MANIPAL SCHOOL OF LIFE SCIENCES
MANIPAL
(A constituent unit of MAHE, Manipal)

Development of gold nanoparticle-based platform for rapid detection of Gram-positive pathogens and their antibiotic resistance genes

Presenter:

Ms. Nupura Manish Prabhune

Roll Number : 201701001

6th Semester B.Sc. Biotechnology

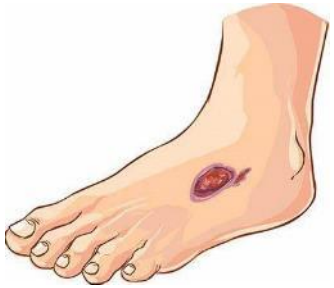
Manipal School of Life Sciences, MAHE, Manipal

Co-authors: **Ms. Yashaswini, Ms. Apoorva Jnana, Dr. K. Satyamoorthy, Dr. T.S. Murali**

Abstract ID: MRCBAS053

Diabetic foot ulcers

- Diabetic foot wound occurs in 25% of patients in India (Soares et al., 2022)
- Polymicrobial nature causes complexity (Thole et al., 2016)
- Rapid diagnostic and antibiotic sensitivity testing method is the need of the hour
- Possible solution – Gold nanoparticle-based colorimetric method
- Development of gold nanoparticle-based platform for rapid detection of Gram-positive pathogens and their antibiotic resistance genes



Gram-positive species (Dominant)
- *Enterococcus* spp.
- *Staphylococcus* spp.
- *Streptococcus* spp.

Gram-negative species

Aim and Objectives

Aim : To develop a gold nanoparticle-based diagnostic platform for the bacterial identification and antibiotic resistance profiling of Gram-positive bacteria involved in DFU.

Objectives :

- To develop gold nanoparticle conjugated probes for identification of selected Gram-positive pathogens (*Enterococcus* spp., *Streptococcus* spp., Methicillin Resistant *Staphylococcus aureus* and Methicillin Sensitive *Staphylococcus aureus*).

- To develop gold nanoparticle conjugated probes for detection of antibiotic resistance genes in selected Gram-positive pathogens.

- Test the efficacy of the designed probes by colorimetric assay (aqueous and solid phase).

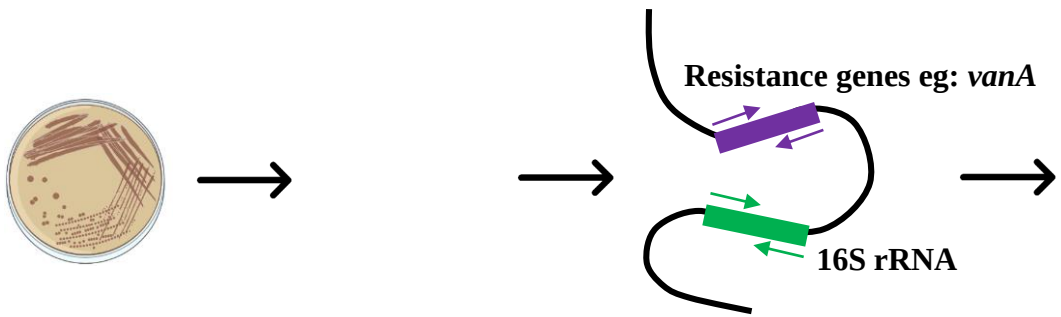
Mechanism

Non-crosslinking approach

Nanoprobe construction

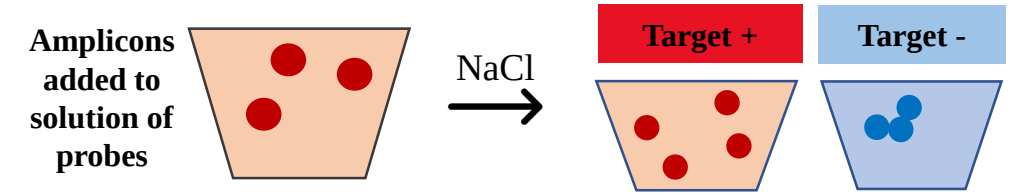
Gold nanoparticle | Thiol-modified probe | Conjugated Au nanoprobes

Hybridization assay

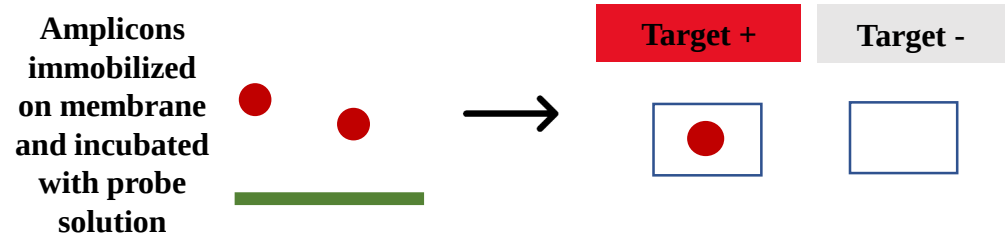


Bacterial sample → Isolation of genomic DNA → PCR amplification

1) Aqueous phase hybridization (Chan et al., 2014)



2) Solid phase hybridization (Bhat et al., 2020)



Work plan

Phase-1

Revival Of Isolates For Three Species – *Enterococcus* spp., *Staphylococcus* spp., *Streptococcus* spp.

