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Research Article

PHENOTYPIC AND GENOTYPIC IDENTIFICATION OF METHICILLIN RESISTANT *STAPHYLOCOCCUS SPECIES* FROM FRUITS

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ABSTRACT

Staphylococcus is one of the most predominant pathogen that impacts major serious health issues. The present study is to evaluate the presence of *Staphylococcus* sp. in locally available fruits such as grape, watermelon, musk melon and gooseberry. About 30 bacterial strains were isolated, enriched and grown in Mannitol Salt Agar (MSA). Biochemical characterization and confirmatory tests like Rapid MRSA agar test and Baird Parker agar tests were screened and tests confirmed as *Staphylococcus* sp. Antibiotic susceptibility test was checked using multiple drug discs (12 antibiotics) and single discs (Methicillin, Mupirocin) by disc diffusion method. Further biofilm acticity was evaluated using crystal violet test tube and congo red agar method. Genotypic identification of isolates were done using gap, nuc, mec for culture identification and eno, icaA, icaD primers for confirmation of biofilm production that resulted in showing all 30 isolates answered for eno, icaA, icaD primers which confirmed as biofilm producers and serious pathogen. Therefore we could elucidate that the pathogenic strains were adhesive on to the fruits due to improper handling; so as the study concluding that serious measures has to be taken while processing and consuming fruits as they tend to be a threat to human beings as a serious food pathogen.

Keywords: Staphylococcus, Genotypic, Biofilm.

1. INTRODUCTION

Bacteria, viruses and parasites are the sources of many food poisoning cases, usually due to improper food handling. Some bacteria, in small amounts, are not harmful to most healthy adults because the human body is equipped to fight them off. The trouble begins when certain bacteria and other harmful pathogens multiply and spread, which can happen when food is mishandled. Foodborne illness (commonly known as food poisoning) is often caused by consuming food contaminated by bacteria and/or their toxins, parasites, viruses, chemicals, or other agents. Foodborne illness occurs when people eat or drink food or beverages contaminated with pathogens, chemicals, or toxins. There are several factors that can contribute to the symptoms and severity of food poisoning, including a weakened immune system and age. While the American food supply is among the safest in the world, the federal government estimates that there are about 48 million cases of foodborne illness each year. Every outbreak of foodborne illness is different. Some general key foodborne pathogens are *E.coli, Salmonella spp., Listeria spp., Cyclospora spp.,* The incidence of foodborne diseases around the world has been recorded in both developing and developed countries likely due to the globalization of food supply and trade. Trends toward greater geographic distribution of fruits, vegetables and herbs from central processing facilities and subsequent storage and handling practices in food preparation areas may also be contributing to an increased frequency of produce-associated infections. Many large outbreaks involving widely consumed commodities such as apple cider, cantaloupe, raspberries, bagged lettuce and spinach, tomatoes, green onions and sprouts have been reported during the past decade [1].

Fresh vegetables and fruits are known to supply several types of health promoting compounds that have been associated with protection from chronic diseases such as cancer, diabetes, hypertension as well as other medical conditions. The surfaces of fruits and vegetables show a large diversity in structure and composition and present a variety of surfaces to which a bacterium may bind. Those surfaces provide a habitat for a variety of microorganisms including bacteria, yeast and molds [2]. The epidermis is covered by an epicuticular wax on aerial organs (leaves, stem, flowers and fruits) or periderm on roots and

tubers. Stomata, lenticels, broken trichomes and scars from detached organs represent natural ways of entry for microorganisms. Since cracks in the surface of fruits and vegetables, as well as in herbs, may occur in certain growing conditions and as post-harvest handling may cause injuries and bruising, microorganisms transferred to fresh produce can enter areas of pre-existing damage [3]. Damage to the cuticular layer can permit microbial proliferation in cellular fluids and moisture released from the damaged sites. Sugars in released juices from damaged tissue attract insects, which can further injure fresh produce and facilitate entry of microorganisms [3]. *Staphylococcus* (staph) is a pathogen commonly found on the skin, throats and nostrils of healthy people and animals. Therefore, it usually doesn't cause illness unless it is transmitted to food products where it can multiply and produce harmful toxins. Staphylococcal symptoms include nausea, stomach cramps, vomiting or diarrhoea. Staphylococcal toxins are heat resistant and cannot be destroyed by cooking. Anyone can develop a staph infection but certain groups of people are at greater risk, including people with chronic conditions such as diabetes, cancer, vascular disease, eczema and lung disease.

S. aureus can gain access to milk either by direct excretion from udders with clinical or subclinical staphylococcal mastitis or by contamination from the environment during handling and processing raw milk [4]. Anti-microbial resistance is an important public health concern worldwide. The development of resistance both in human and animal bacterial pathogens has been associated with the extensive therapeutic use of anti microbials or with their administration as growth promoters in animal production [5].

Methicillin-resistant *S. aureus* (MRSA) was first reported In the United Kingdom (UK) in 1961, and by mid-1990s, it had become a major problem worldwide [6]. Methi-cillin resistance is of particular relevance because it is conferred by the presence of mecA gene, which encodes production of an altered penicillin binding protein (PBP) (PBP2a or PBP2') that has a low affinity for all beta-lactam antimicrobials (penicillins, cephalosporins, carba-penems) [7]. Therefore, methicillin resistant *Staphylococci* are resistant to this broad range of important anti-microbials. The mecA gene resides on a Staphylococcal chromosomal cassette [8].

