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Evaluation of Antimicrobial Susceptibility by *Staphylococcus aureus* Isolated from the Nutrition Service of a Teaching Hospital

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Abstract

The bacteria *Staphylococcus aureus* is found in the nasal passages, mouth and skin of the human population and stands out among foodborne diseases. Some causes of foodborne illness are related with food, handlers, and utensils contamination in a hospital's nutrition service. The aim of this study was to investigate the presence of *S. aureus* in food handlers, equipment, counter-tops and utensils of the nutrition service of a teaching hospital. Samples of the environment, hands and nasal mucosa of employees of a nutrition service were collected with two sterile swabs, in 2016, March and June. A total of 134 samples were obtained, which were submitted to characterization tests Biochemistry and morphotinorial (gram staining, catalase and coagulase tests in tube), phenotypic evaluation by drug diffusion technique and D-test approach, and the biofilm phenotype characterization by culture on Congo Red Agar. Results showed high rates of *S. aureus* colonization, mainly in the kitchen, high frequency of antimicrobial resistance, especially erythromycin and the presence of multi-resistant microorganisms. Large number of positive samples was also found for biofilm production, with totality for the samples of handlers. We highlight the relevance of the data in virtue of the serious consequences and risks that can be triggered in the hospital environment.

Keywords

Staphylococcus aureus, Multiresistance, Contamination, Manipulators

1. Introduction

Diet is present in all phases of human life, and is of extreme importance for maintaining health and well being [1] [2]. Food is essential for life as a basic necessity, however, adequate access to quality products has become a public health problem that can cause damage to health [2] [3]. For this reason, institutions that provide food must necessarily offer a nutritionally balanced and hygienically safe product [4].

One of the possible causes of nosocomial infection is the consumption of contaminated food. Food contamination can begin in the production of primary material or throughout any of the preparation phases, from receipt, through storage, pre-preparation and preparation [5] [6]. As such, employees of hospital Food and Nutrition Units (UANs) have a very important function as they prepare food for individuals with compromised health as well as represent a potential source of contamination with food-borne illnesses [4] [7] [8].

Among food-borne illnesses, those of bacterial origin have been pointed to as the most complicated public health problem in the world. Of the bacteria responsible for food-borne diseases, *Staphylococcus aureus* (*S. aureus*), the gram-positive bacteria that can replicate in a wide range of temperatures, stands out. In favorable conditions these bacteria can produce various toxins including staphylotoxin, an enterotoxin that can resist the high temperatures of cooking. These bacteria are mainly found in food products that require a lot of manipulation, since 20 – 60% of the human population is colonized by *S. aureus*, found mainly in the nasal passages, mouth and skin of food handlers [7] [9] [10]. Another relevant factor in infections caused by these micro-organisms is antibiotic resistance. Research has been developed to better understand the mechanisms involved in antibiotic resistance as it can result in more severe infections and faster evolution of the bacteria [11] [12]. It is believed that antibiotic resistance in *S. aureus* originates in genetic mutation or by acquisition of resistance genes from other bacteria. However, the greatest risk of infection by resistant strains occurs in people who work in or frequent hospitals [11] [12] [13] [14] [15].

Beyond the mechanisms of antimicrobial resistance, *S. aureus* presents diverse virulence factors including bacterial adhesion and production of extracellular polysaccharide slime, which seems to play a relevant role in the dissemination and persistence of these microorganisms in the environment. This slime forms a biofilm on plastic surfaces that contains various layers of bacteria [16]. Understanding the concept of microbial biofilms and aspects inherent to their structure and composition, as well as the process of their formation, are fundamental in developing effective control strategies [17].

Considering this, contamination of the environment, food handler, utensils and equipment is an important link between food, patient and food-born illnesses. The dissemination of *S. aureus* in Food and Nutrition Units in a university hospital in Presidente Prudente, São Paulo has not yet been studied, making

such contamination a threat to that space and the people who occupy it. Considering that there are actions and strategies to control and eliminate microorganisms from health service related spaces, this study set out to investigate the prevalence of *S. aureus* in food handlers, equipment, countertops and milk preparation utensils in enteral diet preparation areas and commissary kitchen of a hospital, as well as evaluate the resistance profile of these bacteria to different antibiotics and phenotypically characterize biofilm formation.