↓

Confirmation of Isolates through MacConkey Agar Colony Character, Gram-Staining, and Biochemical Tests

↓

Phase-2

DNA isolation from finalized strains by Phenol-Chloroform extraction method

↓

PCR amplification of 16S rRNA region and antibiotic resistance gene sequences (Based On MIC)

↓

Phase-3

Synthesis and Characterization of gold nanoparticles

↓

Phase-4

Designing of detection and resistance probes and conjugation to gold nanoparticles

↓

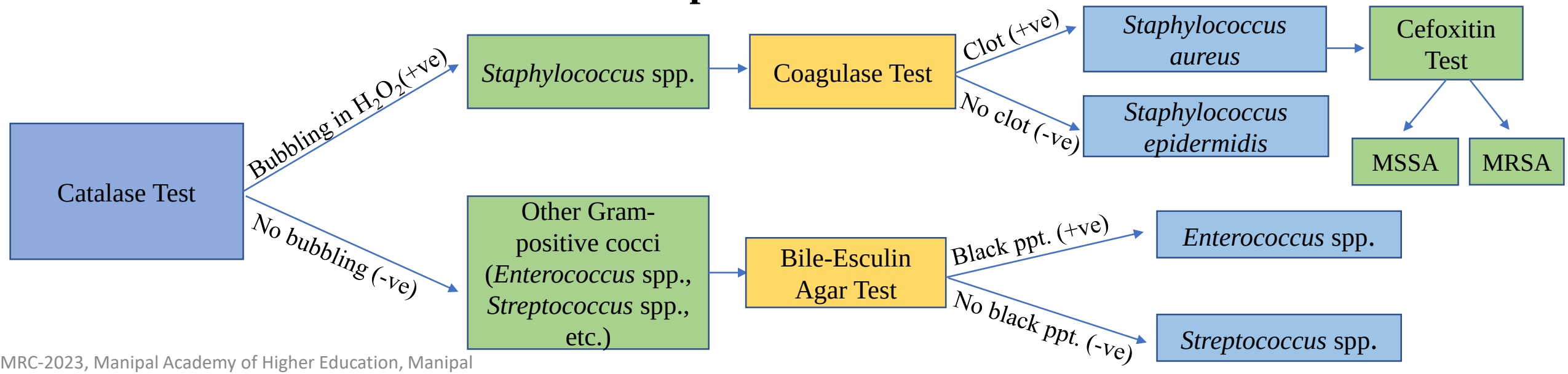
Phase-5

Aqueous phase and Solid phase hybridization assay

1. Colony character, Gram-staining, Biochemical test

- Colony character on MacConkey Agar ; evaluation based on lactose-fermenting ability, colony size, and colony type
- Gram-staining using crystal violet, iodine, decolorizer, and safranin, followed by observation under microscope

Biochemical Test Workflow For Gram-positive cocci



2.(a) DNA isolation protocol (Nishiguchi et al., 2002)

Culture was grown in 5 ml broth, and then cells were pelleted at 3000 xg for 10 minutes

↓

To the pellet, 400 µl TE and 100 µl NaCl (5 M) was added, followed by vortexing

↓

50 µl CTAB was added, followed by vortex, and then incubation at 70ºC for 60 minutes, with occasional mixing by inversion of tube

↓

500 µl of chloroform was added, vortexed and mixed thoroughly, followed by incubation on ice for 30 minutes

↓

The sample was then spun at 10,000 xg in microfuge (cold if possible) for 10 minutes

↓

The upper aqueous phase was collected by avoiding the interphase material and lower phase

↓

500 µl phenol:chloroform was added and vortexed until milky solution was, followed by a spin at top speed in microfuge for 5 minutes

2.(a) DNA isolation protocol (Nishiguchi et al., 2002)

70% ethanol for purification was then added to pellet, followed by the dissolution of isolated DNA in milliQ water

500 µl of chloroform was then added, vortexed, and spun for 5 minutes

To the pellet, 1/10 volume Na-acetate and 2 vol. ice-cold ethanol were added to precipitate the DNA, followed by incubation at -20ºC for at least 1 hour.

The tube was then spun at top speed in microfuge for 10 minutes, after which the pellet was retained

500 µl 70% ethanol was then added, and pellet was resuspended by flicking, followed by another centrifuge round

The tube was inverted on a paper towel with lid open for 5 minutes to drain last bits of 70% ethanol, and then the tube was transferred to 60ºC for 10 min to dry residual ethanol.