Standardized methods of susceptibility test have been used for the detection of MRSA strains. However, phenotypic express of methicillin-resistance can be heterogenous. In addition, methicillin resistance is influenced by culture conditions such as temperature, pH and sodium chloride (NaCl) content in the medium. These factors complicate the detection of methicillin resistance, especially for strains with low level resistance. The polymerase chain reaction (PCR) methods have high sensitivity and specificity and are independent of the physical and chemical conditions of the culture [9, 10]. The ability of bacteria to produce extracellular poly-saccharides on surfaces, which results in the formation of biofilm, enhance bacterial colonization and survival on plant surface and increase their resistance to cleaning and to antimicrobial agents, is well known. Containers used to harvest, transport and display raw fruits and vegetables are often not effectively cleaned and sanitized, which can lead to the development of biofilms. Even single-use containers may hold produce for a sufficient time to allow the formation of biofilms. Contamination of fresh produce with pathogens may result from contact with surfaces harbouring these biofilms. If pathogens attach to biofilms during transport or processing, their survival and growth may be enhanced [3]. The current study is to check the prevalence of *Staphylococcus sp.,* in locally available fruits.

2. MATERIAL AND METHODS

2.1. Sample collection

Spoiled fruit samples were selected as a source for isolating *Staphylococcus* sp., Spoiled fruit samples were collected from the fruit stall and stored at 4°C under sterile conditions.

2.2. Isolation of bacterial strain

Fruit skin samples were thoroughly homogenised in a sterile motor and pestle using 1ml of saline. 1ml of the sample was added to 10ml of sterile nutrient broth prepared and was kept for incubation at 37°C for 24h in a shaking incubator. After incubation 100µl of the inoculum was spread plated in a sterile Mannitol Salt Agar (MSA) plates, and again kept for incubation for 24h at 37°C. From that yellow individual colonies were selected and inoculated into MSA medium and further sub-culturing was done for getting pure colonies.

2.3. Antibiotic Susceptibility Test

2.3.1. Antibiotic test for multidrug resistance

Antimicrobial susceptibilities of the isolates were tested by the agar disc diffusion method on Mueller-Hinton agar. HIMEDIA dodeca G-VI plus multiple antibiotic discs were placed on Mueller-Hinton agar plates, incubated at 37°C for 24 h, and the diameter of each zone was measured in millimetres. The following

antibiotic discs were used: Penicillin, Amoxicillin, Carbenicillin, Methicillin, Azithromycin, Clindamycin, Roxithromycin, Lincomycin, Vancomycin, Rifamycin, Teicoplanin, Lineolid.

2.3.2. Antibiotic test for Methicillin and Mupirocin resistance

Antimicrobial susceptibilities of the isolates were tested by the agar disc diffusion method on Mueller-Hinton agar. Methicillin and Mupirocin antibiotic discs were placed on Mueller-Hinton agar plates, incubated at 37°C for 24 h, and the diameter of each zone was measured in millimetres [11].

2.4. Confirmation of Methicillin Resistant *Staphylococcus*

2.4.1. Rapid MRSA agar plate technique

Rapid MRSA Agar (special peptone-20g/L, Casein peptone-20g/L, sodium chloride-8.5g/L, carbohydrate-14g/L, phenol red- 0.025g/J, chromogenic mix- 6.5g/L, Amina-vitamin mix- 1.2g/L, Agar-15g/L) plates were prepared and isolates were inoculated aseptically and incubated at 30-35°C for 18-24 h [12].

2.4.2. Baird Parker Agar technique

Baird Parker Agar (Tryptone-10g/L, HM peptone-5g/L, Yeast extract-1g/L, Glycine-12g/L, sodium pyruvate-10 g/L, Lithium chloride-5 g/L, Agar-20 g/L) plates were prepared and isolates were inoculated aseptically and incubated at 35-37°C for 24-48 h [13].

2.5. Identification of biofilm activity

2.5.1. Crystal violet test

Nutrient broth was inoculated with loopful of micro-organism from overnight culture plates and incubated for 24 h at 37°C. The tubes were decanted and washed with PBS (pH 7.3) and dried tubes were stained with crystal violet (0.1%). Excess stain was removed and tubes were washed with deionized water. Tubes were then dried in inverted position and observed for biofilm formation.

2.5.2. Congo Red Agar method

This is an alternative method for screening biofilm formation by *Staphylococcus* isolates which requires the use of a specially prepared solid medium -brain heart infusion broth (BHI) supplemented with 5% sucrose and Congo red. The medium was composed of BHI (37 g/L), sucrose (50 g/L), agar no.1 (10 g/L) and congo red stain (0.8 g/L). Congo red was prepared as concentrated aqueous solution and autoclaved at 121°C for 15 minutes, separately from other medium constituents and was then added when the agar had cooled to 55°C. Plates were inoculated and incubated aerobically for 24 to 48 h at 37°C [14].