2. Material and Methods

2.1. Location and Sampling

The study was conducted at the Regional Hospital (HR) of Presidente Prudente "Dr. Domingos Leonardo Cerávolo", located in the west of the State of São Paulo, which is a reference in elective and urgent/emergency medical care for 45 municipalities in the region, in addition to the indirect demand of two other Brazilian states: Paraná and Mato Grosso do Sul. It is a public hospital with 550 beds, including 56 beds of Intensive Care Unit (ICU), 20 adults, 10 coronary, 06 pediatric, 20 Neonatal and has approximately 2000 employees from all over the region.

To evaluate seasonal influence on the presence of *S. aureus*, the present study was conducted in two phases (They were collected in 2016, March and June.) and three distinct food service areas of a teaching hospital (commissary kitchen, milk preparation (lactary) and enteral diet production). In each stage, a total of 67 samples were evaluated, 32 of which were collected from the hands and nasal passages of food handlers (with signed informed consent), and 35 of which were collected from equipment, utensils and surfaces (such as doors, countertops, vats, blender and meat slicer), totaling 134 samples.

It is important to emphasize that sample collection was conducted without prior announcement during busy hours and that all food handlers working in the sector at the time of sampling participated in the study. The months were chosen for their variation in temperature, March having high temperatures and June having low. On the day of the first collection, there was a low of 20°C and high of 35°C, and on the second there was a low of 14°C and high of 28°C.

For material collection, sterile swabs moistened with previously sterilized 0.9% sodium chloride solution were used. For sample collection from food handlers, the swab was inserted into the anterior nasal passage using three delicate circular movements. For hands, collection was performed in the dorsal and anterior area of both hands of each food handler. Utensils, equipment and areas were submitted to the same collection method.

2.2. Seeding and Identification of Colonies

After collection, the swabs were placed in tubes containing BHI (Brain Heart Infusion) broth and transported in a cooler to the microbiology laboratory of the São Paulo Western University (UNOESTE) for future identification.

Culture medium was incubated at 37˚C for 24 hours. Tubes with turbidity were streaked onto mannitol salt agar plates and incubated at 37˚C for 24 hours. Typical colonies showing yellow coloration, indicative of mannitol fermentation, were used for identification of *S. aureus* [18].

Subsequently, biochemical and morphological tests such as Gram stain, catalase and coagulase tests were performed for the identification of *S. aureus* as described by reference [18].

2.3. Susceptibility Tests

Phenotypic determination of resistance profile was performed using the disk diffusion test following the recommended criteria of the Clinical Laboratory Standards Institute (CLSI) [19]. *S. aureus* isolates were inoculated in BHI broth and incubated at 37˚C for 24 hours. The inoculum was then diluted in 0.9% saline and bacterial density was adjusted to 0.5 turbidity on the McFarland scale, corresponding to approximately 10^8 CFU/mL.

Suspensions were spread on plates containing Mueller-Hinton agar using sterile swabs. Filter paper disks containing antimicrobials were applied on the surface of the agar and the plates were incubated at 37˚C for 24 hours. Antimicrobial activity was evaluated by the diameter of inhibition zone and interpreted based on the standards established by CLSI.

The drugs utilized were: oxacillin (1.0 μg), cefoxitin (30.0 μg), clindamycin (2 μg) and erythromycin (15 μg), tetracycline (15.0 μg) and gentamicin (10.0 μg). Induced clindamycin resistance was evaluated by the D-Test, using erythromycin (15 μg) and clindamycin (2 μg) disks placed laterally at a distance between 15 and 23 mm, and incubated under the same conditions. The D-Test was considered positive with the presence of any flattening of the clindamycin inhibition halo in the confluence zone of the two antibiotics. The standard *S. aureus* strain ATCC 25923 was used as a control in the antibiotic sensitivity test.

2.4. Biofilm Formation Test

Congo red agar was used for phenotypic characterization of biofilm, using the method described by reference [20]. Samples were streaked on Congo Red Agar plates and incubated at 37˚C for 24 - 48 hours, with biofilm producing colonies showing black coloration. Colonies with red coloration were considered unable to produce biofilm [21].