The pellet was then redissolved in 100 µl of elution buffer

2.(b) PCR for 16S rRNA region

A 10-microliter reaction mixture was prepared with conventional PCR reagents.
Primers – Forward Primer (27F) – AGA GTT TGA TYM TGG CTC AG
Reverse Primer (534R) – ATT ACC GCG GCT GCT GG

PCR was carried out in a thermocycler at the following conditions :

PCR Reaction Mixture :

MilliQ	2 µl
10x PCR Buffer	1 µl
dNTP (4mM each)	1 µl
Taq (1 unit/ µl)	1 µl
27F primer (1 µM)	2 µl
534R primer (1 µM)	2 µl
DNA	1 µl
Total	10 µl

PCR Conditions :

95ºC	5 minutes
95ºC	30 seconds
60ºC	1 minute
72ºC	1 minute 30 seconds
72ºC	15 minutes
4ºC	Hold

No. Of Cycles - 30

2.(c) Minimum Inhibitory Concentration Test (MIC)

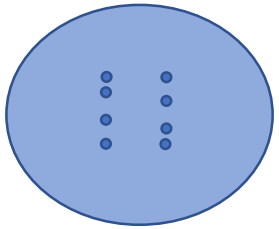
Performed a lawn culture of the strain on a Mueller Hinton Agar Plate

↓

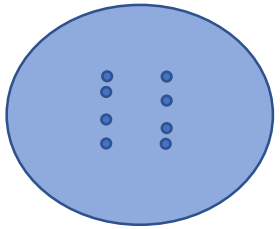
Inoculated the antibiotic strips

↓

After 24 hours of incubation, the plates were observed, and the results were interpreted, to determine the MIC value



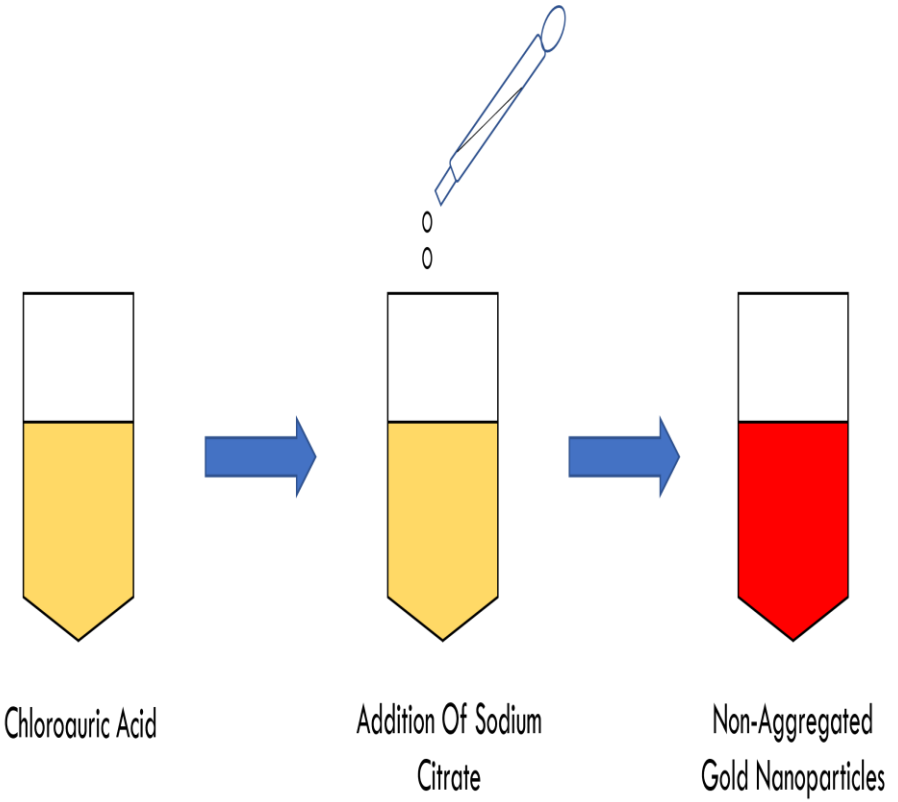
Sensitive strain



Resistant strain

3. Synthesis of Gold Nanoparticles (McFarland, 2004)

1. 1 mM chloroauric acid was prepared by dissolving 0.5 grams of the chloroauric acid powder in 500 ml of autoclaved water.
2. 38.8 mM trisodium citrate was prepared by dissolving 0.5 grams of trisodium citrate powder in 50 ml of autoclaved water.
3. 20ml of chloroauric acid was placed on a magnetic stirrer and heated until boiling.
4. 2 ml of the trisodium citrate solution was added gradually to the boiling chloroauric acid solution.
5. A color change to red wine indicated the formation of the gold nanoparticles.



