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OCCURRENCE AND ANTIMICROBIAL RESISTANCE OF *Salmonella* spp. AND OTHER ENTEROBACTERIA RECOVERED FROM KITCHEN EQUIPMENT OF A UNIVERSITY HOSPITAL IN RIO DE JANEIRO, BRAZIL

MIYAHIRA R.F., SANTOS E.F.A.S., FREITAS-ALMEIDA A.C. AND QUEIROZ M.L.P.*

Dept. de Microbiologia, Imunologia e Parasitologia, Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro -UERJ- Brasil.
*Corresponding Author: Email- qmara6@gmail.com

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Abstract-

Introduction: One of the most significant risk factors identified in food contamination is cross-contamination among the food and the preparation surfaces. We verified the occurrence and the antimicrobial resistance of enterobacteria in equipment used to prepare diets to hospitalized patients in a university hospital in Rio de Janeiro, Brazil.

Methods: A total of 60 samples were collected from semi-industrial equipment (one blender and one mixer) in the hospital's kitchen. Enterobacteriaceae species were identified by classical methods. Antimicrobial susceptibility testing was carried out by the disk diffusion method and minimum inhibitory concentration was determined by the broth microdilution method. The detection of beta-lactamases genes was determined by PCR.

Results: Ninety-seven isolates of Enterobacteriaceae have been identified. We isolated six *Salmonella* spp. The susceptibility test revealed that 77% (n=75) of the isolates presented resistance to at least one antimicrobial agent. The search for extended-spectrum beta-lactamases genes indicated the presence of bla_{SHV} gene in a *Klebsiella pneumoniae* subsp. *pneumoniae* isolate. The sequencing identified the SHV-36 enzyme.

Conclusion: We found important enterobacteria contamination in the hospital kitchen equipment, indicating that the hygiene procedures should be improved. Furthermore, we recovered *Salmonella* spp. isolates from both the blender and the mixer, showing that diets may act as potential vehicles for the dissemination of enteropathogens in this scenario.

Keywords- Hospital diets, *Salmonella* spp., Enterobacteria, Kitchen equipment, Antimicrobial resistance, beta-lactamases

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Introduction

Foodborne diseases, particularly gastrointestinal infections, represent a very large group of pathologies with strong negative impact in public health because of their widespread nature. Little consideration is given to those infections due to the fact that their symptoms are often moderate and self-limiting. However, food contamination can be very dangerous if occurring in a hospitalized patient. Food hygiene in hospital poses peculiar problems, mainly by the presence of patients who may be more vulnerable to microbiological and nutritional risks than healthy subjects [1].

One of the most significant risk factors identified in food contamination is cross-contamination among the food and the preparation surfaces. The failure to effectively remove bacteria from food contact surfaces may have serious implications in the transmission of foodborne diseases [2]. The risk of cross-contamination is further increased if the food temperature is not subsequently controlled or a surface is left uncleaned, permitting bacterial growth [3]. Various bacteria, including *Escherichia coli* and *Salmonella* spp. survive on hands, sponges, utensils and currency for hours or days. Therefore, the relationship between contaminated surfaces and transmission of foodborne pathogens is evident in food processing [4].

Members of the Enterobacteriaceae family are important causes of healthcare-associated infection. Emerging resistance in Enterobacteriaceae is a significant problem that requires immediate attention [5]. Antimicrobial resistance is an increasing threat in hospitalized patients and both mortality and morbidity from infection are higher when caused by antimicrobial-resistant bacteria. The growing prevalence of multiresistant pathogens in nosocomial settings is frequently related to the high selective pressure of antimicrobials commonly used in hospitalized patients [6].

The most common mechanism of resistance among Enterobacteriaceae is the production of extended-spectrum beta-lactamases (ESBL), which can inactivate certain beta-lactam antibiotics [7]. Therefore, most ESBL producers often exhibit multidrug-resistance phenotypes [8]. Considering the high number of immunocompromised patients and the extensive use of antimicrobial agents inside

hospitals, the study of antibiotic resistance in this environment is very important. Therefore, the aims of this work were to verify the occurrence and antimicrobial resistance of enterobacteria recovered from equipment used to prepare diets to hospitalized patients in a university hospital in the city of Rio de Janeiro, Brazil.

