of six residents in whom HGT was documented received antimicrobials, a 7-day course of oral trimethoprim-sulfamethoxazole. The minimal antimicrobial exposure suggests that there may be other factors involved, although a small sample size could also explain these findings.

The following limitations need to be considered. First, only isolates that were fully resistant to ceftazidime were analyzed for ESBL-production and therefore the prevalence of these pathogens may have been underestimated. Second, the study was conducted in a LTCF, a unique setting which allows evaluation of the transmission dynamics over prolonged periods of time, but differs from other healthcare environments. In this study, the average length of stay in the LTCF prior to enrollment was eight years. This prolonged exposure to other residents would provide substantially more opportunities for cross-transmission than in the hospital setting, and therefore the results of this study may not be generalizable. Third, due to logistical reasons, only residents from 4 of 12 wards were enrolled thereby providing a limited sample size. Lastly, the ESBL screening method using 2µg/L of ceftazidime in McConkey agar was designed to identify SHV- and TEM-type ESBLs, which were prevalent at the time of the study. This approach may have missed some CTX-M-type ESBL

producing organisms, which are not resistant to ceftazidime, especially those producing CTX-M-2 or CTX-M-9-group ESBLs, but would have, and did, identify those producing CTX-M-15, which are the most prevalent CTX-M-type ESBL in the U.S. (Johnson *et al.* 2012).

Current efforts to curtail the spread of ESBL-producing gram-negative bacteria focus on preventing cross-transmission and decreasing antimicrobial exposure, both during outbreak and endemic periods. The outcome of interest, in evaluating the efficacy of these interventions, is predominantly the reduction in spread of clonally-related strains. The results of this study suggest that quantification of HGT is also necessary during periods of endemicity.

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Fig. 1.
PFGE dendrogram of SHV-5-producing *E. coli*.

Fig. 2.
PFGE dendrogram of SHV-5/12-producing *K. pneumoniae*.

Fig. 3.

Restriction profiles of SHV-12-encoding plasmids obtained from transformants, with the exception of *K. pneumoniae* R63, which was obtained from its transconjugant. Lanes M, 1 DNA-HindIII digest marker; 1 and 7, *K. pneumoniae* R8; 2 and 8, *K. pneumoniae* R18; 3 and 9, *K. pneumoniae* R36; 4 and 10, *K. pneumoniae* R152; 5 and 11, *K. pneumoniae* R178; 6 and 12, *K. pneumoniae* R63. Lanes 1 through 6 were digested with HpaI; lanes 7 through 12 were digested with PstI.

Fig. 4. Restriction profiles of SHV-5-encoding plasmids obtained from transformants. Lanes M, 1 DNA-HindIII digest marker; 1 and 7, *E. coli* R27; 2 and 8, *E. coli* R31; 3 and 9, *E. coli* R173; 4 and 10, *K. pneumoniae* R173; 5 and 11, *K. pneumoniae* R1RC; 6 and 12, *K. pneumoniae* R215. Lanes 1 through 6 were digested with HpaI; lanes 7 through 12 were digested with PstI.

Table 1

Antimicrobial susceptibility profile of *Klebsiella pneumoniae* and *Escherichia coli* isolates recovered from 28 patients, using standard disk diffusion method

	<i>K. pneumoniae</i> (n = 23)			<i>E. coli</i> (n = 7)		
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
Ceftazidime	0	0	23 (100%)	0	0	7 (100%)
Cefotaxime	0	0	23 (100%)	0	1 (14%)	6 (86%)
Cefoxitin	19 (83%)	4 (17%)	0	6 (86%)	0	1 (14%)
Cefepime	21 (91%)	2 (9%)	0	6 (86%)	0	1 (14%)
Aztreonam	0	0	23 (100%)	0	0	7 (100%)
Ertapenem	23 (100%)	0	0	7 (100%)	0	0
Imipenem	23 (100%)	0	0	7 (100%)	0	0
Gentamicin	22 (96%)	0	1 (4%)	7 (100%)	0	0
Amikacin	4 (17%)	13 (57%)	6 (26%)	6 (86%)	1 (14%)	0
Ciprofloxacin	3 (13%)	0	20 (87%)	6 (86%)	0	1 (14%)
Colistinazolate	10 (43%)	7 (30%)	6 (26%)	6 (86%)	0	1 (14%)