4. Designing of Probes – Detection and Resistance

Detection Probe Designing (Stenberg et al., 2005) –

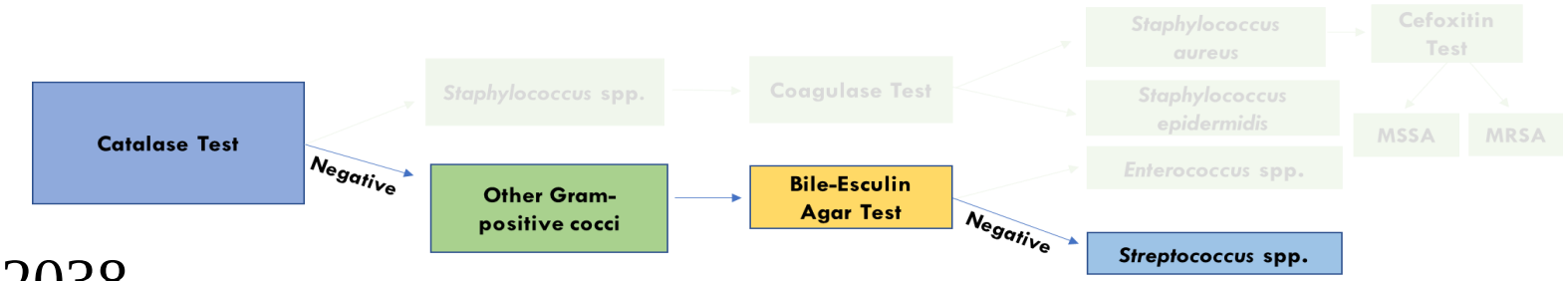
1. To design a detection probe, the 16S rRNA sequence of the given species was retrieved from the Silva database.
2. Around five full length sequences (1500 bp) of different strains of the species were downloaded.
3. Multiple sequence alignment was performed on the BioEdit platform, and then based on consensus sequence, the primers were designed using the Primer3 software (first 500 base pairs).
4. The forward primers were selected as probes, and their specificity was analyzed using test probe software on Silva database.

Resistance Probe Designing (Alcock et al., 2020) –

1. The FASTA sequence of the resistance gene is downloaded from the CARD database.
2. Based on the FASTA sequence, primers are designed using the Primer3 software.
3. Resistance probes designed for – mecA, vanA, tetE, erm

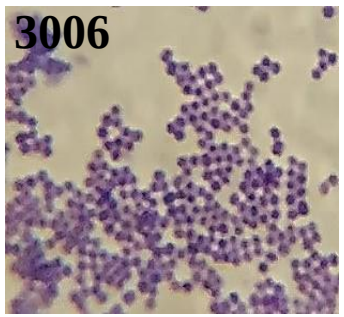
Results - 1

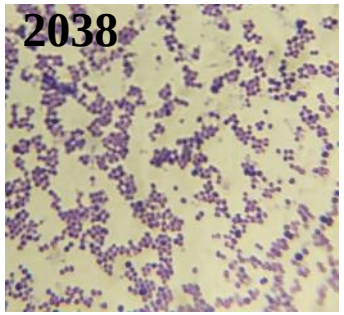
Streptococcus spp.

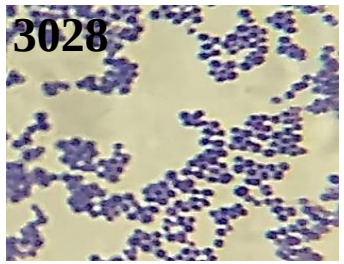


Confirmed isolates – 3006, 3028, 2038

***Streptococcus* strains on MacConkey Agar medium**







Gram-staining of *Streptococcus* strains

Catalase Negative

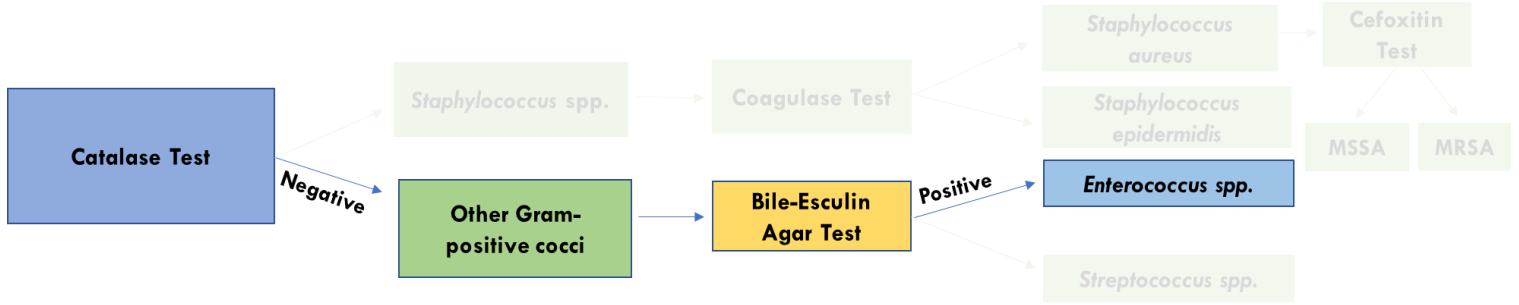
Bile-Esculin Negative

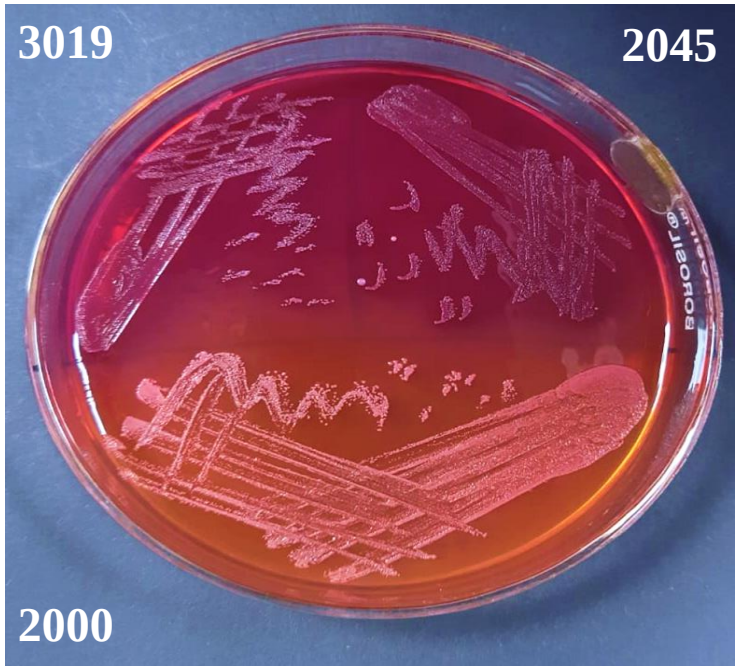
Biochemical test result of *Streptococcus* strains

