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Prevalence of multidrug resistance in methicillin-resistant *Staphylococcus aureus* isolates obtained from raw chicken meat samples and effectiveness of citrox solution against it.

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Abstract: The study was aimed at evaluating the presence of antibiotic-resistant *Staphylococcus aureus* in retailed raw chicken meat from retail stores intended for human consumption and determining the antimicrobial effect of citrox solution to be used as preservative. The presence, characterization, and antibiotic susceptibility of *S. aureus* from 10 retail raw chicken meat samples was performed using standard microbiological methods. All the samples were positive for Staphylococcus species, of which all were positive for S. aureus. The Methicillin Resistant test was carried out on Mueller Hinton Agar (MHA) by the Kirby Bauer disc diffusion Cefoxitin and Oxacillin preparations, then confirmed with the Oxacillin Resistance Screen Agar Base (ORSAB). Antimicrobial susceptibility test using 14 antibiotics showed that all MRSA were resistant to amoxicillin, cefoxitin, vancomycin, tetracycline, optachin, 80% resistant to amoxicillin, 60% resistant to azithromycin, 50% resistant to piperacillin, 40% resistant ciprofloxacin and roxithromycin, 30% reisistant to norflaxacin and amikacin, 20% resistant to oflaxacin while 80% were sensitive to co-trimazole and 30% were sensitive to bacitracin. Natural antibacterial agents such as citrox are effective against many foodborne pathogens and foods contaminated with bacteria. The antimicrobial effects of citrox solutions (1% and 2%) on methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from raw chicken samples using agar diffusion method was studied. The results indicated that citrox is effective in inhibiting the growth of MRSA. Therefore, this study suggests high-level contamination of raw chicken meat with multidrug-resistant *S. aureus* and highlights the public health consequences of consuming such products and to overcome such a problem citrox solution can be used as preservative by inhibiting the growth of MRSA and thus reducing the health hazards that can be caused by consuming such products.

***Keywords*: Citrox solution, Mueller Hinton Agar (MHA), Oxacillin Resistance Screen Agar Base (ORSAB), Antimicrobial susceptibility test, MRSA, Preservative**

I. INTRODUCTION:

The genus *Staphylococcus* is the most important genus present in the family *Micrococcaceae* having in its ambit thirty-two species. The members of this group are Gram-positive, spherical in shape, non-spore forming, non-motile with limited capsule formation (Harris *et al.*, 2002). These bacteria grow well on most routine laboratory media at 37°C. Colonies of the most *Staphylococci* spp. that grow on solid media are circular, smooth, opaque, raised, with white to pigments of different colors. Staphylococci are known to be facultative anaerobes, usually oxidase negative and catalase-positive. Coagulase production by staphylococci organism cause hemolysis of blood, but the pattern of hemolysis depends on both the source of the blood and the staphylococcal strain (Moraveji *et al.*, 2014). The biochemical characters of different species of staphylococci have been well documented. Staphylococci are known to be ubiquitous in nature and are usually isolated from the outer body surfaces of mammals and birds besides also from blood, genitourinary tract, intestines, upper respiratory tract and other organs of the body. Staphylococci are the most common bacteria found in the environment where poultry are hatched, reared, and processed. They are also isolated from the skin and nares, feet and beak of healthy chickens. *Staphylococcus aureus* is one of the major foodborne pathogens in fresh and ready-to-eat products and recognized for causing various infections around the world. There are many foodborne diseases associated with *Staphylococcus* spp. where food handlers who have staphylococcal lesions of the skin, especially of the nasopharyngeal region and the hands, or who are carriers. Most of the contamination of chicken meat due to *S. aureus* was found due to cross-contamination, inadequate heat treatment of the foodstuff and improper storage resulted into outbreaks of food poisoning (Nishchal Dutta *et al*.,2020).

Meat and meat products are among the most consumed foods and are important sources of all the B-complex vitamins, as well as minerals, proteins, and amino acids in humans. Meat of animal origin is the primary source of protein and valuable qualities of vitamins for most people in many parts of the world, thus it is essential for the growth, repair, and maintenance of body cells and necessary for our everyday activities. Meat is the main source of iron in heme form, which is one of the most deficient micronutrients in humans. Due to the chemical composition and biological characteristics, meats are highly perishable foods providing an excellent source of nutrients for the growth of several hazardous microorganisms that can cause infection in humans, resulting in spoilage of the meat and, therefore, economic loss. The microbial pathogens found in meat microorganisms are Listeria monocytogenes, Micrococcus spp, Staphylococcus spp., Clostridium spp., Bacillus spp., Brochotrix thermophacta, Salmonella spp., Escherichia coli, Serratia spp. and Pseudomonas spp. Growth of foodborne pathogens such as Salmonella, and toxin-producing strains of E. coli, L. monocytogenes, C. perfringens, and S. aureus are the main concern with meat and poultry products. These bacteria are the most common cause of foodborne illnesses. Besides poultry meat, S. aureus as well as Methicillin-resistant S. aureus can be found in swine and cattle
Meat (Erinda Lika *et al*., 2021).

The most significant Gram-positive organism that has gained attention because of its associated hospital- and community-acquired infections is S. aureus. This bacterium multiplies quickly at room temperature to produce toxins that cause food poisoning. Naturally, its distribution is very common globally, but the most important infection origin of S. aureus is food. According to Scallan et al., S. aureus has come into the spotlight as a foodborne pathogen with more than 200,000 estimated yearly infections domestically acquired within the US. Staphylococcal food contamination represents the greatest economically significant foodborne illness and produces gastrointestinal illness through a wide variety of toxins, including staphylococcal enterotoxins characterized by vomiting and diarrhea within 2 to 6 h after the consumption of contaminated food. A large number of daily consumed foods serve as an optimum growth medium for S. aureus, and this varies from country to country, especially due to different habits in food consumption. S. aureus and other pathogens in meat result from improper hygienic practices at the point of handling by slaughter personnel during meat processing, and other faulty abattoir processes such as improper evisceration of animals which increases the chances of cross-contamination of gut pathogens to meat. Residues from medicines, insecticides, herbicides, and other compounds used in daily agricultural practice could be detected in minor quantities in food of animal origin. A few hundred compounds, mainly antibiotics, have been used to cure animals and protect their health, however, some of them have also been used to enhance food animal production. Among many unethically used compounds, the most often used are antimicrobials, adrenoreceptor blocking agents, ivermectin, sedatives, coccidiostats,