2.6. Genotypic Identification of Methicillin Resistant *Staphylococcus sp.,*

2.6.1. DNA isolation

DNA was extracted from the bacterial cultures grown in Brain heat infusion agar for overnight culture. DNA was harvested from 1loop of bacterial colony inoculated in 200µl of Lysis buffer (1% Tritone X-100µl, 0.5% Tween 20-50 µl, 10mM Tris Hcl-100 µl, 1mM EDTA-20 µl and make up to 1L) and the mixture was kept for incubation at 70°C for 10 min. The whole lysate was centrifuged at 6000 rpm for 3 min.

2.6.2. PCR amplification

For identifying the isolated bacterium and confirming its biofilm activity by gene amplification using selected primers

3. RESULTS AND DISCUSSION

Earlier reports were undertaken to study regarding pathogens in fruits, vegetables and common food. But *Staphylococcus* spp., being an important pathogen is mostly studied from human skin and nasal samples and least studied from fruits. In the present study, the fruits were used to evaluate the prevalence of *Staphylococcus* sp., and 30 bacterial strains were isolated the locally available fruits like grape, water melon, musk melon and gooseberry were used. The Mannitol Salt Agar (MSA) is the growth and enrichment medium for *Staphylococcus*. *Staphylococcus* sp., that has the ability to cause throat, skin infections and other deadly diseases.

MRS is a resistant variation of the common bacterium *Staphylococcus*. It is an invasive pathogen that can cause disease in almost any tissue or organ in the human body, primarily in compromised individuals. Patients with breaks in their skin due to wound, in dwelling catheters or burns are those with certain risk of developing MRS infection. The major confirmatory test is Rapid MRSA agar test. In that medium, special peptone, Casein peptone and amino-vitamin mix provides essential nutrients for growth. Carbohydrate is the source of carbon and energy. Phenol red is the pH indicator. The chromogenic mixture incorporated in the medium is specifically cleaved by *Staphylococcus* (MRS) to give greenish yellow coloured colonies [15]. The thirty bacterial isolates when streaked on Rapid MRSA agar plates, fifteen isolates showed greenish yellow colonies as shown in Fig 1.

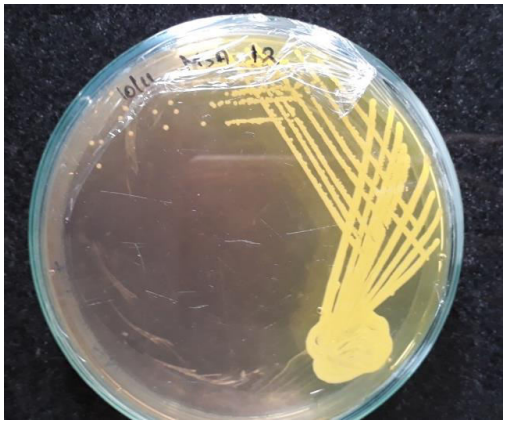

Fig. 1: MRS bacteria grown on MSA Agar

The identity of *Staphylococcus* isolates on Baird-Parker Agar must be confirmed with a coagulase reaction. Baird-Parker Agar can also be used to detect coagulase activity by adding fibrinogen plasma. Fibrinogen Plasma Trypsin Inhibitor supplement (FD195) dissolved in 10 ml sterile distilled water added to 90 ml sterile molten media kept at 45-50°C. On this medium coagulase positive colonies appear white to grey-black surrounded by an opaque zone due to coagulase activity within 24-48 hours incubation at 37°C. The tellurite additive is toxic to egg yolk-clearing strains other than *Staphylococcus* and imparts a black colour to the colonies. Glycine, pyruvate enhances growth of *Staphylococcus*. With the addition of egg yolk, the medium becomes yellow, opaque. The egg yolk additive, in addition to provide enrichment, aids in the identification process by demonstrating lecithinase activity (egg yolk reaction). A clear zone and grey-black colonies on this medium are diagnostic for coagulase negative *Staphylococci* [13]. Among thirty bacterial isolates, all of them were coagulase negative and showed a clear zone, grey-black colonies in the medium.

Staphylococcal infections were earlier treated using Penicillin. But over the years, resistance to this drug developed. Methicillin was the next drug of choice. While methicillin is very effective in treating most *Staphylococcus* infections some strains have developed resistance to methicillin and can no longer be killed by this antibiotic. These resistant bacteria are called Methicillin Resistant *Staphylococcus* (MRS). Patients with breaks in their skin due to wound, indwelling catheters or burns are those with certain risk of developing MRS infection. Here further many antibiotics are also used to find the antibiotic susceptibility of the bacterial isolates [15]. Seventeen isolates showed resistance to Methicillin and nine showed resistance to Mupirocin among thirty isolates. Twelve different antibiotics were present in the multiple drug discs among the thirty bacterial isolates only eight isolates were observed with resistance to four or more than four antibiotics and those were *S. equorum, S. lentus and S. haemolyticus* strains. Most of the isolates were sensitive to almost all antibiotics as shown in Table 1.