Results - 1

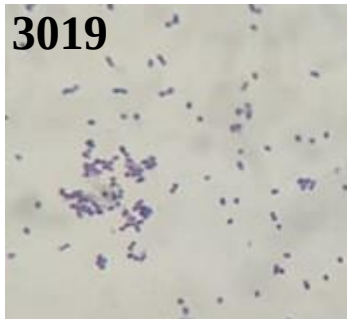
Enterococcus spp.

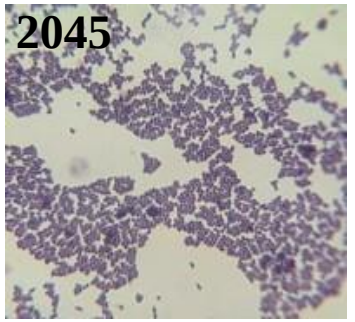
Isolates – 2045, 3019, and 2000

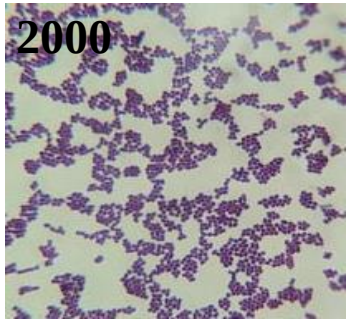




***Enterococcus* strains on MacConkey Agar medium**







Gram-staining of *Enterococcus* strains

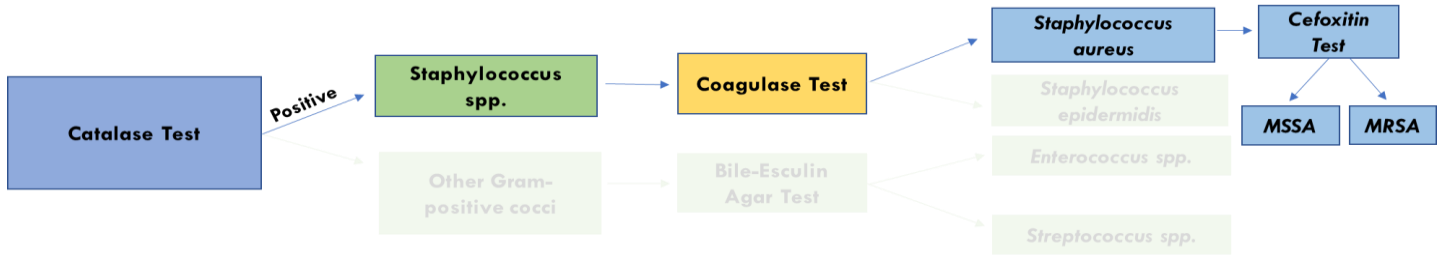
Catalase Negative

Bile-Esculin Positive

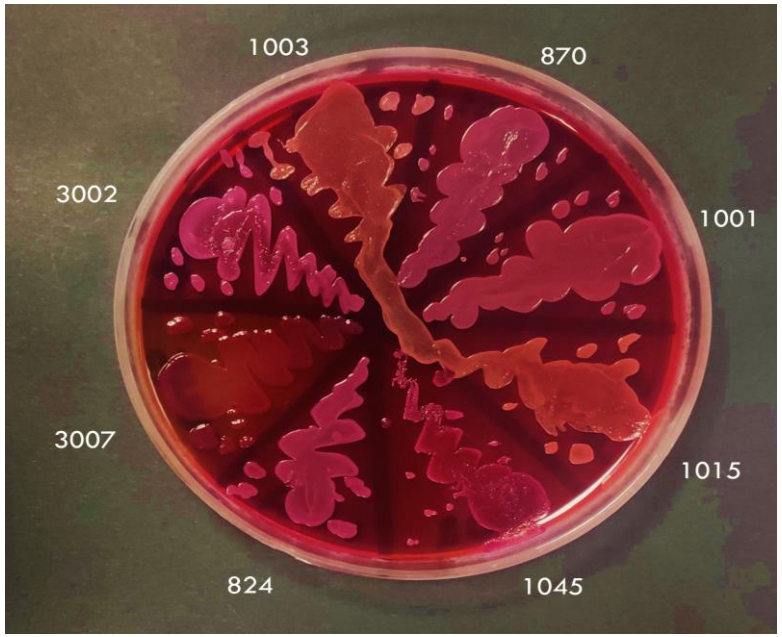
Biochemical test result of *Enterococcus* strains

