



Extraction of Staphyloxanthin from *MRSA* Isolated from Raw Chicken Meat and to Determine its Antibacterial Activity Against Other Bacteria.

1 Rohini Shetty, 2 Sahrish Ansari.

1 Professor in department of Microbiology, Chikitsak Samuha's S.S. and L.S. Patkar-Varde College, Mumbai, India

2 Postgraduation student in department of Microbiology, Chikitsak Samuha's S.S. and L.S. Patkar-Varde College, Mumbai, India.

Abstract: Ten isolates characterized as *Staphylococcus aureus*, were isolated from 10 different raw chicken meat samples from different retail stores in Mumbai. The staphyloxanthin (STX) production of *S. aureus* isolate was studied. The milk agar medium revealed the highest production of pigment at pH 8 for 72 hr at 37 degrees C. The Staphyloxanthin pigment was extracted using methanol and its Rf value was determined using Thin Layer Chromatography (TLC). Antibacterial activity of STX was detected against the bacteria used in this study namely *Pseudomonas aeruginosa*, *Salmonella typhi*., *Shigella* spp, *Escherichia coli*, *Klebsiella* spp, *S.pyogenes*, *S.luteus* out of it showed its antibacterial activity against *Klebsiella* spp., *Escherichia coli*, , *S.luteus*, *Shigella* spp, *Pseudomonas aeruginosa* only. Consequently, the aim of the present study was to detect the role of staphyloxanthin pigment production from *S. aureus* isolates from different raw chicken meat samples as antibacterial agent against some pathogenic bacteria used in this study.

Keywords: *Staphylococcus aureus*, Staphyloxanthin (STX), Milk agar medium, Methanol, Thin Layer Chromatography (TLC), Antibacterial activity.

I. INTRODUCTION

Staphylococcus aureus has been tested in meat and poultry products to assess microbiological safety, sanitation conditions during processing, and storage quality of products (Tompkin, 1983). *S. aureus* is a frequent etiological agent of food poisoning (Halpin-Dohnalek and Marth, 1989; Lo'pez et al., 1993; Jablonski and Bohach, 1997). Its presence in poultry (Waldroup, 1996) emphasizes the need for laboratory surveillance for this bacterial pathogen. However, not all *S. aureus* strains present in processed poultry carcasses are a cause for public health concern. The animal strains make a very small contribution to human food poisoning (Hajek and Marsalek, 1971; Shiozawa et al., 1980; Parker, 1983; Isigidi et al., 1990). On the other hand, the *S. aureus* isolates from human sources may be considered the most dangerous strains of public health significance (Isigidi et al., 1992). In fact, poultry meat has been frequently associated with foodborne illness in which initial contamination is traceable to food handlers (Halpin-Dohnalek and Marth, 1989).

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).

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-methicillin-resistant *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 technique 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 pathogen *Staphylococcus aureus* is a gram-positive, gold-colored colony. The yellow-to-orange colony color of *S. aureus* is one of the classical criteria for identification of this species. Staphyloxanthin is membrane-bound carotenoid which plays a role in the environmental fitness of *S. aureus*. Membrane pigments have also been hypothesized to be virulence factors in *S. aureus*, potentially by detoxifying reactive oxygen species produced by phagocytes. Carotenoids may also stabilize the *S. aureus* membrane during infection and pathogenesis. Staphyloxanthin is a typical secondary metabolite. It is not necessary for the growth and reproduction of *S. aureus* but might serve a role in survival in infected hosts and in combating the immune system, staphyloxanthin is mainly produced in stationary phase, its chemical formula (C₅₁H₇₈O₈). Staphyloxanthin is a neutral molecule and light had no effect on its synthesis (Al-Kazaz *et al.*, 2014).