Antibiotic susceptibility is widely used to measure the susceptibility of bacteria to antibiotics. Because may bacteria have resistance to some antibiotics. Susceptibility is determined by measuring the diameter of the zones of bacterial inhibition around the antibiotic disks and comparing the diameter with disk diffusion method. The isolates were assayed for their resistance to antibiotic activity against 12 different antibiotics using multidrug discs. The isolates were tested using the disc diffusion method where cultures are swabbed in the medium and discs are placed above it to observe the zone formation and in turn resistance to antibiotics is found. Antibiotic susceptibility was found for all the 30 isolates using multidrug disc. The result is as shown in the fig 2 and table 2.

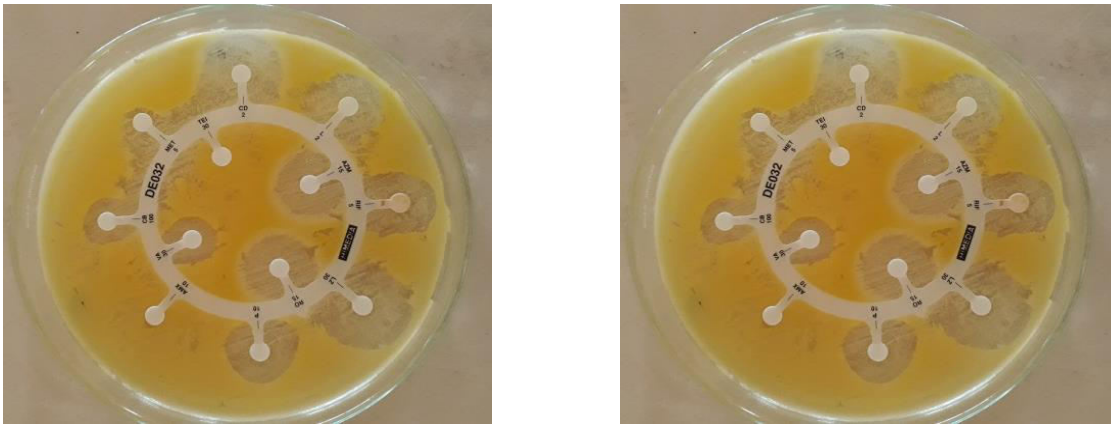

Fig. 2: Antibiotic test for Multidrug Resistance

Table 1: Biochemical characteristics of 30 isolates

S. NO	Culture No	Gram's Staining	Catalase	Oxidase	Coagulase	DNase
1	GRA01	+	-	-	-	+
2	GRA02	+	-	-	-	+
3	GRA03	+	-	-	-	+
4	GRA04	+	-	-	-	+
5	AML05	+	+	-	-	+
6	GRA06	+	-	-	-	+
7	GRA07	+	-	+	-	+
8	GRA08	+	-	-	-	+
9	WAM09	+	-	-	-	+
10	MUM10	+	+	+	-	+
11	GRA11	+	-	-	-	+
12	GRA12	+	-	-	-	+
13	WAM13	+	+	-	-	+
14	MUM14	+	+	-	-	+
15	MUM15	+	-	-	-	+
16	MUM16	+	-	-	-	+
17	GRA17	+	+	-	-	+
18	MIX18	+	+	-	-	+
19	MUM19	+	-	-	-	+
20	MUM20	+	+	-	-	+
21	MUM21	+	-	-	-	+
22	GRA22	+	-	-	-	+
23	GRA23	+	+	-	-	+
24	MIX24	+	+	-	-	+
25	GRA25	+	+	-	-	+
26	MIX26	+	-	-	-	+
27	WAM27	+	-	-	-	+
28	WAM28	+	+	-	-	+
29	WAM29	+	+	-	-	+
30	WAM30	+	-	-	-	+

Table 2: Antibiotic susceptibility of 30 isolates

S. NO	Culture No	P	AMX	CB	MET	AZM	CD	RO	L	VA	RIF	TEI	LZ
1	GRA01	S	S	S	S	S	S	S	S	S	S	R	S
2	GRA02	S	R	R	R	S	S	S	S	S	S	R	S
3	GRA03	S	S	S	S	S	S	S	S	S	S	R	S
4	GRA04	S	R	R	S	R	S	S	S	S	S	R	S
5	AML05	S	R	S	S	S	S	S	S	S	S	R	S
6	GRA06	S	S	S	S	S	S	S	S	S	S	R	S
7	GRA07	S	S	S	S	S	S	S	S	S	S	R	S
8	GRA08	S	S	S	S	S	S	S	S	S	S	S	S
9	WAM09	S	S	S	S	S	S	S	S	S	S	S	S
10	MUM10	S	S	S	S	S	S	S	S	S	S	S	S
11	GRA11	S	S	S	R	S	S	S	S	S	S	R	S
12	GRA12	S	R	S	R	S	S	S	S	S	S	R	S
13	WAM13	S	S	S	S	S	S	S	R	S	R	R	S
14	MUM14	S	R	S	R	S	S	S	S	S	R	S	S
15	MUM15	R	R	S	R	S	R	S	S	S	R	R	S
16	MUM16	R	R	S	R	S	S	S	S	S	S	R	S
17	GRA17	S	S	S	R	S	S	S	S	R	S	R	S
18	MIX18	S	S	S	R	S	S	S	S	S	S	S	S