Results - 1

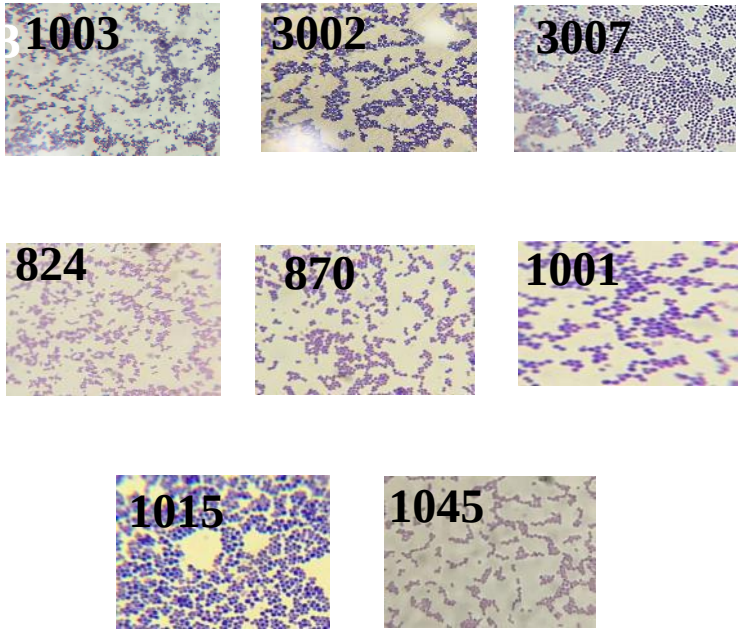
Staphylococcus spp.



Confirmed Isolates – 1003, 3002, 3007, 824, 1045, 1015, 1001, and 870



***Staphylococcus* strains on MacConkey Agar medium**

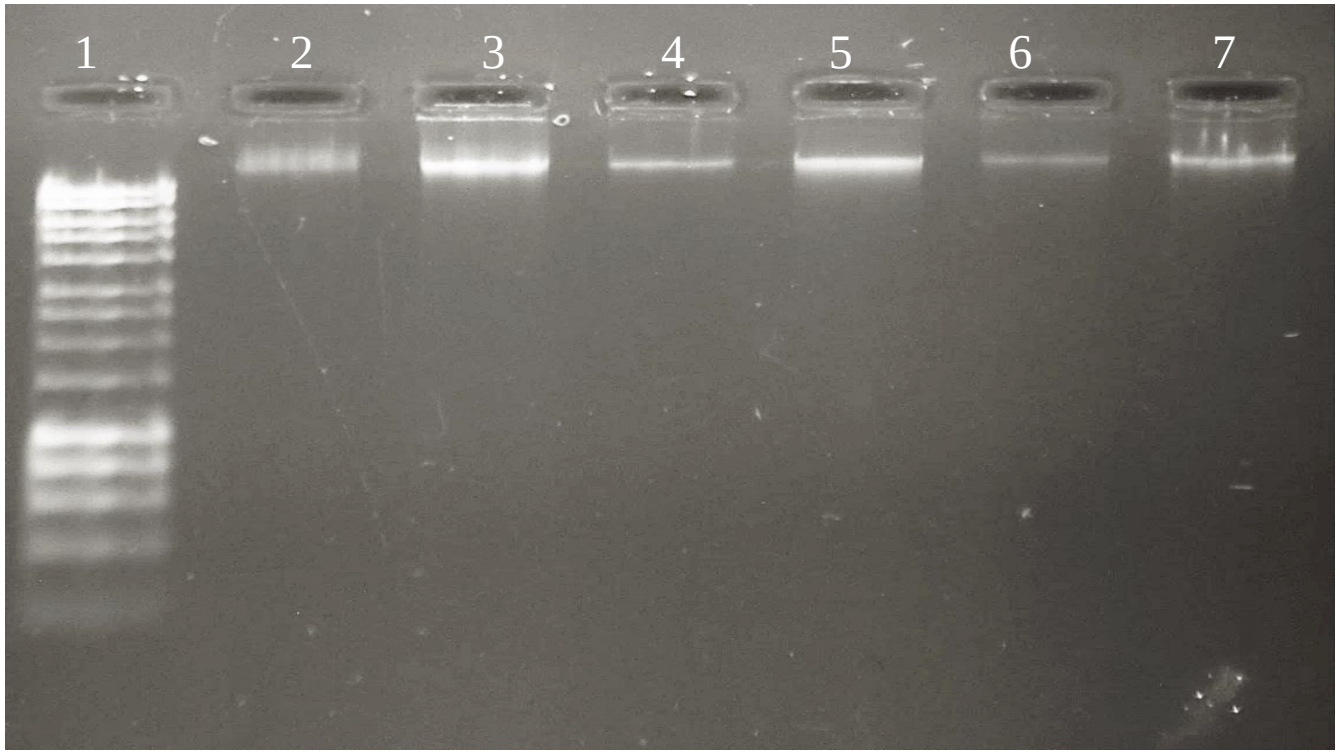


Gram-staining of *Staphylococcus* strains

Catalase Positive | Coagulase Positive

Biochemical test result of *Staphylococcus* strains

Isolated DNA – Gel electrophoresis image

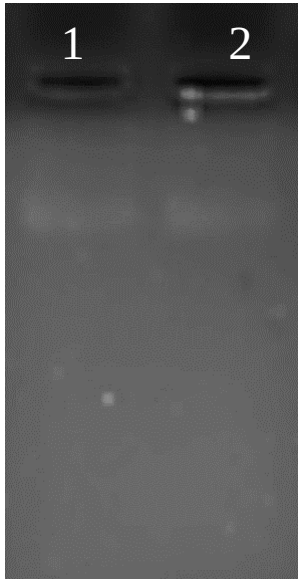


1 – 1 kb DNA ladder
2 – Gram-negative sample
3 – Gram-negative sample
4 – Gram-negative sample
5 – 3006 (*Streptococcus*)
6 – 2045 (*Enterococcus*)
7 – 3007 (*Staphylococcus*)