2.5. Data Analysis

Presence of *S. aureus* and antimicrobial resistance were descriptively analyzed, such that the verification of differences between frequency of sample resistance was performed by chi-squared test (χ^2) (P < 0.05).

3. Results

Identification of *S. aureus* was carried out as described in the methods. In the first sampling, *S. aureus* was identified in 51 of the 67 samples. Of these, 32 sam-

ples were from food handlers, with 28 samples positive for *S. aureus*, a positive rate of close to 87%. With respect to samples from equipment and utensils, 66% positivity was found. In the second sampling, *S. aureus* was identified in 25 samples, with 53% positive samples from food handlers and 23% positive samples from equipment and utensils. The number of samples positive for *S. aureus* was analyzed separately according to nutrition preparation area, with the percentage for food handlers and equipment and countertops shown in Table 1.

As shown in Figure 1, the values for positive samples were statistically different between the first and second sampling, considering that they were analyzed in the same environments ($p < 0.05$).

Table 1. Number of samples positive for *S. aureus* from food handers and equipment and countertops in different nutrition preparation areas.

	1st Sampling			2nd sampling		
	Number of Samples	Number positive	%	Number of Samples	Number of Samples	%
LACTARY						
Equipment/Countertops	14	07	50	14	02	14.28
Handler	08	04	50	08	06	75
KITCHEN						
Equipment/Countertops	14	13	92.85	14	04	28.57
Handler	24	24	100	24	11	45.83
ENTERAL						
Equipment/Countertops	07	03	42.85	07	02	28.57
Handler	*					

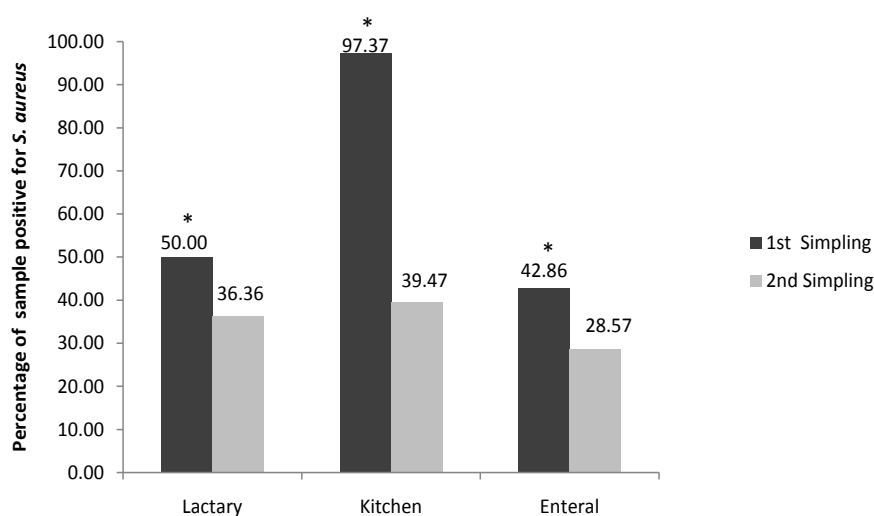*Handlers in the enteral diet preparation area were the same as the lactary

Figure 1. Percentage of the presence of *S. aureus* in samples collected in the lactation, kitchen and enteral of a Teaching Hospital (*$p < 0.05$).