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 hygiene 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)

The results of identifying the ability of *S.aureus* isolates (43 isolate) for staphyloxanthin production on different culture media found that milk agar medium was the best medium for the staphyloxanthin production which gave the highest percentage of orange pigment production 44.1 %. While, trypticase yeast medium and trypticase soya medium revealed percentage 37.2%. Peanut seeds medium and sesame seed medium appeared percentage 30.2 % for orange staphyloxanthin production. No production of orange pigment was observed in several media such as sunflower seeds medium, brain heart infusion agar, carotinoid expression medium, muller hinton agar and nutrient agar. These results suggested that specific nutrients are required for staphyloxanthin production. According to the results pigment producer isolate AE36. Medium components may be critical for production of staphyloxanthin. The fatty acid as a carbon source is a better substrate for the growth of bacteria than sugars. Based on the comparison between the composition of different fatty acid containing seeds and oils. The saturated form of fatty acid could be a better choice of carbon source for the maximum production of pigment. *Milk: Milk agar medium, TYA Trypticase yeast medium, TSA: Trypticase soya medium, PNSA: Peanut seeds medium, SSA: Sesame seed medium, SFSA: Sunflower seeds medium, BHIA: Brain-Heart Infusion Agar, N.A: Nutrient Agar MHA: Mueller-Hinton Agar CEM: Carotinoid expression medium. The antibacterial activity of the pigment was examined against several bacterial genera (*Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Salmonella spp.*, *Shigella spp.*, *Escherichia coli*, *Klebsiella spp*, *Proteus spp*. *Pseudomonas fluorescens*, *Pseudomonas putida*, *Staphylococcus aureus* isolate AE36, *Staphylococcus aureus* isolate AE32 and *Staphylococcus aureus* isolate AE38. The results revealed that staphyloxanthin has no activity against tested bacteria that used in the study. Other study by reported the staphyloxanthin pigment extraction did not show antibacterial activities against several gram positive and gram-negative bacteria at concentration 0.25 mg/ml, the results in the study confirmed by results of which revealed that the staphyloxanthin pigment extract has no activity against *Klebsiella spp.*, due to that STX pigment is a virulence factor help bacteria to cause a disease after inter human body (Al-Kazaz et. al.,2014).

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 containing 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 streaking the organism on Tributyrin Agar Plates and incubated at 37 degrees C for 24 hrs, after incubating clear zones around colonies indicates 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 shaking 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 stabbing 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 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 37°C for 24 hours and measured the inhibition zone. In Cefoxitin disc diffusion test the ≤ 21 mm 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.

Production of staphyloxanthin pigment from MRSA isolates.

In order to identify the ability of *S.aureus* isolates for the staphyloxanthin production , 10 ml of Brain-Heart Infusion Broth (BHIB)was inoculated with 100µl of *S.aureus* isolate (10 isolates) and incubated at 37°C for 18 hour in order to get 10⁸ cell/ml (Bacterial growth was determined by measuring the absorbency at 620 nm). A volume of 100 µl of the inoculum from each isolate was streaked on Milk agar medium incubated at 37°C for two days and then incubated at 20°C for two days. Appearance of growth with pigment (orange, yellow) indicates a positive result.

Extraction and determination of Rf value of staphyloxanthin pigment by TLC.

The pigment of *Staphylococcus aureus* (STX) was extracted by using methanol, as follows:

Bacterial cells were recovered from the growth on milk agar plate for 72hr at 37 degrees C. Agar surfaces were rinsed with sterile double distilled water (each rinse with 3 ml). Then the bacterial cells were centrifuged at 6000rpm for 15 min. The supernatant was discarded and the pellet was resuspended with double distilled water and then centrifuged again at 6000 rpm for 15 min. Staphyloxanthin extraction from the pellet containing the bacterial cells was collected. The pellet was mixed with 8 ml of 99.9% methanol wrapped with aluminum foil to prevent exposure to light. The packed cells were resuspended in 3 ml of methanol, held at 55°C in water bath for 5 min and cooled for 10 min , and then, the extract was collected by centrifugation (6000 rpm for 15 min). The extraction was repeated twice, until no further pigment could be extracted. Then Rf value of extracted pigment was determined by Thin-layer chromatography (TLC). Solvent systems were used. The TLC plates were spotted with staphyloxanthin extracts and developed with the following: benzene-methanol-acetic acid 87:11:2 (vol/vol/vol).