vasodilatory drugs, and painkillers. Residues from such compounds in food products are a major public health concern, especially with rising interest and increased awareness of the potential deposits of drugs and their metabolites in the meat and meat products consumed by humans, as well as the development of antimicrobial resistance (AMR). Treatment of S. aureus infections involves the use of antibiotics. However, the use and misuse of antibiotics prophylactically or sub-therapeutically to prevent bacterial infections in livestock and the resultant residue, in general have been responsible for the development of multi drug resistant bacterial isolates and a significant public health issue. Several microorganisms have developed resistance to various antibiotics, which have triggered the expansion of novel antibiotics with a higher resistance level. Numerous studies have shown the presence of S. aureus in raw meat and meat products from retail stores with a prevalence below 1% in Asia, up to around 12% in Europe (Erinda Lika *et al*., 2021).

Before the availability of antibiotics, invasive infections caused by *S. aureus* were often fatal. The introduction of penicillin greatly improved the prognosis for patients with severe staphylococcal infections, but after a few years of clinical use resistance appeared in *S. aureus* due to production of beta-lactamases. Methicillin was designed to resist hydrolysis by beta-lactamases, but soon after methicillin was introduced into clinical practice, resistant *S. aureus* strains were identified and designated as Methicillin-resistant *Staphylococcus aureus* (MRSA). The term MRSA has been retained, although oxacillin has now replaced methicillin for susceptibility testing in laboratories and is the marker for classifying *S. aureus* as MRSA. Until recently, MRSA was predominantly a nosocomial pathogen causing hospital acquired as well as community-acquired infections (Mera *et al*., 2011; Tong *et al.*,2015; Boswihi and Udo, 2018). Due to the increase of MRSA strains every decade, these bacteria were identified in the early 1980's as a major cause of nosocomial infections (Boyce *et al*., 2004). The possibility of transmission of healthcare associated MRSA (HAMRSA) to the community was obvious. Since 1987, MRSA was increasingly found in the community associated-methicillinresistant *S. aureus* (CA-MRSA) presented with severe skin and soft tissue infections as well as necrotizing pneumonia (Hayani *et al*., 2008). MRSA infections account for one fifth of all hospital-acquired infections, costing the UK National Health Service approximately £1 billion per year (Cepeda *et al.,* 2005). The problem has been aggravated by the rapid spread and high incidence of MRSA in intensive-care units (Cepeda *et al.*,2005). The continuing rise in MRSA infection rates and its spread worldwide has led to calls for action to control infection and develop novel anti-MRSA agents and vaccines (Cutler and Wilson, 2004; Hancock, 2007). In a recent letter, Simor et al. reported results obtained by using oxacillin resistance screening agar base (ORSAB; Oxoid Limited, Basingstoke, England) for the detection of methicillin- resistant *Staphylococcus aureus* (MRSA) from clinical specimens indicating it to be the most suitable techmique to detect MRSA (A. E. Simor, J. Goodfellow, L. Louie, and M. Louie, Letter, J. Clin. Microbiol. **39:**3422, 2001). ORSAB is a modification of a mannitol-salt agar supplemented with oxacillin, in which mannitol-positive isolates turn blue due to an acid-dependent chromogenic component (aniline blue).

Methicillin-resistant Staphylococcus aureus (MRSA) was identified in 1962 and, together with certain species of Enterococcus, is currently considered a global pandemic threat. Methicillin-resistant S. aureus is classified into three groups, of which healthcare-associated methicillin-resistant S. aureus (HA-MRSA) is considered a major causative agent of chronic diseases and is present in catheters among other places. After two decades from its discovery, the first case of acquired community-associated S. aureus MRSA (CA-MRSA) was reported in many countries, as was livestock-associated S. aureus (LA-MRSA). LA-MRSA has also been reported to be associated with companion animals. The HA-MRSA and CA-MRSA infections that generally affect humans are not involved in livestock infections. However, LA-MRSA may affect humans, especially in the case of occupational contact with livestock. Although many foods containing CA-MRSA, LA-MRSA, and HA-MRSA have been documented, it is not clear whether MRSA can be classified as a food-borne pathogen. MRSA is found in several species of animals, such as pigs, poultry, and cattle, and their meat products. Poultry meat is highly perishable and provides a high nutritive medium for the growth of bacteria and other spoilage and pathogenic microorganisms. The increasing production and global demand for poultry meat has also increased the importance of poultry meat hygiene and safety worldwide. The preservatives employed to inhibit the growth of spoilage and pathogenic microorganisms have high acidity. However, consumers consider their use undesirable, and their demand has reduced the levels of such additives in foods. Although the safety of these foods is supposed to be ensured primarily by the low pH of these additives, several pathogens, namely, Escherichia coli O157:H7,

Listeria monocytogenes, and Salmonella spp., have been reported to survive or even grow in these foods. Usually, the carriers of these pathogens are the raw ingredients, as well as any contaminations from the processing environment and packaging operations (Hany Mohamed Yehia *et al*., 2019).

The aim of this work was to assess the source of MRSA isolates as well as describe the antimicrobial susceptibility patterns of isolates in retail raw chicken meat intended for human consumption and to evaluate the effect of different concentrations of citrox (1% and 2%) on MRSA in vitro.