Materials and Methods

Samples Analysis

During a 15-week period, a total of 60 samples were collected from semi-industrial equipment (one blender and one mixer) from the kitchen of a tertiary care teaching hospital with 525 beds located in the city of Rio de Janeiro. The choice of the blender and the mixer was made based on the frequency of use and the level of sanitization. The samples were collected from the equipment surfaces after the sanitization processes with sterile swabs twice a week. The swabs were transported in sterile saline solution (0.85% NaCl) under refrigeration and immediately processed in the laboratory. The quantitative microbiological analysis of the equipment was performed by two different methods: (1) aerobic colony counts at 35°C and (2) determination of most probable number (MPN) of coliform bacteria at 35°C and 45°C, according to the recommendations of the American Public Health Association (APHA) [9]. After the determination of MPN of coliforms, the presence of Enterobacteriaceae was evaluated by plating 0.1 mL of the growth in EC broth onto Eosin Methyl Blue Agar (EMB, OXOID, LTD., Basingstoke, Hampshire, England). The characteristic colonies were stored in glycerol GC 20% at -20°C.

Physiological Characterization of Enterobacteriaceae

After storage, strains were inoculated into 3 mL of Brain Heart Infusion (BHI, Difco, Becton Dickinson, Maryland, USA) and incubated at 35 ± 2°C for 24 hrs. Growth was plated on EMB agar plates and incubated at 35 ± 2°C for 24 hrs. Up to five different colony types from each sample were identified by classical methodology as a species of the Enterobacteriaceae family [10,11].

Antimicrobial Susceptibility Testing and Screening of β-lactamases

Isolates were tested by the disk diffusion method, according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) [12]. The following antimicrobial drugs were tested (OXOID): cefepime (30 µg), aztreonam (30 µg), chloramphenicol (30 µg), cephalotin (30 µg), cefoxitin (30 µg), imipenem (10 µg), tetracycline (30 µg), gentamicin (10 µg), cefotaxime (30 µg), amikacin (30 µg), sulfamethoxazole/trimethoprim (1.25 µg/ 23.75 µg), ceftazidime (30 µg), ampicillin (10 µg), tobramycin (10 µg), ciprofloxacin (5 µg), ampicillin/sulbactam (10µg/10µg) and nalidixic acid (30 µg) (*Salmonella* spp.). Disk diffusion assays with ceftazidime (30 µg), ceftazidime+clavulanic acid (10/10µg), cefotaxime (30 µg) and cefotaxime+clavulanic acid (30/10µg) were performed for the detection of ESBL among strains showing positive results on the initial screening test for ESBL recommended by the CLSI [12]. For the detection of AmpC beta-lactamases, the approximation disk test with cefoxitin (30 µg) and cefotaxime (30 µg) was used [13]. Reference strains *E. coli* ATCC 25922, *E. coli* ATCC 35218 and *Klebsiella pneumoniae* ATCC 700603 were used as control.

Minimum Inhibitory Concentration (MIC)

Isolates with positive results on the screening tests for ESBL and AmpC beta-lactamases had their MICs determined for ceftazidime/ cefotaxime and cefoxitin, respectively. MIC was determined by the broth microdilution method using cation-adjusted Mueller-Hinton broth (Difco Laboratories), according to CLSI guidelines [12]. Reference strain *E. coli* ATCC 25922 was used as control.

Detection of Beta-lactamase Genes

The detection of beta-lactamase genes was determined by Polymerase Chain Reaction (PCR). Specific primers were used for the detection of the bla_{TEM}, bla_{SHV}, bla_{OXA-1}, bla_{CTX-M} and bla_{CMY-2} genes [14]. DNA was extracted by thermal lysis. The PCR was performed in a 50 µL reaction mixture containing: 31 µL of ultra pure water, 1x Taq polymerase buffer, 1.5 mM $MgCl_2$, 20 µM of each primer, 2mM of each dNTP, 0.5 U Taq DNA polimerase (Invitrogen, Carlsbad, California, USA) and 5 µL of DNA template. Positive control strains were kindly provided by the Enterobacteria Laboratory of the Oswaldo Cruz Institute (Rio de Janeiro, Brazil). *E. coli* DH5-α was used as negative control for all reactions. PCR products were separated by electrophoresis in 1 % agarose gels (1 x TBE) at 100 V, stained with ethidium bromide and photographed under UV light.

Sequencing of β-lactamase Genes

Before sequencing, all PCR products were purified using the AxyPrep Bacterial Genomic DNA Miniprep Kit (Axygen Biosciences). Sequencing reactions were performed with the same primers used for PCR detection using the DYEnamic ET terminator cycle sequencing kit (GE Healthcare). Chromatograms were transformed into FASTA format with Phred software [15]. The obtained nucleotide sequences were analysed by BLAST [16] searches in GenBank database (http://www.ncbi.nlm.nih.gov) and were aligned with representative bacterial sequences obtained from the public databases using ClustalX software [17].