0.8% agarose gel | 30 minutes | 100 V

PCR Results For 16S rRNA Sequence

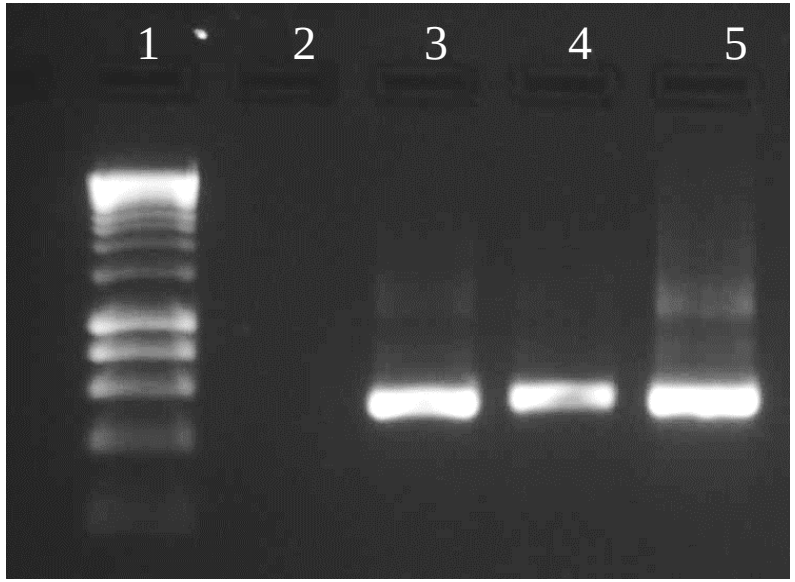
Primer run



1 – 27F
2 – 534R

1% agarose gel
7 minutes
100 V

PCR run

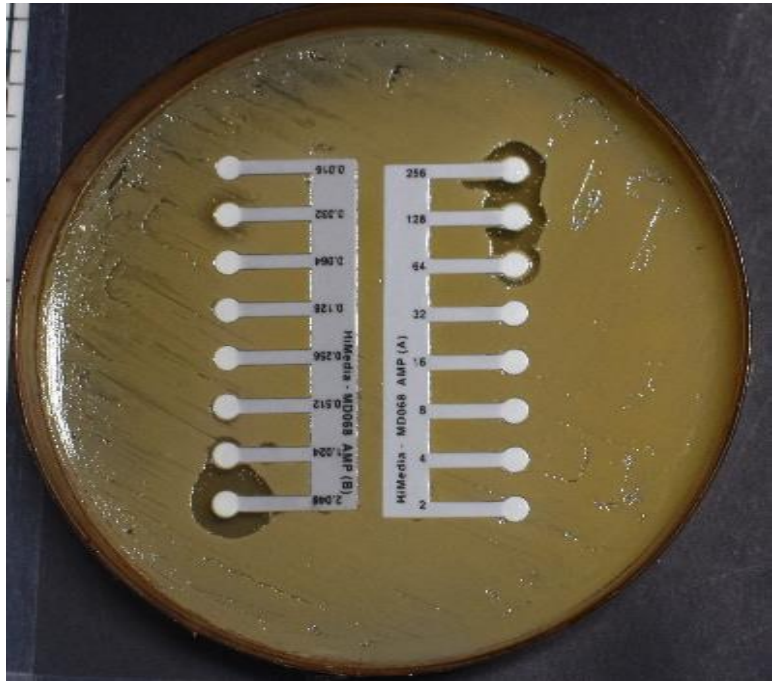


1 – 1 kb Ladder
2 – Negative Control
3 – 2038 (*Streptococcus*)
4 – 2045 (*Enterococcus*)
5 – 3007 (*Staphylococcus*)

1.5% agarose gel
30 minutes
100 V

MIC Results

Staphylococcus spp.