II. Literature Survey

Test of Methicillin resistant using disc diffusion method on Muller Hinton Agar (MHA) media showed that the total results of isolates resistant to Oxacillin preparations were 32 (28.1%) *S. aureus* and 14 (13.2%) CNS, while the results of isolates resistant to Cefoxitin were 19 (16.7) %) *S. aureus* and 4 (3.8%) CNS. The results showed that there were no isolates that were only resistant to Cefoxitin in the disc diffusion method, all isolates that were detected Test of Methicillin resistant using disc diffusion method on Muller Hinton Agar (MHA) media showed that the total results of isolates resistant to Oxacillin preparations were 32 (28.1%) *S. aureus* and 14 (13.2%) CNS, while the results of isolates resistant to Cefoxitin were 19 (16.7) %) *S. aureus* and 4 (3.8%) CNS. The results showed that there were no isolates that were only resistant to Cefoxitin in the disc diffusion method, all isolates that were detected. *Staphylococci* strains are bacteria that are often found on the surface of the respiratory mucosa and urogenital tract in humans and animals. The strains are divided into two large groups which are distinguished from their coagulation ability, where each CPS represented by *S. aureus* and CNS are commensal bacteria that are opportunistic infections in humans and animals. In the study of 220 samples of cow's milk, 100% were found to have *Staphylococci* strains contamination with the proportion of 114 (51.8%) being S. aureus whereas 106 (48.2%) were CNS. It is different from the study conducted by research in Czech Journal of Food Sciences, which isolated 47.5% of *Staphylococci* strains from dairy cow milk, while the dominant strain was *S. aureus* with 46.4% of the total *Staphylococci* strains isolates. In a result conducted by The Pharma Innovation Journal also only isolated 57% of *Staphylococci* strains, of which 73.6% were *S.aureus*. That is because the study had a specific purposive design to detect the presence of *Staphylococci* strains in dairy cow milk on farms that have low hygine milking thereby increasing the potential for contaminants in dairy cows. In line with the research in North India stated that differences in the number of isolates found could be influenced by differences in study design such as population and geographic distribution of samples, types of antibiotics used and infection control practices. In 1959, the preparations for methicillin and semisynthetic penicillins such as oxacillin were used as anti-staphylococcal therapy, but in 1961 a study from Trends in Microbiology Journal stated that there have been cases of MR. Research conducted by a researcher in Clinical Microbiology and Infection Journal convey the existence of MR in the United Kingdom which was then spread in several regional hospitals in Europe. Resistance to *Staphylococci* strains against methicillin is a global problem, where various studies to demonstrate resistance and distribution schemes are carried out intensely in Europe, Africa, America and Asia. Research in International Journal of Current Research Journal suggested that the nature of MR indicates the nature of resistance to all β-lactam antibiotics including cephalosporins, monobactams and antibiotic groups for the treatment of *Staphylococcal* infection. Infection with methicillin resistance strains will have a significant impact on therapeutic problems and the potential for increased spread, so the urgency of clinical microbiology laboratories identify is very fundamental. Basically, polymerase chain reaction (PCR) is the gold standard in detecting MRSA, but often has cost and technical constraints so that phenotypic methods are still an option in detecting. The study used 2 methods in evaluating MRS namely Cefoxitin disc diffusion test and Oxacillin disc diffusion test referring to the Kirby Bauer method combined with ORSA confirmation. The Cefoxitin disc diffusion test method detected 19 *S.aureus* isolates and 4 CNS isolates had resistance properties, while the Oxacillin disc diffusion test method detected 32 *S.aureus* isolates and 14 CNS isolates had resistant properties which were later confirmed to have 28 *S.aureus* isolates and 12 MR positive CNS isolates with ORSAB test. Research in Indian Journal of Pathology and Microbiology Journal suggested that Cefoxitin has a higher sensitivity and specificity to mecA gene expression compared to Oxacillin which is weak in inducing PBP2a

production. European Journal of Clinical Microbiology & Infectious Diseases Journal and Journal of Antimicrobial Chemotherapy reported that the results of detection with Cefoxitin disc diffusion test were better in detecting the presence of mecA gene compared to the results of detection with Oxacillin disc diffusion test. Strengthened by research report in International Journal of Current Research which states that Cefoxitin is better at detecting MR compared to Oxacillin. In the study all isolates detected resistant by Cefoxitin disc diffusion test were also detected by Oxacillin disc diffusion test, whereas not all isolates by Oxacillin disc diffusion test had positive results detected by Cefoxitin disc diffusion test. Oxacillin disc diffusion test has a high false positivity level, it can be influenced by the presence of hyperproduction of β-lactamase so that it appears phenotypic expression but does not have a genotype resistance mechanism. Researcher in Memorias do Instituto Oswaldo Cruz Journal suggested that the comparison of the number of isolates detected by Cefoxitin disc diffusion test was more selective than the results of Oxacillin disc diffusion test and ORSA, it showed that Cefoxitin disc diffusion test had higher specificity compared to Oxacillin disc diffusion test and ORSA. Positive MRSA and MR-CNS screening results confirm that healthy dairy cows can carry MRSA and MR-CNS without clinical symptoms. Harijani et al. stated that MRSA could be identified in healthy dairy cows without showing clinical evidence manifestation. MRSA and MR-CNS colonization will not cause serious illness if it occurs in humans and animals with normal (healthy) conditions, but can cause serious life-threatening disease if it occurs in an individual have low immunity. Transmission of MRSA and MR-CNS in dairy cattle occurs through direct contact with bacteria found in other animals, humans, and contaminated environments. Interaction between animals and humans has an important role in the spread of MRSA and MR-CNS because most dairy cows get MRSA and MR-CNS through human contact. However, there are other factors like that as genetics and the environment can influence transmission of infection. European studies have found MRSA and MR-CNS suitable for dairy cows that are identical to the farmer and infected animals. Human MRSA and MR-CNS predominance the strains on the farm indicate that the animal becomes infected through contact with an infected person, and this demonstrated that livestock can propagate MRSA and MRCNS to humans or other species. The results of the study suggest that MRSA and MR-CNS can found in healthy livestock samples. This is in accordance with the results of Tyasningsih's research et al., who stated that MRSA was increasing identified in healthy cows. The consequences are often physical contact between humans and pets that can facilitate the occurrence of MRSA transmission. This is a potential health problem due to MRSA from humans can cause infection in livestock, and livestock can be the source of MRSA and MR-CNS for infections in humans. The spread of MRSA and MR-CNS itself occurs both through direct contact with an infected person or through direct previous contact with a surface or object contaminated by an infected individual. Animals with MRSA and MR-CNS infections can serve as reservoirs of bacteria or human transmission by bacteria, therefore control and prevent transmission of MRSA and MRCNS animal to animal, as well as from animal to human must be done by adopting clean habits prevent outbreaks of MRSA and MR-CNS bacteria in animals and humans can be prevented by early detection (Mustofa Helmi Effendi et al., 2021)