19	MUM19	S	S	S	R	S	S	S	S	S	S	S	S
20	MUM20	S	S	S	R	S	R	R	S	S	S	R	R
21	MUM21	S	S	S	S	S	R	S	R	S	S	R	S
22	GRA22	S	S	S	R	S	R	S	R	S	S	R	S
23	GRA23	S	S	S	S	S	S	S	S	S	S	R	S
24	MIX24	S	S	S	R	S	S	S	S	S	S	S	S
25	GRA25	S	S	S	R	S	S	S	S	S	S	R	S
26	MIX26	S	S	S	R	S	S	S	S	S	S	R	S
27	WAM27	S	S	S	R	S	R	S	R	S	S	R	S
28	WAM28	S	S	S	R	S	R	S	R	S	S	R	S
29	WAM29	S	S	S	S	S	S	S	S	S	S	R	S
30	WAM30	S	S	S	R	S	S	S	S	S	S	R	S

Methicillin and Mupirocin antibiotic discs were separately done to list out MRS cultures specifically. Many showed resistance towards Methicillin and sensitivity towards Mupirocin. The result is as shown in the fig 3 and table 3.

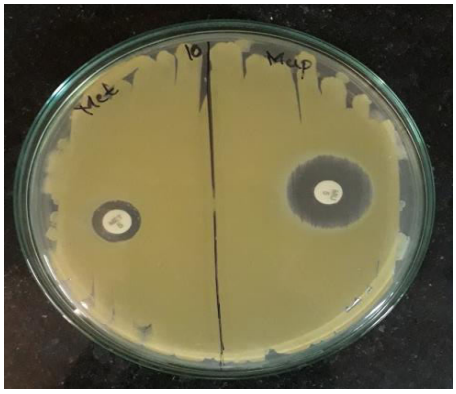

Fig. 3: Antibiotic test for Methicillin and Mupirocin resistance

Confirmatory tests for MRS are Rapid MRSA agar plate technique and Baird Parker agar method. In MRSA agar plate method Methicillin Resistant Staphylococcus aureus cultures will grow in a greenish yellow colour. In Baird Parker agar method MRS cultures will grow as black colonies. Fifteen isolates showed positive result for MRSA agar plate method and 29 cultures showed positive result for Baird Parker agar method. The result is as shown in fig. 4, 5 and table 4.

Presence of more strains of *Staphylococcus* with many drugs resistance is a great threat to society and all living beings. Biofilm (a slimy layer producing) bacteria pose a severe threat as they possess the ability to act as a persistent source of microbial contamination that may lead to food spoilage as well as transmission of diseases (Brooks & Flintio, 2008). Biofilm formation is a manifestation of bacterial quorum sensing and depends on an interaction between the bacterial cells, the attachment surface and the surrounding medium [16, 17]. As a result of these interactions a ring formation occurs in the test tubes when tested with crystal violet stain. Twenty isolates showed ring formation in the test tubes bt confirming biofilm formation as shown in fig. 6 and tabulated in table 2.

Table 3: Antibiotic tests of 30 isolates

S. NO	Culture No	MET	MUP
1	GRA01	S	S
2	GRA02	R	S
3	GRA03	S	S
4	GRA04	S	S
5	AML05	S	R
6	GRA06	S	R
7	GRA07	S	S
8	GRA08	S	S
9	WAM09	S	S
10	MUM10	S	S
11	GRA11	R	S
12	GRA12	R	S
13	WAM13	S	S
14	MUM14	R	S
15	MUM15	R	S
16	MUM16	R	S
17	GRA17	R	S
18	MIX18	R	R
19	MUM19	R	S
20	MUM20	R	S
21	MUM21	S	S
22	GRA22	R	R
23	GRA23	S	S
24	MIX24	R	R
25	GRA25	R	R
26	MIX26	R	S
27	WAM27	R	R
28	WAM28	R	R
29	WAM29	S	S
30	WAM30	R	R