Within the environments analyzed, the kitchen was subdivided into the butchery, pre-preparation and preparation areas. In the lactary, the dirty (bottle cleaning) and clean (preparation and portioning) areas were analyzed separately. Separation in the enteral diet preparation area was not established. The specific positivity for each area is shown in Figure 2 and Figure 3.
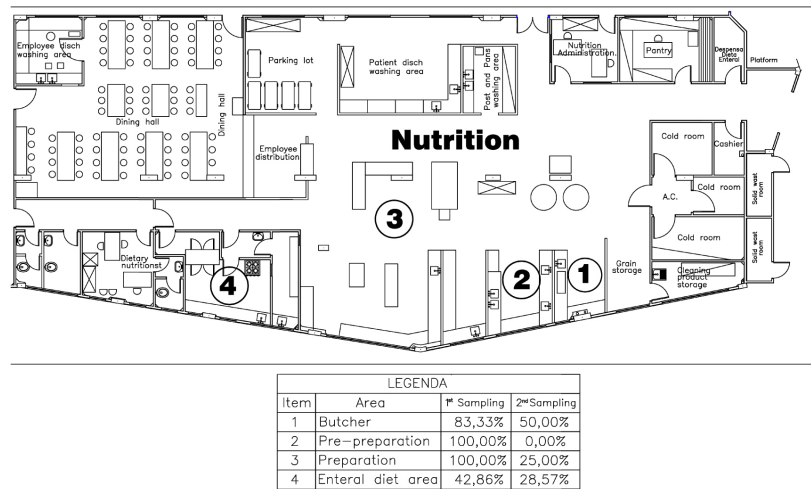

LEGENDA			
Item	Area	1ª Sampling	2ⁿᵈ Sampling
1	Butcher	83,33%	50,00%
2	Pre—preparation	100,00%	0,00%
3	Preparation	100,00%	25,00%
4	Enteral diet area	42,86%	28,57%

Figure 2. Positivity in areas, benches and equipment in sectors identified in the layout of the nutrition service.

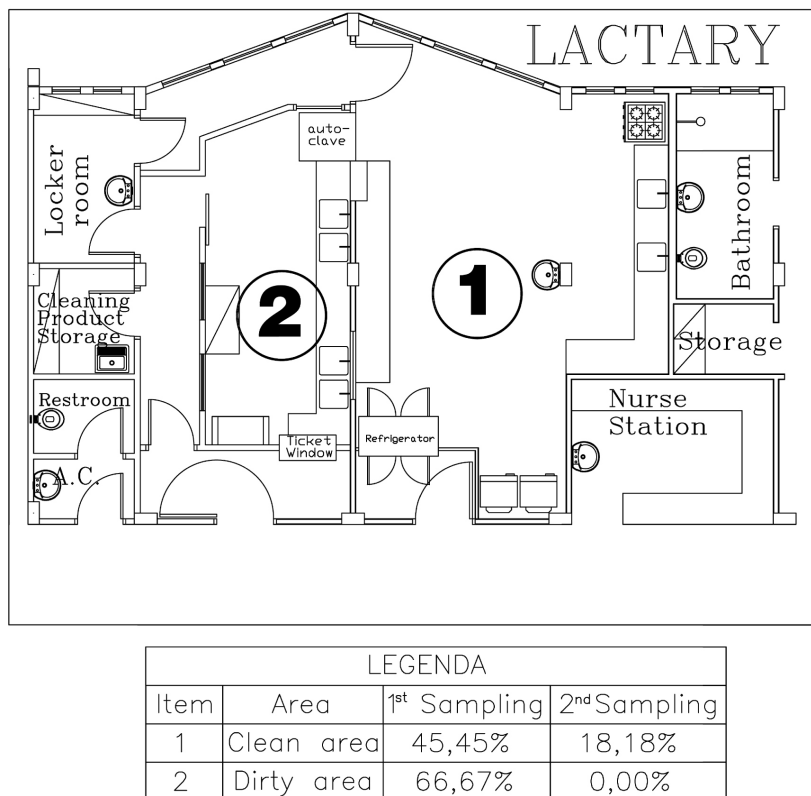| LEGENDA			
Item	Area	1st Sampling	2nd Sampling
1	Clean area	45,45%	18,18%
2	Dirty area	66,67%	0,00%

Figure 3. Positivity in areas, benches and equipment of the lactario identified in the layout of the service itself.

After identification of isolates, anti-microbial resistance was evaluated. Furthermore, positivity for D Test was evaluated. As shown in Table 2, all preparation areas had microorganisms with at least one type of antimicrobial resistance. Although no statistical difference in antimicrobial susceptibility was found, it was possible to observe in the first and second samplings that microorganisms showed the highest percentage of resistance to Erythromycin, followed by Oxacillin (P > 0.05).