Results

The Brazilian legislation does not establish microbiological standards to swab tests in kitchen equipment. Dancer suggests that microbial counts on food-processing equipment should be < 5 CFU/ cm^2 [18]. We found colony counts above 2 x 10 CFU/cm^2 in 70% of the equipment. Coliform counts higher than 10 MPN mL^{-1} at 35°C were observed in the blender (76.6%) and the mixer (73.3%). Similarly, the percentage of samples presenting coliform contamination at 45°C was 66.6% (blender) and 63.3% (mixer). The physiological characterization indicated 97 Enterobacteriaceae isolates: 58 from the mixer and 39 from the blender [Table-1]. *Enterobacter* spp. was the most frequent isolate (37%; n=36/97), followed by *Klebsiella* spp. (19%; n=18/97) and *Citrobacter* spp. (19%; n=18/97). We isolated six *Salmonella* spp. (6%), four from the mixer and two from the blender.

The susceptibility test revealed that 77% (n=75) of the isolates showed resistance to at least one antimicrobial agent tested and 38% (n=37) presented multiple resistance (three or more drugs). Resistance or intermediate resistance to seven antimicrobial agents was observed in two isolates of *Enterobacter cloacae*. We found 12 isolates with ceftazidime, cefotaxime or aztreonam inhibition zone diameters compatible with the initial screening test for ESBL recommended by the CLSI [12]. From these 12 isolates, seven were isolated from the blender and five from the mixer. Ten of these isolates presented multiple antimicrobial resistance, while eight were resistant to cefotaxime and four to ceftazidime. The expression of AmpC beta-lactamases was observed in 32 isolates; however, 31 were identified as belonging to the ECSM species (*Enterobacter* spp., *Citrobacter* spp., *Serratia* spp., and *Morganella morganii*), with known chromosomally encoded AmpC beta-lactamases. One of the

isolates showing this phenotype was characterized as *K. pneumoniae* subsp. *pneumoniae*. Three *Salmonella* spp. isolates were resistant to nalidixic acid and susceptible to ciprofloxacin.

Table 1- Enterobacteriaceae isolates recovered from a mixer and a blender used to prepare diets for hospitalized patients

Genus	Species or Serotypes	Equipment		Total
		Mixer	Blender	
Citrobacter	*C. braakii*	2	1	18
	C. gillenii	9	5	
	C. murliniae	0	1	
Enterobacter	*E. aerogenes*	4	0	36
	E. amnigenus Biogroup 1	4	4	
	E. asburiae	1	0	
	E. cloacae	7	5	
	E. cowanii	2	0	
	E. intermedium	1	2	
	E. kobei	5	1	
Escherichia	*E. coli*	4	2	6
Klebsiella	*K. pneumoniae* subsp. *ozaenae*	2	0	18
	K. pneumoniae subsp. *pneumoniae*	7	9	
Pantoea	*P. agglomerans*	0	2	2
Raoultuella	*R. planticola*	1	0	11
	R. terrigena	5	5	
Salmonella	*S.* Give	2	0	6
	S. Enteritidis	1	1	
	S. Typhimurium	1	1	
Total		58	39	97

MIC results indicated that all AmpC beta-lactamase producing isolates were resistant to cefoxitin (MIC ≥ 32 µg/mL). However, the *bla*$_{CMY-2}$ gene was not found in these isolates. From the 12 isolates with ceftazidime, cefotaxime or aztreonam inhibition zone diameters compatible with the initial screening test for ESBL, 11 were found to be resistant or intermediate resistant to cefotaxime (MIC > 1 µg/mL) and three were found to be resistant or intermediate resistant to ceftazidime (MIC > 4 µg/mL) [Table-2]. The search for ESBL genes in these 12 Enterobacteriaceae isolates indicated the presence of the *bla*$_{SHV}$ gene in a *K. pneumoniae* subsp. *pneumoniae* isolate recovered from the blender [Fig-1]. Sequence results identified as the SHV-36 enzyme.