MET-Methicillin; MUP-Mupirocin

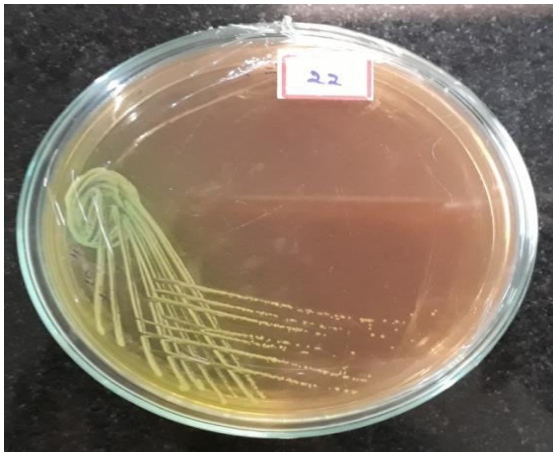

Fig. 4: Rapid MRSA agar plate

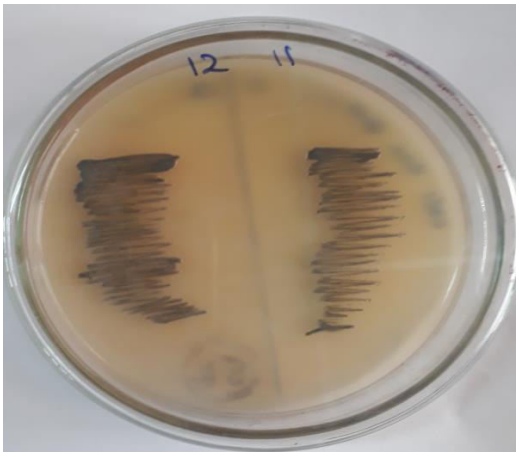

Fig. 5: Baird Parker agar plate

Table 4: MRSA Confirmation of 30 isolates

S. No	Culture No	MRSA plate	BPA plate
1	GRA01	+	+
2	GRA02	+	+
3	GRA03	+	+
4	GRA04	+	-
5	AML05	+	+
6	GRA06	-	+
7	GRA07	-	+
8	GRA08	-	+
9	WAM09	-	+
10	MUM10	-	+
11	GRA11	-	+
12	GRA12	-	+
13	WAM13	+	+
14	MUM14	-	+
15	MUM15	-	+
16	MUM16	-	+
17	GRA17	-	+
18	MIX18	-	+
19	MUM19	+	+
20	MUM20	-	+
21	MUM21	+	+
22	GRA22	+	+
23	GRA23	-	+
24	MIX24	+	+
25	GRA25	+	+
26	MIX26	-	+
27	WAM27	+	+
28	WAM28	+	+
29	WAM29	+	+
30	WAM30	+	+

- Negative result; + Positive result

Another biofilm confirmatory test is modified congo red agar test as shown in fig. 7. The strains were cultured on CRA plates. The production of rough black colonies by slime producing strains was used to differentiate them from non-slime producing *Staphylococcus* strains (red smooth colonies) and to confirm biofilm activity [18]. Twenty nine bacterial isolates showed rough black colonies.

Thus biofilm activity is confirmed completely. These isolates are biofilm producers as well as they are multi drugs resistant too. Therefore these are a great threat to human beings. These isolates as they were isolated from fruit samples, there must be at most care taken when using or consuming these fruits raw. They can cause serious health issues in human beings and as they are very resistant to many drugs, it is quite difficult to treat infections and diseases.

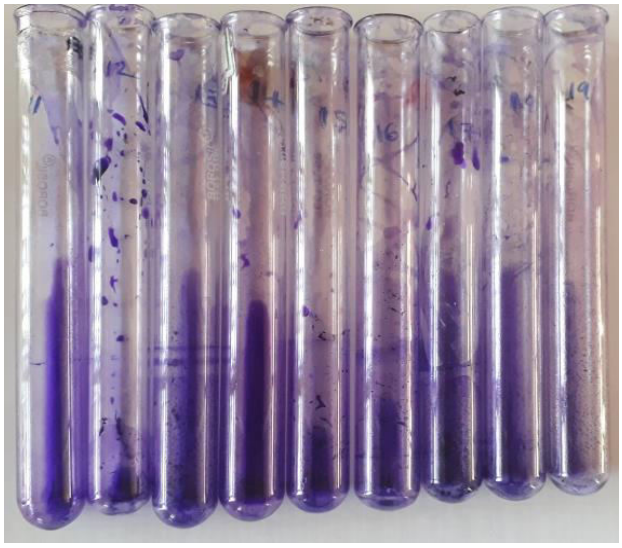

Fig. 6: Crystal violet tube test

Fig. 7: Congo red agar plate of bacterial isolate WAM09 showing black colonies indicating biofilm formation

Table 5: Biofilm activity of all thirty bacterial isolates isolated from fruit skin

S.No	Culture No	Crystal violet test	Congo red test
1	GRA01	-	+
2	GRA02	+	+
3	GRA03	-	+
4	GRA04	-	+
5	AML05	-	+
6	GRA06	+	+
7	GRA07	+	+
8	GRA08	-	+
9	WAM09	+	+
10	MUM10	+	+
11	GRA11	+	+
12	GRA12	-	+
13	WAM13	+	+
14	MUM14	+	+
15	MUM15	+	+
16	MUM16	+	+
17	GRA17	+	+
18	MIX18	+	-
19	MUM19	-	+
20	MUM20	-	+
21	MUM21	+	+
22	GRA22	+	+
23	GRA23	+	+
24	MIX24	+	+
25	GRA25	+	+
26	MIX26	-	+
27	WAM27	+	+
28	WAM28	+	+
29	WAM29	-	+
30	WAM30	+	+

Here the genotypic identification and confirmation was done using primers like gap, nuc and mec primers for *Staphylococcus, Staphylococcus aureus* and Methicillin Resistant *Staphylococcus* respectively. For confirmation of biofilm production eno, icaA and icaD primers were used. PCR amplification of the nuc gene (270bp), a "gold standard" was used for the identification of *S. aureus* [19]. This gene encodes an extracellular thermostable nuclease, which is one of the main distinctive features differentiating *S. aureus* from other *staphylococci*, making it an important tool for prompt detection of *S. aureus* infections [20,21]. In this study, we did not observe any S. aureus culture as shown in Table 6. Hence was negative for all the bacterial isolates.