It was also possible to observe the percentage of microorganisms with multi-resistance to antimicrobials (Figure 4). It was shown that the presence of microorganisms resistant to at least one antimicrobial (45% and 44%) is higher than those sensitive to all antimicrobials tested (31% and 24%) both in the first and second sampling, respectively (P > 0.05). Furthermore, microorganisms resistant up to 5 antimicrobials were found in both the first and second samplings.

As shown in Figure 5, the presence of resistant microorganisms in the kitchen was higher than that found in other areas studied, such that resistance to four or five antimicrobials occurred exclusively in the kitchen in both samplings.
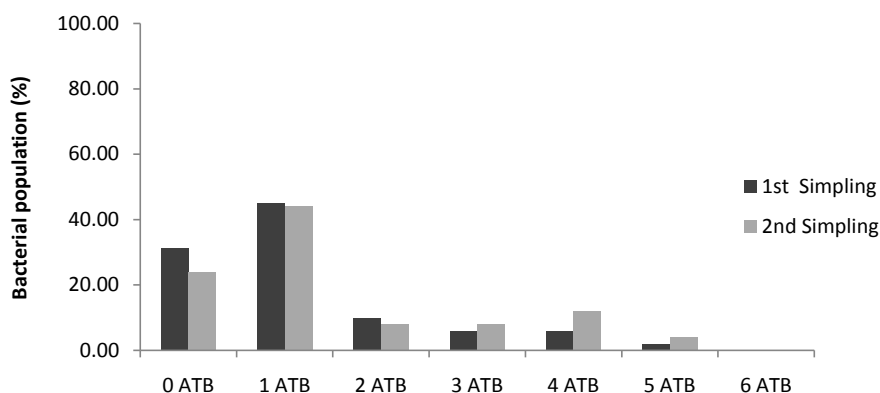

Figure 4. Percentage of bacterial population resistant to more than one type of antimicrobial.

Table 2. Percentage of resistance to antimicrobials and positivity for D-test by preparation area in the first and second samplings.

Antibiotic (%)	1st Sampling			2nd Sampling		
	Lactary	Kitchen	Enteral	Lactary	Kitchen	Enteral
CLIN	0	13.51	0	12.50	33.33	0
ERI	27.27	45.94	100	75	53.33	0
OXA	9.09	29.72	33.33	12.50	33.33	50
GEN	9.09	5.40	0	0	13.33	0
TET	18.18	13.51	0	25	20	0
CFO	9.09	16.21	0	0	26.66	0
D TEST	18.18	10.81	0	12.50	0	0