The Rf value was calculated according to the following equation:

$$Rf = \text{Distance of spot sample movement} / \text{Distance of spot solvent movement.}$$

Staphyloxanthin as an antibacterial agent

The activity of staphyloxanthin as antibacterial agent was tested by the well-diffusion as follows:

The Mueller-Hinton Agar (MHA) plates were inoculated with 10⁸cell/ml of indicator isolates (*Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella* spp, *Escherichia coli*, *Klebsiella* spp, *S.pyogenes*, *S.luteus*.) (obtained from department of Microbiology / university of Mumbai). Wells were prepared in the plates with 6 mm sterile cork borer. The wells for each culture of indicator bacteria were filled with 100 µl of staphyloxanthin solutions extracted from different isolate. The plates were incubated at 37 degrees C for 24 hr. Inhibition was detected by a zone of clearing around the staphyloxanthin extract.

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.

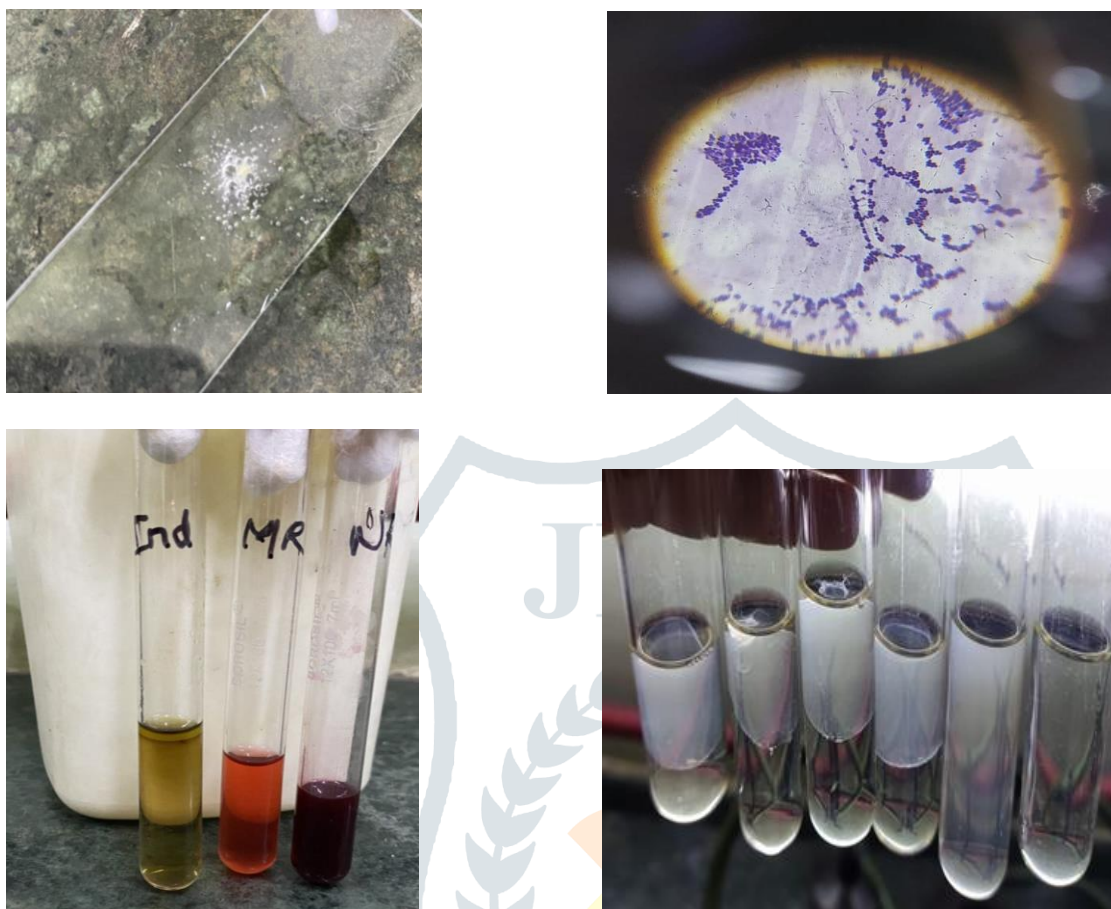


Figure 1. Positive Catalase test showing effervescence on addition of Hydrogen Peroxide.