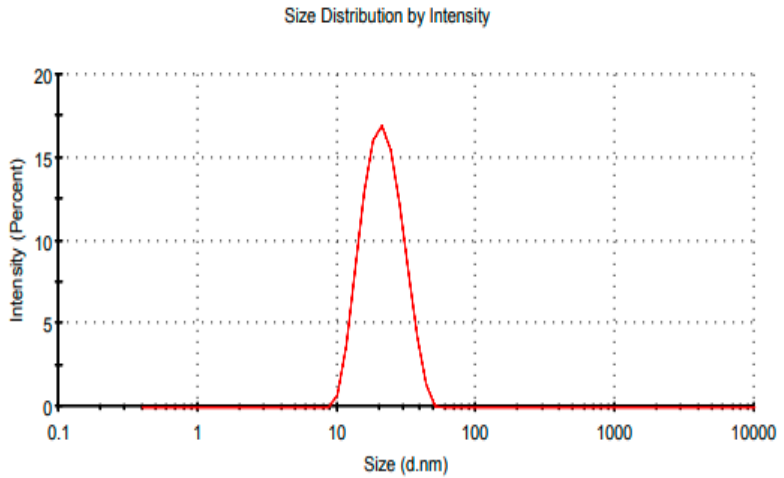


Ampicillin (64 mcg/ml)

Erythromycin (2 mcg/ml)

Synthesized Gold Nanoparticles (Characterization)

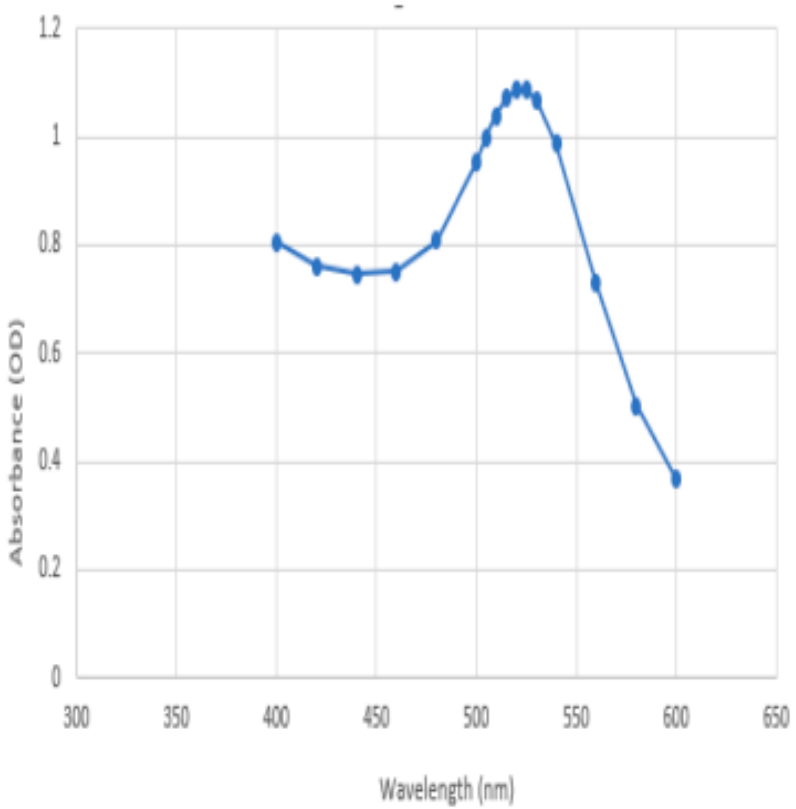
Zeta Sizer Analysis



Results

		Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 17.09	Peak 1:	22.20	100.0	7.140
PdI: 0.296	Peak 2:	0.000	0.0	0.000

Spectrophotometric Analysis



Detection Probes Designed

1. *Enterococcus faecalis*
2. *Streptococcus pyogenes*
3. *Staphylococcus aureus*

Conclusion

- High accuracy, cost-effective and rapid nature
- Solid phase better suited as a Point-Of-Care device
- Clinical Application

References

- Alcock BP, Raphenya AR, Lau TT, Tsang KK, Bouchard M, Edalatmand A, et. al. CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. *Nucleic Acids Res*. 2020;48:D517-525.
- Bhat AI, Rao GP. Dot-Blot Hybridization Technique. In: Characterization of Plant Viruses. *Springer Protoc. Handb.* Humana, New York, NY; 2020.
- Chan WS, Tang BSF, Boost MV, Chow C, Leung PHM. Detection of methicillin-resistant Staphylococcus aureus using a gold nanoparticle-based colorimetric polymerase chain reaction assay. *Biosens Bioelectron.* 2014;53:101-111.
- Jnana A, Muthuraman V, Varghese VK, Chakrabarty S, Murali TS, Ramachandra L et al. Microbial community distribution and core microbiome in successive wound grades of individuals with diabetic foot ulcers. *Appl. Environ. Microbiol.* 2020;86:1-14.
- McFarland AD, Haynes CL, Mirkin CA, Duyne RPN, Godwin HA. Color my nanoworld. *J Chem Educ.* 2004;81:544A.
- Nishiguchi M, Doukakis P, Egan M, Kizirian D, Phillips A, Prendini L. DNA isolation procedures. In DeSalle R, Giribet G, and Wheeler WC, editor. Techniques in molecular systematics and evolution. Basel: Birkhäuser Verlag; 2002, p. 247-287.
- Soares MM, Santos JV. Diabetes-related foot ulcers and amputations. *IDF Diabetes Atlas*. 2022;10:5-9.
- Stenberg J, Nilsson M, Landegren U. ProbeMaker: an extensible framework for design of sets of oligonucleotide probes. *BMC Bioinform.* 2005;6:1-6.
- Thole MV, Lobmann R. Neuropathy and diabetic foot syndrome. *Int. J. Mol. Sci.* 2016;17:1-11.

Thank You