CLIN: Clindamicin; ERI: Erythromycin; OXA: Oxacillin; GEN: Gentamicin; TET: Tetracycline; CFO: Cefoxitin. Zone diamter according CLSI (2018).
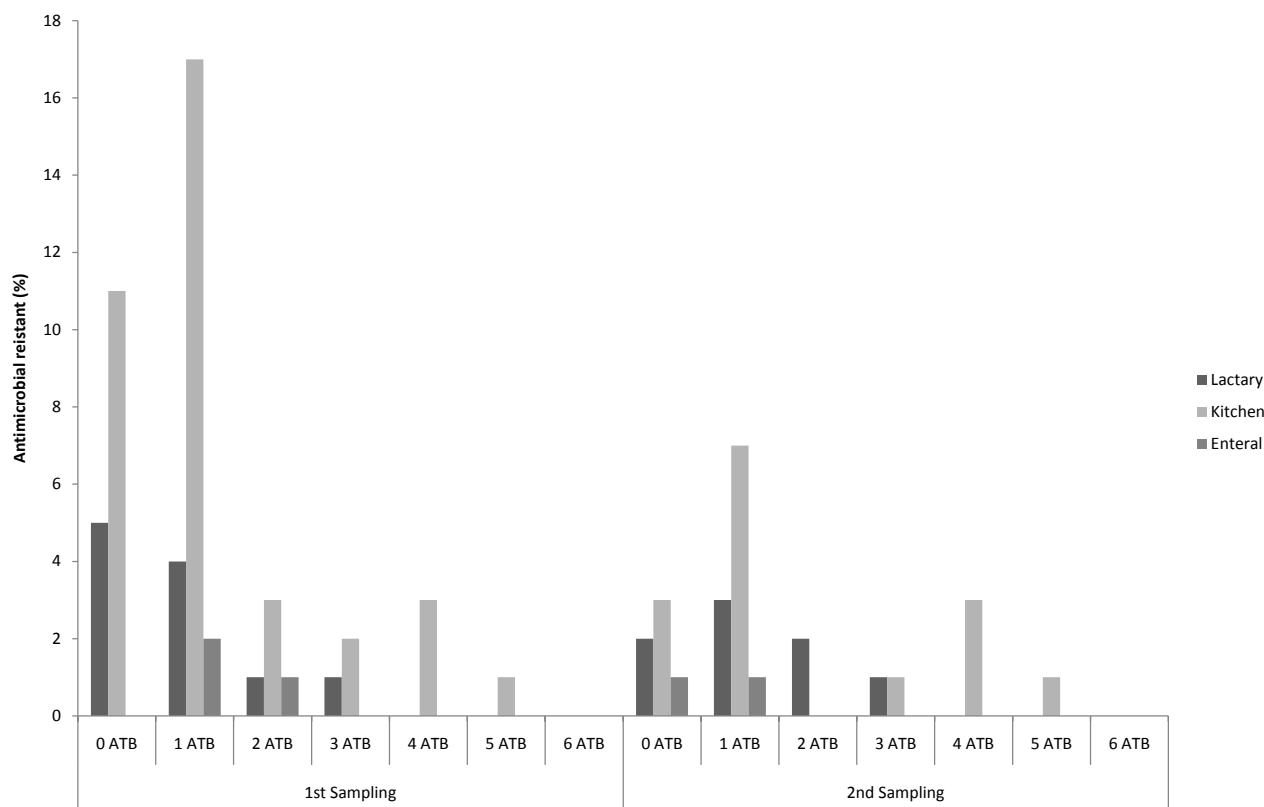
Figure 5. Percentage of bacterial population resistant to more than one type of antimicrobial, according to each sector of the nutrition service

Of the 76 samples isolated for *S. aureus* in both samplings, it was possible to observe that 97.4% were positive for biofilm production. It was further found that the only non-biofilm producing sample was isolated from the environment and that all samples isolated from food handlers were biofilm producing.

4. Discussion

Food-borne illnesses occur frequently and are considered a public health problem in Brazil. Multiple factors could be involved in this process such as improper hygiene of equipment, utensils and food handlers. Hospitalized individuals are an at-risk group as they have a compromised immune system, and as such a small number of pathogens can be sufficient to cause food-borne intoxication or infection and further debilitate their health [4] [7] [8] [22].

S. aureus comprises part of the normal mucosal, mouth and skin microbiota of food handlers. However, the high frequency of these bacteria in handlers is an imminent risk as it can be transmitted to food by direct or indirect contact. Given adequate conditions, these microorganisms can multiply and produce staphylococcal toxin, which can consequently cause food intoxication [7] [11] [12] [14] [15].

Reference [23] found that close to 88.8% of enteral diet preparation utensils in a hospital were contaminated with *S. aureus*. Reference[24] estimated the preva-

lence of *S. aureus* carriers in 20 food handlers in a day care center and found the extremely high colonization level of 95%. Reference [18] showed that *S. aureus* was present in 46.7% of nasal mucosa samples and 34.4% of hand samples from food handlers from industrial, hospital and restaurant Food and Nutrition Units (UANs). Compared to the study of reference [18] the present study identified a much higher number of samples positive for *S. aureus* among those collected from food handlers, close to 87% in the first sampling and 53% in the second.

These results show that even with the knowledge necessary to correctly carry out the job, there are still aspects that must be prioritized with respect to the personal hygiene of food handlers such as proper hand washing. Thus, strategies for awareness and periodic training of employees are relevant aspects in food safety and control.