Our PCR assay showed accuracy, speed and simplicity for the simultaneous detection of the investigated *Staphylococci* species and their methicillin resistance. The PCR combined with an appropriate DNA-extraction method developed in this study is a rapid, simple and reliable strategy for discrimination of prevalent *Staphylococcus species* in fruits, using gap primer and with simultaneous determination of their methicillin resistance using mec primer and could be useful in laboratory routine. Twenty eight isolates had answered for gap primer and species found using PCR RFLP. The different strains of Staphylococcus obtained are *S. haemolyticus, S. lentus, S. delphini, S. homonis, S. saprophyticus and S. equorum.* And all the thirty isolates were confirmed with biofilm activity [19].

Biofilm activity is confirmed by the detection of icaA, icaD and eno genes. The ability of *Staphylococcus* to form biofilms helps the bacterium to survive in hostile environments within the host and is considered to be responsible for chronic or persistent infections [22, 23]. Several studies have shown that the formation of slime and biofilms by *S. haemolyticus* and *S. lentus* strains causing catheter-associated and nosocomial infections is associated with the presence of the *ica*A, *ica*D and eno genes [24, 25]. In this research, the results of a PCR test for the *ica*A, *ica*D and eno genes were important to detect biofilm-producing microorganisms.

Staphylococcus lentus and Staphylococcus delphini are gram-positive and coagulase-negative *staphylococci*. It typically lives on the human skin and mucosa and the most common infections on catheters and implants. *S. lentus* is one of five most common organisms that cause noscomial infections due to the increase in usage of biomaterials in the clinical environment. It is also the most frequent organism found in the blood of bone

marrow transplant patients and on central venous catheters for patients of total parenteral nutrition [26]. *S. lentus* strain from healthy adult nares show that there are many kinds of the organism in each individual. A common *S. lentus* strain RP62a (ATCC 35984) was isolated in Memphis, Tennessee during 1979-1980 in a wide spread of intravascular catheter sepsis. RP62a is a strain that produces slime, grows collectively and forms biofilm. The treatment of *S. delphini and S. lentus* infections caused by biofilm with antibiotics is often ineffective and results in the necessity to remove the implants.

Table 6: PCR of 30 bacterial isolates

S. No	Culture No	gap	Nuc	mec	eno	icaA	icaD
1	GRA01	+	-	-	+	+	+
2	GRA02	+	-	+	+	+	+
3	GRA03	+	-	-	+	+	+
4	GRA04	-	-	-	+	+	+
5	AML05	+	-	-	+	+	+
6	GRA06	+	-	-	+	+	+
7	GRA07	+	-	-	+	+	+
8	GRA08	+	-	-	+	+	+
9	WAM09	+	-	-	+	+	+
10	MUM10	+	-	-	+	+	+
11	GRA11	+	-	+	+	+	+
12	GRA12	+	-	+	+	+	+
13	WAM13	+	-	-	+	+	+
14	MUM14	+	-	+	+	+	+
15	MUM15	+	-	+	+	+	+
16	MUM16	+	-	+	+	+	+
17	GRA17	+	-	+	+	+	+
18	MIX18	+	-	+	+	+	+
19	MUM19	+	-	+	+	+	+
20	MUM20	+	-	+	+	+	+
21	MUM21	+	-	+	+	+	+
22	GRA22	-	-	-	+	+	+
23	GRA23	+	-	-	+	+	+
24	MIX24	+	-	+	+	+	+
25	GRA25	+	-	+	+	+	+
26	MIX26	+	-	-	+	+	+
27	WAM27	+	-	-	+	+	+
28	WAM28	+	-	-	+	+	+
29	WAM29	+	-	-	+	+	+
30	WAM30	+	-	-	+	+	+

Numerous coagulase-negative staphylococci appear commonly on the skin of human. Of these species, *S. lentus* and *S. hominis* are the most abundant. While *S. lentus* tends to colonize the upper part of the body, *S. hominis* tends to colonize in areas with numerous apocrine glands, such as axillae and the pubic region. They, on average, stay on the skin for only several weeks or months [14].

The highly antibiotic-resistant phenotype and ability to form biofilms make *S. haemolyticus* a difficult pathogen to treat. *Staphylococus haemolyticus* is a coagulase-negative member of the genus *Staphylococcus*. The bacteria can be found on normal human skin flora and can be isolated from axillae, perineum, and ingunial areas of humans. *S.haemolyticus* is also the second most common coagulase-positive staphylocci presenting in human blood [27].

Staphylcoccus saprophyticus is not naturally found in healthy humans. It infects humans through sexual intercourse or through contact with animals. *S. saprophyticus* colonizes in the urinary tract of young women and men of all ages. The infection can spread to rectal and vaginal areas. Alterations to the genital area are effected by spermicides and candidal infection

increases the susceptibility of *S. saprophyticus* infection. Urease activity is known to be an infection causing factor in UTIs. Kidney and uretal stones are associated with *S. saprophyticus* infection. The more severe diseases caused by infection are pyelonephritis, septicemia, nephrolithiasis, and endocarditis.