Simor et al. reported results obtained by using oxacillin resistance screening agar base (ORSAB; Oxoid Limited, Basingstoke, England) for the detection of methicillin-*resistant Staphylococcus aureus* (MRSA) from clinical specimens (A. E. Simor, J. Goodfellow, L. Louie, and M. Louie, Letter, J. Clin. Microbiol. 39:3422, 2001). ORSAB is a modification of a mannitol-salt agar supplemented with oxacillin, in which mannitol-positive isolates turn blue due to an acid-dependent chromogenic component (aniline blue). When specimens from persons thought to be at high risk for MRSA colonization were screened with this agar base, 102 of 104 MRSA-positive clinical specimens (98%) were correctly identified. In total, 138 clinical specimens yielded blue, mannitol-fermenting colony types; therefore, the positive predictive value of ORSAB-positive specimens for MRSA has been 74%. During a period of 8 months, 4,200 *staphylococcal* isolates, including ca. 2,200 isolates of *S. aureus* from unselected clinical specimens was tested, on ORSAB. A total of 131 strains of MRSA were included; all but one (99%) of the MRSA isolates were detected with ORSAB, the one exception being negative for mannitol fermentation, as assessed with the ATB ID 32 Staph kit (bioMe´rieux, Marcy-l'Etoile, France). In total, 266 isolates were blue on ORSAB. The results of identification of these isolates (using the ATB ID 32 Staph kit) obtained from the three different predominant specimen types were presented. The overall predictive

value of ORSAB-positive isolates was 48.9% (130 of 266 isolates were positive). Predictive values with respect to specimen types were as follows: for specimens from nares and throat, 65.2% (60 of 92 isolates were positive); for skin and soft tissue specimens, 47.8% (32 of 67 isolates were positive); and for urine specimens, 33.3% (23 of 69 isolates were positive). While the sensitivity of ORSAB observed by us was as high as that reported by Simor et al., predictive values in our study were much lower. This may be explained by two facts: (i) the study was not restricted to persons at high risk for MRSA colonization but included all submitted clinical specimens and (ii) there were only a few urinary tract specimens (1% of all specimens) included in the study of Simor et al.; in the study, where these specimens were about 20% of all specimens, the predictive value for these specimens was much lower than that for specimens from nares and throat. Simor et al. state that, in contrast to specimens planted on ORSAB, specimens planted on mannitol salt agar supplemented with 2 g of oxacillin per ml "required additional work-up of mannitol-fermenting colonies that were subsequently determined not to be MRSA." Taking into account that in the study the second most predominant ORSAB positive species was *Staphylococcus haemolyticus* (133 of 2 specimens [46.2%]), selectivity would be appreciably enhanced if other mannitol fermentation-positive oxacillin resistant *staphylococcal* isolates, especially those of *S. haemolyticus*, were ruled (Andrea Becker et al., 2002)

Inoculation with 1% and 2% citrox solutions inhibited the growth of MRSA. However, the zone of inhibition was observed to be larger with 2% citrox. Since both concentrations were effective, we decided to treat the chicken fillets with both (1% and 2%) to limit or stop the growth of MRSA in chicken fillet samples during the storage period. Chitosan mixed with citrox at both concentrations (1% and 2%) did not appear to have any effect on the growth of MRSA, so there was no need to use it in our experiments (Hany Mohamed Yehia et al., 2019).

III. METHODOLOGY

Collection and processing of samples:

A total of 10 samples of poultry meat (raw chicken meat) samples were collected from different retail shops in vicinity of Mumbai. About 100 grams of meat samples were collected in dry, clean and sterile polythene bags and transported to the laboratory for microbiological analysis within one hour or refrigerated at 4°C till further analysis was carried out.

These samples were then processed no later than 48 hours after purchase. These samples were then swabbed with sterile cotton swabs and inoculated onto the Brain Heart Infusion broth (BHI) and then incubated overnight at 37°C. On the next day, the swabs were streaked onto the different media plates like Baird Parker Agar (BPA) and Mannitol Salt Agar (MSA) for isolation of *Staphylococcus* spp.

Identification of bacterial isolates

The bacterial colonies were isolated after incubation. These colonies were subjected to Gram's staining and observed for gram positive cocci. Beta- hemolysis was performed by streaking the organism on Blood Agar Plates and incubating at 37 degrees C for 24 hrs, complete clear zones (hemolysis) around colonies after incubation indicates positive result. Oxidase was performed by using oxidase disc, organism was spot inoculate on the oxidase disc and a quick purple colour development indicates positive result. Coagulase was performed by inoculating the culture in Walbum's medium containg 1:10 diluted plasma and incubated at 37 degrees C, clotting was examined after 1 hr and at 30 mins interval upto 6 hrs if not examined at 1 hr with positive and negative controls. Lipase was performed by by streaking the organism on Tributyrin Agar Plates and incubated at 37 degrees C for 24 hrs, after incubating clear zones around colonies indatices positive result showing lipase activity. DNase was performed by streaking the organism on the DNase Test Agar Base and incubating at 37 degrees C for 24 hrs, after incubation the plates were flooded with 1N HCl and checked for clear zones around the colonies which indicates positive result for DNase activity. Gelatinase activity was performed by streaking the organism on the Nutrient Gelatin Medium and incubated at 37 degrees C for 24 hrs, after incubation the plates are flooded with saturated ammonium sulfate to precipitate the unhydrolyzed gelatin making clear zones easier to be seen within 5-10 mins indicating positive result for gelatinase activity. Later for identification and requisite biochemical tests were carried out to further confirm the presence of the pathogen.