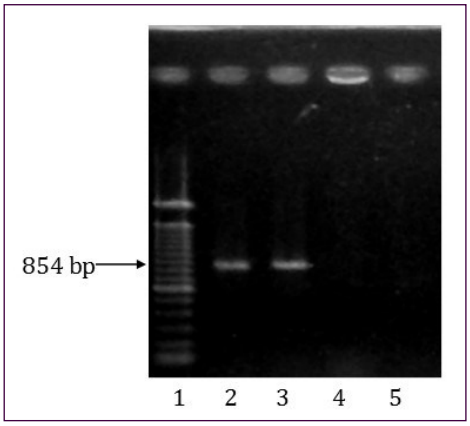

Fig. 1- PCR amplification of *bla*$_{SHV}$ gene. Lane 1: 100bp ladder; lane 2: *E. coli* 18 (positive control); lane 3: *Klebsiella pneumoniae* subsp. *pneumoniae* K42; lane 4: *E. coli* DH5-α (negative control) and lane 5: reaction mix.

Table 2- Minimum Inhibitory Concentration (MIC) values, antimicrobial susceptibility and presence of beta-lactamase genes in isolates with inhibition zone diameters compatible with the initial screening test for ESBL.

Isolate	Origin	Species	Ceftazidime		Cefotaxime		Presence of β-lactamase genes
			MIC (µg/mL)	Antimicrobial susceptibility (mm)	MIC (µg/mL)	Antimicrobial susceptibility (mm)	
E100	blender	*Enterobacter cloacae*	< 0.5	26	64	6	none
EC99	mixer	*Escherichia coli*	< 0.5	25	2	26	none
C90	blender	*Citrobacter gillenii*	< 0.5	27	8	28	none
S84	mixer	*Salmonella* Give	< 0.5	24	8	27	none
S79b	mixer	*Salmonella* Typhimurium	< 0.5	21	8	21	none
S76b	mixer	*Salmonella* Give	< 0.5	6	4	10	none
E66	mixer	*Enterobacter cloacae*	16	6	> 128	6	none
K42	blender	*K. pneumoniae* subsp. *pneumoniae*	< 0.5	26	1	26	*bla*$_{SHV}$
E29	blender	*Enterobacter cloacae*	16	6	16	6	none
E27	blender	*Enterobacter kobei*	8	8	> 128	12	none
E26	blender	*Enterobacter cloacae*	1	20	2	10	none
K6a	mixer	*K. pneumoniae* subsp. *ozaenae*	4	25	4	6	none

Discussion

The importance of microbial surface contamination for the epidemiology of infectious diseases has long been recognized. Environmental surface contamination has also been specifically implicated in a number of hospital-acquired infections. The potential for contaminated surfaces to contribute to the transmission of pathogens depends on several factors, including the ability of pathogens to remain viable on different environmental surfaces, the frequency with which they contaminate surfaces and whether the levels of contamination are high enough to result in transmission to patients [19,20].

Nevertheless, few studies have evaluated the contamination of surfaces in hospital kitchens. We found colony counts above 2 x 10 CFU / mL in 70% of the samples analyzed. A study developed in two hospitals in Egypt examined the contamination of utensils used in the preparation of diets. The authors performed aerobic mesophilic counts by swabbing the surfaces of the following food utensils: meat knives, vegetable knives, meat chopping boards, salad chopping boards, cooking pan, roasting pan, patient tray and food distribution containers. Similarly to our results, the authors found aerobic mesophilic counts exceeding 10^2 CFU / mL in all food utensils, except the food distribution containers. They also highlighted

that the contaminated surfaces of equipment and utensils in a hospital kitchen may be vehicles of infection and may also promote the spread of pathogens in the nosocomial environment [21].

Healthcare-associated infections are increasing in prevalence due to a number of factors, including aging populations, increasing number of immunocompromised patients, as well as increasing use of invasive interventions [22]. During the last few decades, the frequency and the spectrum of antibiotic resistant infections have increased steadily within the United States, Europe and the developing world. This increase has been attributed to a combination of microbial characteristics, the selective pressure of antimicrobial use, and social and technical changes [23].

Our results indicated the presence of many multiresistant Enterobacteriaceae in the hospital kitchen equipment. Therefore, the diets prepared in the blender and the mixer may potentially disseminate isolates with important resistance profiles inside the hospital, especially because the food does not suffer heat treatment after the use of the equipment. Disk diffusion tests indicated higher rates of antimicrobial resistance in *Enterobacter* spp., *Citrobacter* spp., *Raoultella* spp, *Klebsiella* spp. and *Salmonella* spp. isolates.