The first samples collected showed higher positive rates for *S. aureus*, principally from the kitchen, where 100% of samples from food handlers were positive. This could be related to factors such as high temperatures and humidity in the area during the period analyzed, which favors greater replication and colonization by microorganisms [22] [25]. Another important factor in this process is planning menus adequate for the production capacity of the service [26]. Such menus must meet nutritional requirements with respect to eating habits, hygiene and sanitation as well as equipment and time available for preparation. Menus must also be planned based on the capacity and availability of the workforce [27] [28]. It is worth noting that the menu served on the date of the first sampling was comprised of highly complex dishes compared to the date of the second sampling, which was less complex. This could justify an overload of work that resulted in less time spent on personal and material hygiene.

Multiple studies have been conducted with the objective of verifying the antimicrobial sensitivity profile of *S. aureus*, and a great variability between different geographic regions and strains isolated from communities and hospitals has been observed [29] [30] [31] [32] [33]. Antimicrobial resistance develops naturally as the bacterial population adapts, and a large part of its rise is due to the indiscriminate use of antibiotics. Antimicrobial resistance mechanisms are based on biosynthesis of enzymes or other molecular phenomena that are able to degrade, inactivate, block or expel the antibiotic from the cell [31] [34] [35] [36].

The majority of *S. aureus* isolates in this study showed resistance to at least one antimicrobial. The susceptibility profile was evidence of the greater rate of erythromycin resistance. Reference [37] evaluated the resistance profile of *S. aureus* isolated from the nasal cavity of medical students from the São Paulo Western University (Universidade do Oeste Paulista). 204 samples positive for *S. aureus* were evaluated, and of those around 95.2% were resistant to erythromycin, corroborating the data of this study. Erythromycin is an antibiotic from the macrolide family whose mechanism of action is to reversibly bind to the 50S portion of the ribosome and inhibit the microorganism's protein synthesis [38] [39]. The data found show that the use of these antibiotics should be restricted as an

alternative in the prophylaxis or treatment of infections caused by *S. aureus*, even those originating from the community.

Macrolide resistance occurs due to three mechanisms: modifications in the binding site of the ribosome, active efflux or enzymatic inactivation of the drug. Binding site modification results in a resistance phenotype known as MLSB [38] [39]. This phenotype is coded by the gene erm (erythromycin methionine methylase) found in various micro-organisms [32] [33].

Expression of the MLSB phenotype can be constitutive (MLSBc) or inducible (MLSBi). Inducible resistance requires the presence of an inducer. Normally the samples that carry the inducible erm gene are resistant to the inducer and remain susceptible to non-inducer macrolides and lincosamides. Low levels of erythromycin are used as an inducer of the MLSBi phenotype in D Test [32] [33] [38].

In this study, the test for inducible resistance to clindamycin, or D Test was used. In the D Test, the microorganisms that show flattening of the clindamycin halo adjacent to the erythromycin disk (called the "D" halo) indicate inducible resistance to clindamycin, that is, a positive MLSB phenotype. Resistance to erythromycin and clindamycin can be related to the presence of the ermA gene [31] [32] [33] [40].

Despite the high presence of strains resistant to erythromycin, few were positive for the D Test. Reference [38] evaluated inducible resistance to clindamycin among clinical isolates of *S. aureus* and found close to 6.12% were positive for the phenotype. Reference [33] evaluated 190 *S. aureus* isolates to determine their antimicrobial susceptibility by means of routine tests and D test. The results showed that 10% of isolates showed inducible resistance to clindamycin and 9% showed constitutive resistance. Furthermore in that study, higher levels of inducible and constitutive resistance were found in methicillin-resistant *S. aureus* (MRSA) compared to methicillin susceptible *S. aureus* (MSSA).

Hospital acquired infections are considered a global problem and have stimulated the rise of multi-resistant (MR) microorganisms [31] [34]. Multi-resistant microorganisms are those resistant to different classes of antimicrobials tested for in microbiological exams [31] [34] [35]. Various consequences are related to the presence of multi-resistant microorganisms such as a longer period of hospitalization, a higher number of invasive procedures and a greater risk of morbidity and mortality. Examples of these microorganisms are methicillin-resistant *Staphylococcus aureus* (MRSA) as well as *S. aureus* with reduced sensitivity to vancomycin, known as vancomycin-resistant *Staphylococcus aureus* (VRSA) [14] [31] [34] [35].