Biochemical characterization

S. aureus suspected colonies were subjected to various biochemical tests like the Catalase test by adding a drop of hydrogen peroxide solution to a colony and checking for effervescence for positive result , VP (Voges-Proskauer) test was performed by inoculating 18-24 hrs pure culture and incubating at 37 degrees C, after incubation 6 drops of 5 % alpha- naphthol was added and mixed then 2 drops of 40% potassium hydroxide was added and mixed, development of pink-red colour at surface within 30 mins vigorously shanking indicates a positive result. Citrate test was performed by inoculating and incubating the agar slant at 37 degrees C for 24 hrs and a colour change from green to blue indicates positive result. MR (Methyl Red) test was performed by inoculating 18- 24 hrs old culture in broth and incubating at 37 degrees C for 24 hrs, after incubation 2-3 drops of methyl indicator was added and an immediate red colour development gives a positive result. NR (Nitrate Reduction) test was performed by inoculating the nitrate broth and incubating at 37 degrees C for 24 hrs, after incubation N2 gas was checked before addition of reagents then add 6-8 drops of nitrite reagent A and reagent B, if no colour change is observed zinc powder was added, positive result is noted by development of a cherry red colouration on addition of reagent A and B or absence of red colour development on addition of zinc powder. OF (Oxidative-Fermentative) test was performed by stabing the organism in citrate agar slant and apply mineral oil in one of the two tubes and incubating at 37 degrees C for 14 days, a development of yellow colour in open tube indicates positive result for oxidative condition and development of yellow color in both the tubes indicate positive result for the fermentative condition. TSIA (Triple Sugar Iron Agar) test was performed by by inoculating and incubating agar slant at 37 degrees C for 24 hrs and a colour change of agar slant to black indicates positive result. Indole test was performed by inoculating and incubating the tryptophan broth at 37 degrees C for 24 hrs, after incubation addition of 0.5 ml of Kovac's reagent is added to the broth culture and formation of a pink to red colour ring in the reagent layer on the top of the medium within seconds of adding the reagent indicates positive result and Fructose, Galactose, Glucose, Lactose, Maltose, Mannitol utilization test was performed by inoculating and incubating the broth at 37 degrees C for 24 hrs and colour change was observed.

Phenotypic detection of Methicillin resistant

Test of Methicillin Resistant to S.aureus isolate was carried out with Disc-diffusion test using 2 preparations of Cefoxitin 30 and Oxacillin on Muller Hinton agar plates. All positive samples of S. aureus were tested with Cefoxitin and Oxacillin using the Disc-diffusion test method. Isolates that have been isolated and identified will be purified on Mannitol Salt Agar and Baird Parker Agar and incubated at 37 degree C for 24 hours. Sterile Cotton Swab was put on 0.5 Mc Farland's suspension from positive isolate, then Swab was streaked evenly on the surface of Muller Hinton agar. Cefoxitin and Oxacillin were placed side by side with a distance of 4.5 cm on Muller Hinton agar medium which had been inoculated with isolate and then incubated 37oC for 24 hours and measured the inhibition zone. In Cefoxitin disc diffusion test the ≤21mm inhibition zone is an MR isolate, whereas in Oxacillin the disc diffusion test isolate with a zone of 10mm inhibition zone is MR. Confirmation of Phenotypic detection of Methicillin resistant was carried out by tested on the Oxacillin Screen Agar Base.

Antimicrobial susceptibility test

Antimicrobial susceptibility of MRSA isolates against 14 antibiotics was carried out using agar disc diffusion method according to CLSI (2017). The antibiotics used were vancomycin (VA 30 µg), amikacin (AK 30 µg), amoxicillin (AX 25 µg), chloramphenicol (C 30 µg), ciprofloxacin (CIP 5 µg), ofloxacin (OFX 5 µg), tetracycline (TE 30 µg), bacitracin (B 130 µg), piperacillin (PIP 75 µg), roxithromycin (RXM 15 µg), co-trimazole (COT 25 µg), azithromycin (AZI 15 µg), norfloxacin (NOX 10 µg), optachin (OP 5 µg).

Citrox Solution

The citrox solution was prepared in the laboratory by mixing 18 g of citric acid, 18 g of malic acid,
and 5 g of ascorbic acid in 100 mL of water. The pH of the solution was adjusted to ~2.7. The solution was yellow in color. The citrox solution was subsequently diluted to 1% and 2% and sterilized at 121 degree C for 15–20 min along with the addition of 0.85% NaCl.

Activity of Citrox

The effect of citrox on MRSA was evaluated through an antimicrobial activity technique by inoculating one colony of S. aureus in brain heart infusion broth at 37 degree C for 24 h. A total of 100 µL of culture was inoculated into Muller Hinton Agar medium using the agar well diffusion method. Then, a hole with a diameter of 6 mm was punched aseptically with a sterile cork borer, and two different volumes (50 mL and 100 mL) of

1% and 2% citrox solution was introduced into each well. For comparison of its effects on MRSA, undiluted and with added NaCl citrox solution was also studied. The plates were then incubated at 37 degrees C for 24 h, and the zone of inhibition was observed.

IV. RESULTS AND DISCUSSION

A total of 10 raw chicken meat samples were examined for the presence of *S. aureus*. The *S. aureus* was isolated from a total of 10 samples in raw meat with a prevalence of 100% which were Catalase positive and later confirmed by other biochemical tests.

Isolation and identification for Staphylococcus

The samples following the standard protocol were streaked on Mannitol Salt Agar (MSA) for selective culture of *S. aureus* and yellow colonies with yellow zones in the media were obtained and on Baird Parker Agar selective culture of *S. aureus* was observed as black color colonies with opaque zones. Gram's staining performed on suspected colonies showed the presence of Gram-positive cocci organisms (Fig.2) discrete or in groups typical of *S. aureus*. The colonies picked from Mannitol Salt Agar (MSA) were subjected to Catalase test, which showed positive reactivity (Fig. 1). Furthermore, biochemical test was performed in the study for confirming the presence of *S. aureus* with the help of 12 tests for identification of *S. aureus* namely MR test (Fig. 3), Voges Proskauer, Citrate utilization, Indole (Fig. 3), OF, Nitrate reduction (Fig. 3), TSIA, Glucose utilization tests and 5 different carbohydrates utilization tests namely Fructose, Galactose, Lactose, Maltose and Mannitol and other tests performed for bacterial identification namely Lipase, Beta-hemolysis, Coagulase (Fig. 4), DNase and Gelatinase were positive except Oxidase. The results from these tests confirmed the presence of *S. aureus.* All the samples were confirmed primarily with the help of its growth characteristics on selective media and then with the help of biochemical testing.