Enterobacter cloacae was one of the 10 most common blood culture pathogens identified in Canadian hospitals between 2007 and 2009 [24]. ESBL-producing *E. cloacae* are being increasingly reported worldwide. In a study conducted at a hospital in Tunis, of the 66 *E. cloacae* isolates tested, 44 (67%) were ESBL producers [25]. In our investigation, *Enterobacter* spp. was the most frequent microorganism (37%) and two *E. cloacae* isolates presented resistance or intermediate resistance to seven antimicrobial agents.

K. pneumoniae, one of the most important nosocomial pathogens, has demonstrated the ability to develop and/or acquire new resistance determinants and is thought to be a reservoir of antimicrobial resistance genes [26]. In our research, we detected the bla_{SHV-36} gene in a *K. pneumoniae* subsp. *pneumoniae* isolate recovered from the blender. However, according to the classification of beta-lactamases (www.lahey.org/studies), it is still not clear whether the SHV-36 is an ESBL.

Conversely, Munday et al. classified the SHV-36 beta-lactamase as an ESBL [27]. They identified the bla_{SHV-36} gene in a *Klebsiella* spp. isolate recovered from faeces submitted for the diagnosis of diarrheal disease from a hospital-based patient in York, United Kingdom. The MIC results showed resistance to cefotaxime (MIC = 4 mg/L) and susceptibility to ceftazidime (MIC < 0.25 mg/L) [27]. Our results indicated that the *K. pneumoniae* isolate was susceptible to both antimicrobials (ceftazidime = 1 µg/mL and cefotaxime = 2 µg/mL). A study conducted in Portugal also detected the bla_{SHV-36} gene in *K. pneumoniae* clinical isolates and considered this enzyme as a broad-spectrum beta-lactamase [28].

The *K. pneumoniae* isolate also showed a presumptive AmpC-producing phenotype. However, we could not find the bla_{CMY-2} gene. Many nosocomial isolates of *Klebsiella* spp. producing plasmid-mediated AmpC β-lactamases have been involved in several worldwide outbreaks of infection. Often, genes encoding plasmid-mediated AmpC beta-lactamases coexist in the same plasmid with genes encoding mechanisms of resistance to other classes of antibiotics, leaving clinicians with limited therapeutic options [29].

Of the twelve Enterobacteriaceae strains with positive results on the initial screening test for ESBL, eleven were resistant to cefotaxime and three to ceftazidime. These findings are of great concern, since these drugs are widely used in hospitals. Although the beta-lactamase encoding genes have not been found in these strains, we believe that the observed phenotype is probably related to the presence of other genes that have not been investigated and/or the primers used in this study [14].

Over the last decade, the high incidence of multidrug resistance in Enterobacteriaceae has become a serious public health problem worldwide. Because of their critical importance for human and veterinary medicine, resistance to extended spectrum beta-lactams, especially third- and fourth-generation cephalosporins and penems, is of special interest. The increasing presence of these beta-lactamases in pathogenic bacteria limits the therapeutic use of these drugs [8]. They are spread among bacterial species by plasmids, often carrying multiple antibiotic resistance genes [30]. In a prospective study of 455 consecutive episodes of *K. pneumoniae* bacteremia in 12 hospitals in seven countries, 85 episodes were attributed to an ESBL-producing organism. Failure to use an antibiotic active against ESBL-producing *K. pneumoniae* was associated with extremely high mortality [26].

Conclusion

We found significant contamination in the kitchen equipment used to prepare diets to hospitalized patients. Our findings showed that the safety hygiene procedures in handling the hospital kitchen equipment should be improved. Importantly, reports of our results were sent to the Division of Nutrition of the University Hospital and measures to control the cleaning processes of kitchen equipment were reviewed. Moreover, trainings were offered for food handlers, with the aim of preventing cross-contamination in kitchen environments. Considering that we recovered *Salmonella* spp. isolates from both the blender and the mixer, our results indicate that diets may act as potential vehicles for the dissemination not only of enteropathogens and enterobacteria, but also of resistance genes.

List of Abbreviations

APHA: American Public Health Association

ATCC: American Type Culture Collection

BHI: Brain Heart Infusion

CFU: Colony-Forming Unit

CLSI: Clinical and Laboratory Standards Institute

ECSM: *Enterobacter* spp., *Citrobacter* spp., *Serratia* spp. and *Morganella morganii*

EMB: Eosin Methylene Blue Agar

ESBL: Extended-spectrum beta-lactamases

MIC: Minimum Inhibitory Concentration

MPN: Most Probable Number

PCR: Polymerase Chain Reaction

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Conflict of Interest : None declared.

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