Among the *S. aureus* isolates from the first sampling, 23.52% showed resistance to 2 to 5 antimicrobials concomitantly while for those of the second sampling, 32% were found resistant to 2 to 5 antimicrobials, with samples resistant to 4 or 5 antimicrobials found only in the kitchen. None of the isolates showed simultaneous resistance to 6 antimicrobials. The rise in multi-resistant microor-

ganisms creates an important public health problem as they render many of the traditional treatments for infections ineffective. To counter the spread of MRs, hospitals have implemented monitoring policies principally with regards to the rational use of antimicrobials and efficient infection control practices to avoid emergency and spread of microbial resistance [31] [34] [35].

The present study found 97.3% positivity for biofilm production among samples isolated from both samplings, showing concordance with the study by reference [41], which despite having a higher number of samples, also noted the prevalence of biofilm producing strains using Congo Red Agar.

It is noteworthy that all samples isolated from food handlers in the present study were biofilm producers, and that only two samples (one from each sampling, same source) were not. This fact can be explained since boitic and abiotic surfaces in contact with non-sterilized water and organic substances can serve as a nutrient conducive to bacterial adhesion. A biofilm is composed of multiple bacterial layers, from the same or different species, forming three dimensional structures with channels or niches of concentration gradients where water, nutrients and air can pass, essential for survival. The presence of lipids, phospholipids, carbohydrates and vitamins, as well as polysaccharide, allows for cellular multiplication [17].

Reference [42] evaluated adhesion and biofilm formation capacity in *S. aureus* samples isolated from food service areas, revealing an elevated capacity in these bacteria to adhere and form biofilm on polypropylene and stainless steel surfaces under different growth conditions. Furthermore, the authors noted that the few studies relating to *S. aureus*' ability to form biofilms employed standard strains inoculated by synthetic means, and little is known about the ability of wild *S. aureus* strains to adhere and form biofilms when exposed to environmental conditions found in food processing locales.

Reference [43] evaluated the adhesion and biofilm formation capacity of *S. aureus* with respect to the influence of environmental stress factors during seafood processing. The data from that study suggest that the prevalence of *S. aureus* strains on food processing surfaces is dependent on the ability to adapt to environmental stress conditions present during food production.

Showing further concern about *S. aureus* colonization among health service professionals in contact with hospitalized patients, reference [44] showed in their project that 25% of nurses in the surgery wing were colonized by *S. aureus* on the palm of their hands or nasal mucosa, or both, with a large percentage of MRSA. Reference [45] noted in their study as well that the vast majority of blood samples collected from patients, samples from medical devices, as well as other locations in the hospital environment, was positive for *S. aureus*. Beyond being resistant to beta-lactam antibiotics, they noticed that the MRSA samples were strongly associated with stable biofilm production.

These findings are relevant to food safety and can be important for choosing the safest materials and environmental conditions during the processing, packing and storage of food.

5. Conclusion

By the obtained results it is shown that utensils, equipment and food handlers in the investigated hospital showed high rates of *S. aureus* colonization, especially in the kitchen area (production). We further reemphasize the high frequency of antimicrobial resistance, especially resistance to erythromycin. The study also calls attention to the presence and persistence of multi-resistant microorganisms in both samplings, due to the risks and consequences that they can cause in the hospital environment. The large number of samples positive for biofilm production also stands out, as well as their ubiquity in samples isolated from food handlers. Educational and awareness programs were proposed and carried out in the institution, considering the safety of the patient, as these steps will avoid food contamination and transmission of different diseases at the hospital level.

Author's Contribution

MMS and GN drafted the manuscript; LWKE and MVPR coordinated the study; BDL, CHM and JMS collected data and participated in its design. All the authors read and approved the final manuscript.

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Ethics Approval

Collection of samples was carried out after approval by the Ethics Committee under protocol number CAAE 49832615.9.0000.5515 following the standards of CONEP Resolution CNS 466/2012, by the São Paulo Western University and the Regional Hospital of Presidente Prudente, São Paulo, Brazil.

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