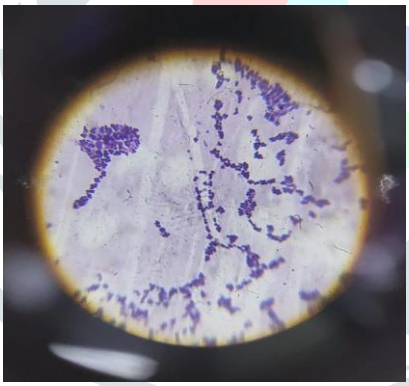

Figure 1. Positive Catalase test showing effervescence on addition of Hydrogen Peroxide.

Figure 2. Gram's staining results of suspected colonies showed the presence of Gram-positive cocci organisms.

Figure 3. Positive results of Indole, MR and NR biochemical tests (refer materials and methods).

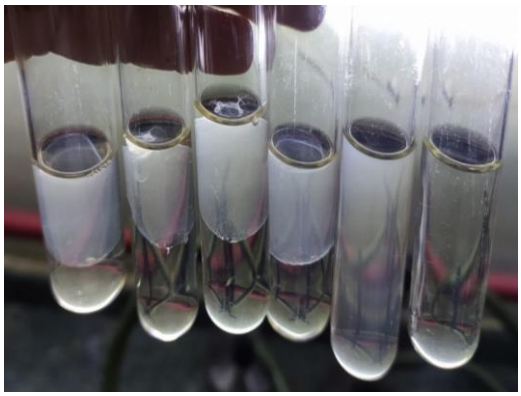

Figure 4. Plasma clots showing positive Coagulase test.

Phenotypic detection of Methicillin resistant

Test of Methicillin resistant using disc diffusion method on Muller Hinton Agar (MHA) media showed that the all the S. aureus isolates were resistant to Oxacillin and Cefoxitin. The results in Table 1 showed that there were no isolates that were only resistant to Cefoxitin in the disc diffusion method, all isolates that were detected resistant to Cefoxitin and Oxacillin. MR test confirmation was carried out using Oxacillin Resistance Screen Agar Base (ORSAB), where from a total of 10 S. aureus isolates resistant to Oxacillin the disc diffusion method was found to be positive confirmed as MR-SA. It is analyzed in Table 2 that from a total of 10 *S. aureus* isolates that were positively resistant Cefoxitin showed positive results on ORSAB.

Table 1. Results for Methicillin resistant of S. aureus isolates using disc diffusion method on Muller Hinton Agar (MHA) media to Oxacillin and Cefoxitin.

S. aureus isolates from raw chicken meat sample.	Zone of inhibition for Oxacillin (mm).	Zone of inhibition for Cefoxitin (mm).
S. aureus isolate 1	10	21
S. aureus isolate 2	10	20
S. aureus isolate 3	10	21
S. aureus isolate 4	10	21
S. aureus isolate 5	10	21
S. aureus isolate 6	10	20
S. aureus isolate 7	10	20
S. aureus isolate 8	10	21
S. aureus isolate 9	10	21
S. aureus isolate 10	10	20

* In Cefoxitin disc diffusion test the ≤21mm inhibition zone is an MR isolate, whereas in Oxacillin disc diffusion test isolate with a zone of 10mm inhibition zone is MR.

Table 2. Results for MR test confirmation using Oxacillin Resistance Screen Agar Base (ORSAB).

S. aureus isolates from raw chicken meat sample.	*S. aureus* colony color on ORSAB.
S. aureus isolate 1	Blue
S. aureus isolate 2	Blue
S. aureus isolate 3	Blue
S. aureus isolate 4	Blue
S. aureus isolate 5	Blue
S. aureus isolate 6	Blue
S. aureus isolate 7	Blue
S. aureus isolate 8	Blue
S. aureus isolate 9	Blue
S. aureus isolate 10	Blue

*MRSA colonies on ORSAB appears blue in color whereas non-MRSA colonies appear colorless.

In 1959, the preparations for methicillin and semisynthetic penicillins such as oxacillin were used as anti-staphylococcal therapy, but in 1961 a study from Trends in Microbiology Journal stated that there have been cases of MR. Research conducted by a researcher in Clinical Microbiology and Infection Journal convey the existence of MR in the United Kingdom which was then spread in several regional hospitals in Europe. Resistance to Staphylococci strains against methicillin are a global problem, where various studies to demonstrate resistance and distribution schemes are carried out intensely in Europe, Africa, America and Asia. Research in International Journal of Current Research Journal suggested that the nature of MR indicates the nature of resistance to all beta-lactam antibiotics including cephalosporins, monobactams and antibiotic groups for the treatment of Staphylococcal infection. Infection with methicillin resistance strains will have a significant impact on therapeutic problems and the potential for increased spread, so the urgency of clinical microbiology laboratories identifies is very fundamental. Basically, polymerase chain reaction (PCR) is the gold standard in detecting MRSA, but often has cost and technical constraints so that phenotypic methods are still an option in detecting (Sancaka Chasyer Ramandinianto *et al*., 2021).

This study uses 2 methods in evaluating MRS namely Cefoxitin disc diffusion test and Oxacillin disc diffusion test referring to the Kirby Bauer method combined with ORSA confirmation. The Cefoxitin disc diffusion test method which were later also confirmed with ORSAB test. Research in Indian Journal of Pathology and Microbiology Journal suggested that Cefoxitin has a higher sensitivity and specificity to mecA gene expression compared to Oxacillin which is weak in inducing PBP2a production. European Journal of Clinical Microbiology & Infectious Diseases Journal and Journal of Antimicrobial Chemotherapy reported that the results of detection with Cefoxitin disc diffusion test were better in detecting the presence of mecA gene compared to the results of detection with Oxacillin disc diffusion test. Strengthened by research report in International Journal of Current Research which states that Cefoxitin is better at detecting MR compared to Oxacillin (Sancaka Chasyer Ramandinianto *et al*., 2021).