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.

<i>S. aureus</i> isolates from raw chicken meat sample.	Zone of inhibition for Oxacillin (mm).	Zone of inhibition for Cefoxitin (mm).
<i>S. aureus</i> isolate 1	10	21
<i>S. aureus</i> isolate 2	10	20

<i>S. aureus</i> isolate 3	10	21
<i>S. aureus</i> isolate 4	10	21
<i>S. aureus</i> isolate 5	10	20
<i>S. aureus</i> isolate 6	10	21
<i>S. aureus</i> isolate 7	10	21
<i>S. aureus</i> isolate 8	10	20
<i>S. aureus</i> isolate 9	10	21
<i>S. aureus</i> isolate 10	10	21

* In Cefoxitin disc diffusion test the ≤ 21 mm 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).

<i>S. aureus</i> isolates from raw chicken meat sample.	<i>S. aureus</i> colony color on ORSAB.
<i>S. aureus</i> isolate 1	Blue
<i>S. aureus</i> isolate 2	Blue
<i>S. aureus</i> isolate 3	Blue
<i>S. aureus</i> isolate 4	Blue
<i>S. aureus</i> isolate 5	Blue
<i>S. aureus</i> isolate 6	Blue
<i>S. aureus</i> isolate 7	Blue
<i>S. aureus</i> isolate 8	Blue
<i>S. aureus</i> isolate 9	Blue
<i>S. aureus</i> 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).

Production of staphyloxanthin pigment from MRSA isolates.

The results of production of staphyloxanthin pigment from *S. aureus* on milk agar medium gave orange pigment production for all the 10 isolates. These results suggested that specific nutrients are required for staphyloxanthin production. According to the results pigment producer isolate. Medium components may be critical for production of staphyloxanthin. The fatty acid as a carbon source is a better substrate for the growth of bacteria than sugars (Al-Kazaz *et al.*, 2014).

Extraction and determination of Rf value of staphyloxanthin pigment by TLC.

The best results of extraction the pigment was with methanol. The RF value was determined with TLC and was found to be 0.625.

Anti-bacterial activity of staphyloxanthin against bacteria

The antibacterial activity of the pigment was examined against several bacterial genera (*Pseudomonas aeruginosa*, *Salmonella typhi*., *Shigella* spp, *Escherichia coli*, *Klebsiella* spp, *S.pyogenes*, *S.luteus*). The results revealed that staphyloxanthin has antibacterial activity against tested bacteria that used in this study namely *Klebsiella* spp., *Escherichia coli*, *S.luteus*, *Shigella* spp, *Pseudomonas aeruginosa* only indicating that it can be used as treatment against infection caused by these organisms.

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 examined for production of staphyloxanthin pigment production. All the MRSA isolates showed good pigment production on Milk Agar which was extracted using methanol method and its Rf value was determined by TLC.

The antibacterial activity of this pigment against different organisms studied using Agar diffusion Method indicated that the pigment has the ability to inhibit the growth of the organisms and can be used against the infection caused by the organisms. 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 MRSA 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.

Focusing mainly on the presence of MRSA on raw chicken meat and helping eliminate other organisms using staphoxanthin pigment extracted as an antibacterial agent as an ointment or in cosmetics to avoid infections caused by some pathogenic organisms by inhibiting the growth.

As we are focusing on MRSA and the staphyloxanthin as an application. The main concept of while developing the application was the usefulness of the application towards its inhibition of growth of some pathogenic organisms. Staphyloxanthin pigment can be used as an antibacterial agent so that it will help inhibiting growth and reduce infections that can be hazardous.

So, our main motive is to find an effective antibacterial agent to inhibit growth of some pathogenic organisms by extracting staphyloxanthin pigment from MRSA found on raw chicken meat as the application in different ointment and cosmetics.

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