It was found that risk of infection increases in summer and spring months, with contact to domesticated animals (cows, sheep, pigs), and through swimming outdoors. The virulence factors of *S. saprophyticus* include adherence to urothelial cells by means of a surface-associated protein, lipoteichoic acid; a hemagglutinin that binds to fibronectin, a hemolysin; and production of extracellular slime [28]. Therefore it is found that all these strains of *Staphylococcus* have reached fruits through mishandling or improper handling of fruits during harvesting, packaging or supply. Thus, the overall phenotypic and genotypic identification and analysis of *Staphylococcus* isolates obtained are done and are confirmed as pathogens.

4. CONCLUSION

Staphylococcus, one of an important pathogen which causes serious health issues and even death has become a major threat to people nowadays. It is mainly present in human nasal, blood like samples. There are a different strains in *Staphylococcus* in which almost all strains are pathogenic in nature. The current study is to check the prevalence of *Staphylococcus* sp., in locally available fruits. Here, we have selected locally available fruit samples to isolate *Staphylococcus*. Fruits like grape, water melon, musk melon and gooseberry were selected for strain isolation and were enriched and grown in Mannitol Salt Agar (MSA). About thirty bacterial isolates were isolated.

These well maintained and grown cultures were subjected to a series of biochemical tests and confirmatory tests. In biochemical characterisation, bacterial isolates were found to be Gram positive, cocci. And almost all showed positive result for catalase, DNase and showed negative result for oxidase and coagulase tests. These characterisations were verified using Bergey's manual. Methicillin resistant *Staphylococcus* (MRS) are becoming a major threat among *Staphylococcus* sp., so in order to confirm the bacterial strains to be Staphylococcus and specifically Methicillin resistant *Staphylococcus,* phenotypic and genotypic identifications are done. Phenotypic identification includes confirmatory tests like Rapid MRSA agar plate method, Baird Parker agar method, in which bacterial isolates showed greenish blue colonies and black colonies respectively. Thus, all bacterial isolates were confirmed to be *Staphylococcus* sp., among them, some isolates might be Methicillin resistant *Staphylococcus* so to identify them and to find the antibiotic susceptibilty of all isolates, antibiotic test is done. It is done using multidrug discs with 12 different antibiotic and separately resistance to Methicillin and Mupirocin is found out. This is done by disc diffusion method and resistance and sensitivity of bacterial isolates against antibiotics are found out. Apart from these, Staphylococcus sp., has the ability of slime formation, that is, biofilm formation, as they are highly pathogenic. To confirm the biofilm activity of bacterial isolates Crystal violet tube test and modified congo red agar test is done, which shows ring formation in tubes and black matte colony formation respectively, which confirms the biofilm activity of bacterial isolates. All the thirty isolates isolated showed biofilm activity.

The next stage was the genotypic identification of the bacterial isolates. For that gap, nuc and mec primers were used to identify *Staphylococcus, Staphylococcus aureus* and Methicillin resistant *Staphylococcus* respectively. The bacterial isolates which answered gap primer in 933bp were further taken for RFLP-Restriction Fragment Length Polymorphism using the Alu I restriction enzyme. Isolates answered nuc primer at 270bp were confirmed as Staphylococcus aureus and isolates answering mec primer at 310bp were confirmed as Methicillin resistant *Staphy-lococcus.* Using these primers all the thirty isolates were identified genotypically and different strains like *S.homini, S.equorum*, *S.haemolyticus, S. lentus, S. saprophyticus and S. delphini* were found out. Along with this biofilm activity was also confirmed genotypically using eno, icaA and icaD primers and all isolates answered for all primers at 302bp, 188bp and 198bp respectively.

From the locally available fruits like grape, gooseberry, water melon and musk melon, thirty isolates were isolated. Among them *S. lentus, S. delphini, S. haemolyticus, S. hominis, S. equorum* and *S. Saprophyticus* were identified through different phenotypic and genotypic identification methods. The primers used for this identification were gap, nuc and mec for the confirmation of *Staphylococcus, S. aureus* and Methicillin Resistant *Staphylococcus* respectively. All the 3 primers were standardised by our self. For these primers 28 cultures were observed with positive result for gap primer and species identification was done using PCR RFLP by Alu I restriction enzyme. None of the cultures

showed positive result for nuc primer, because no cultures were *S. aureus*. And 13 isolates were observed with positive result for mec primer, that is, those 13 cultures are highly resistant to methicillin antibiotic. All these isolates were resistant to almost all antibiotics too. To find the biofilm activity of bacterial isolates, phenotypic and genotypic methods were done. And all the 30 isolates were observed with biofilm forming capacity and confirmed as highly pathogenic. It is clearly understood that all these strains have reached fruits due to improper handling of fruits by human beings. Therefore, we conclude that serious measures should be taken before processing or consuming these fruits or else there would be higher threat to human beings itself, as all these isolates are serious food pathogens. More studies can be conducted to eradicate the biofilm formation of pathogens.

Conflict of interest

None declared

5. REFERENCES

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