In this study all isolates detected resistant by Cefoxitin disc diffusion test were also detected by Oxacillin disc diffusion test, all isolates by Oxacillin disc diffusion test had positive results detected by Cefoxitin disc diffusion test. Oxacillin disc diffusion test sometimes has a high false positivity level, it can be influenced by the presence of hyperproduction of β-lactamase so that it appears phenotypic expression but does not have a genotype resistance mechanism. Researcher in Memorias do Instituto Oswaldo Cruz Journal suggested that the comparison of the number of isolates detected by Cefoxitin disc diffusion test was more selective than the results of Oxacillin disc diffusion test and ORSA, it showed that Cefoxitin disc diffusion test had higher specificity compared to Oxacillin disc diffusion test and ORSA (Sancaka Chasyer Ramandinianto *et al*., 2021).

The results of this study suggest that MRSA can be found in healthy livestock samples. This is a potential health problem due to MRSA from humans can cause infection in livestock, and livestock can be the source of MRSA for infections in humans. The spread of MRSA itself occurs both through direct contact with an infected person or through direct previous contact with a surface or object contaminated by an infected individual. Animals with MRSA infections can serve as reservoirs of bacteria or human transmission by bacteria, therefore control and prevent transmission of MRSA animal to animal, as well as from animal to human must be done by adopting clean habits prevent outbreaks of MRSA and MR-CNS bacteria in animals and humans can be prevented by early detection (Sancaka Chasyer Ramandinianto *et al*., 2021).

Antimicrobial susceptibility test

The presence of antimicrobial-resistant bacteria in meat has been widely reported from different parts of the world. The use of antibiotics in livestock and the resultant residue contribute to high antibiotic resistance levels of *S. aureus* found in meat products. All the S. aureus isolates in this study were resistant to tetracycline, Vancomycin, Optochin, 20% resistant to Ofloxacin and Co-trimazole, 30% resistant to Norfloxacin and Amikacin, 40% resistant to Ciprofloxacin and Roxithromycin, 50% resistant to Piperacillin, 60% resistant to Azithromycin, 70% resistant to Bacitracin, 80% resistant to Amoxicillin and 100% sensitive to Chloramphenicol (Fig. 5). Varying resistance of S. aureus from raw meat has been reported by many authors, ranging from 25.00% to 73.30%.

S. aureus strains were least resistant to Ofloxacin (20%) and cotrimoxazole (20%). Some authors have reported that Ofloxacin-resistant isolates from raw meat can range up to 19.40%. This may lower percentage

may be because it is in injection form and hardly used, unlike a vast majority of antibiotics that come in capsule or tablet forms. For cotrimoxazole, contrary to the findings of this study, Effah et al. reported a 57.80% resistance of Methicillin-resistant *S. aureus* isolated from raw meat. Other authors, however, reported varying resistances (8.00 to 34.2%) to Methicillin-resistant *S. aureus* (MRSA) from humans (Erinda Lika *et al*., 2021). *S. aureus* is among the most prevalent cause of clinical infections globally and has garnered substantial public attention due to the increased mortality associated with the multidrug resistance phenomenon. The findings also show the potential dissemination of multidrug-resistant *S. aureus* strains in the raw chicken meat samples examined. *S. aureus* isolates were multidrug-resistant to all antibiotics tested. The presence of multidrug-resistant strains poses a severe public health risk, as well as other emerging novel diseases (Erinda Lika *et al*., 2021).

Table 3. Multidrug resistance of S. aureus isolates in raw chicken meat samples.

Antibiotics	**Isolates Resistance (n)**	**Isolates Sensitive(n)**
Tetracycline	10	-
Chloramphenicol	-	10
Azithromycin	6	4
Norfloxacin	3	7
Optochin	10	-
Ciprofloxacin	4	6
Ofloxacin	2	8
Vancomycin	10	-
Amikacin	3	7
Bacitracin	7	3
Piperacillin	5	5
Roxithromycin	4	6
Amoxicillin	8	2
Co-trimazole	2	8

Ljubojevic et al. pointed out significant problems of widespread use of tetracyclines in poultry farming. Irregular and unprescribed usage of antibiotics may have resulted in the development and transmission of resistant strains from poultry to humans via the food chain. Furthermore, Puvaca and de Llanos have explained mechanisms of transmission and resistance via the fecal-oral route between humans, environmental sources, and food and pet animals in their review. The significant impact on drug resistance could also be due to inappropriate antibiotic medical decision therapy (Erinda Lika *et al*., 2021).

In the research of Thorburn et al., post-antibiotic and post-Beta-lactamase inhibitor effects of amoxicillin were investigated. The effects of AMX were investigated on several bacteria including S. aureus and E. coli and a necessity for antibiotic dosage reduction was observed. Also, Sader et al. highlighted that the usage of third-generation antibiotics exhibits more balanced spectrums of activity against pathogens and infections when compared with other antibiotics, but only in strictly controlled therapy (Erinda Lika *et al*., 2021).

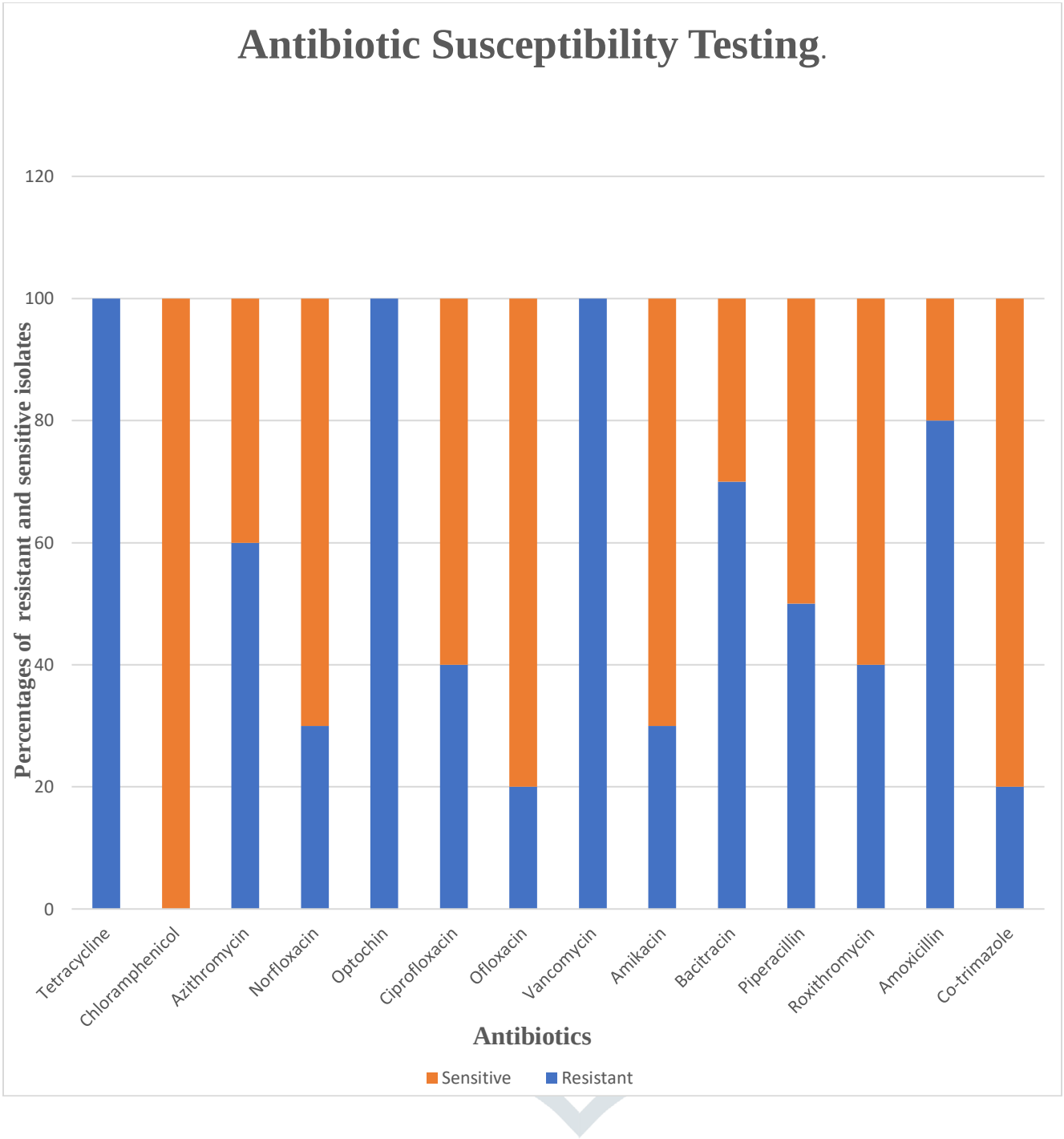

Figure 5. Percentage of *S. aureus* isolates resistant to common antibiotics.

Activity of Citrox

Inoculation with 1% and 2% citrox solutions inhibited the growth of MRSA (Table 4). However, the zone of inhibition was observed to be larger with 2% citrox. This indicates that citrox solution can be used a preservative to inhibit MRSA isolated from raw chicken meat samples.

Table 4. Antibacterial activity of citrox solution against S. aureus isolates.

S. aureus isolates from raw chicken meat sample.	Zone of inhibition observed with 1% citrox solution (mm).	Zone of inhibition observed with 2% citrox solution (mm).
S. aureus isolate 1	7	10
S. aureus isolate 2	11	13
S. aureus isolate 3	11	16
S. aureus isolate 4	11	12

S. aureus isolate 5	11	13
S. aureus isolate 6	11	15
S. aureus isolate 7	11	12
S. aureus isolate 8	15	16
S. aureus isolate 9	13	15
S. aureus isolate 10	14	15

V. CONCLUSION AND FUTURE SCOPE

In conclusions, the present study revealed high proportion of *Staphylococcus* species in raw chicken meat. Isolation rate of *Staphylococcus* species from retail shops signals the existence of poor hygienic practices and consequently, its public health implication. This study has shown that based on its ORSAB test results the strains of *Staphylococci* that were isolated from raw chicken meat prevalence for MRSA. It can be concluded that detecting for Methicillin Resistant is easily epidemiological tool for prevention the spreading of antimicrobial resistance from raw chicken meat to public health. The role of food in the spread of pathogens cannot be over-emphasized in public health. Based on the results, raw chicken meat from retail stores remains a potential source in transmitting pathogenic foodborne bacteria. All the samples were positive for Staphylococcus species, of which all were positive for S. aureus. The S. aureus isolates were most resistant to tetracycline (100%), Vancomycin (100%), and Amoxicillin (80%), while decreased resistance toward Co-trimazole (20%) and Ofloxacin (20%) was recorded. Therefore, there is the need for adequate food processing, especially at a suitable temperature, to reduce the possible microbial contamination in the food products, as well as surveillance of and good hygiene practice by meat handlers in the face of an increasing threat of multidrug-resistant S. aureus both in animals and humans. From findings, it was determined that raw chicken meat from retail stores can be classified as “very high additional risk” or even as “high additional risk”. This highlights the importance of continued surveillance and the need to take measures in the primary sector to minimize the risk for the consumer. This study has also shown effectiveness of citrox solution against MRSA isolated from raw chicken meat samples which indicates it to be a useful and effective preservative against the same.

Focusing mainly on presence of MRSA on raw chicken meat and helping eliminate them by using a suitable preservative.

As we are focusing on MRSA and the preservative as an application. The main concept of while developing the application was the usefulness of the application towards its inhibition of MRSA. The citrox solution can be used as a preservative so that it will help inhibiting MRSA growth and reduce food related diseases caused by MRSA when chicken meat with infected MRSA is consumed.

So, our main motive is to find an effective solution as a preservative to inhibit MRSA on raw chicken meat by developing citrox solution as the preservative application.

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