ISOTHERMAL AMPLIFICATION BASED IDENTIFICATION OF MULTIDRUG RESISTANCE STAPHYLOCOCCUS SPECIES

THESIS SUBMITTED TO THE
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

MASTER OF SCIENCE
IN
ANIMAL BIOTECHNOLOGY
BY

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KARNAL-132001 (HARYANA), INDIA

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This is to certify that the thesis entitled **"ISOTHERMAL AMPLIFICATION BASED IDENTIFICATION OF MULTIDRUG RESISTANCE STAPHYLOCOCCUS SPECIES"** submitted by **SACCHIDANANDA BERA** in partial fulfillment of the requirement for the award of the degree of **Master of Science** in **Animal Biotechnology** of the **National Dairy Research Institute (Deemed University),** Karnal (Haryana)**,** India, is a bonafide research work carried out by him under my supervision and guidance, and no part of the thesis has been submitted for any other degree or diploma.

Dated: 20/7/2019

20/7/2019
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Major Advisor
(Guide)

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

15	Agarose (2%) gel with different concentrations of template DNA of a single isolate tested for optimization in LAMP. 100ng template DNA of LAMP product gave better amplification.	45
16	Agarose 2% gel with non specific amplification observed for cattle, buffalo and E.coli DNA at 100ng per 25 µl reactions. No amplification was observed in case of non template.	46
17	Agarose (2%) gel with different concentrations of *Bst* polymerase were optimized with single DNA isolate (6275) and Non template control (NTC).	47
18	(A) Agarose (2%) gel with different concentrations of $MgSO_4$ were optimized with single DNA isolate (6275) & non template control.(B) Non specific amplification was seen in non template with 4mM.	48
19	Agarose 2% gel with different concentrations (2%, 3% and 4%) of glycerol was added to reaction mixture with 100ng of template DNA.	49
20	Agarose (2%) gel with 0.1 molar of 5M betaine was added to the raction mix containing 0.4mM $MgSO_4$ and 3% glycerol.	50
21	Agarose (2%) gel showed positive amplification for species specific *Mec A* gene performed at 65^0C for 1 hour.	51
22	(A) Detection of ten LAMP products positive for *Mec* A gene was visualized using 100µM HydroxyNaphthol Blue at 65^0C for 1 hour. (B) Agarose gel (2%) showed positive amplification for LAMP products that were visualized as positive isolates by HNB. Negative controls like NTC and without Bst tube showed no visualisation with HNB and in agarose gel electrophoresis.	53

LIST OF ABBREVIATIONS & SYMBOLS

%	Percentage
µg	Microgram
µl	Microlitre
^{0}C	Degree centigrade
B3 primer	Backward outer primer
BIP	Backward inner primer
bp	Base pairs
Bst	*Bacillus stearothermophilus*
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
F3 primer	Forward outer primer
FIP	Forward inner primer
HDA	Helicase dependent amplification
LAMP	Loop mediated isothermal amplification
Coa	Coagulase
ml	Milli litre
mM	Milli Molar
NASBA	Nucleic acid sequence based amplification
nm	nano meter
OD	Optical density
PCR	Polymerase chain reaction
pg	Pico gram
pmol	Picomole
RCA	Rolling circle amplification
RPA	Recombinase polymerase amplification
SDA	Strand displacement activity
HNB	Hydroxy naphthol blue
MSA	Mannitol salt agar
NAM	Nutrient agar media
AST	Antimicrobial susceptibility test

ABSTRACT

The present study aims to identify multidrug resistant *Staphylococcus species* specific genes by rapid detection platform. Many culture based approaches were developed for identification of *Staphylococcus species* morphologically but it has limitations for bacterial growth, antibiotic interference and growth of other micro organisms. To overcome such drawbacks several DNA based methods were developed from the past three decades. Mostly PCR was performed for 16S rRNA gene that proves to be gold standard method for detection of *Staphylococcus species* but they required to be improvised further in terms of specificity, sensitivity and simplicity. Four *S.spp* specific genes responsible for causing virulence (*coa, hlg* & *pvs)* and antimicrobial resistance (*mec* A) were analyzed in PCR by visualizing in 2% agarose gel. Results obtained were better as specific genes were targeted but it is limited by a doubling by cycle amplification and requires different gradients of temperature that can only be provided by thermal cycler. LAMP is an isothermal nucleic acid based amplification method that overcomes limitations shown by PCR. It provides rapid and easy detection based on amplified products at a constant temperature of 60-65^{0}C. LAMP primers were designed using online bio-software and amplification reactions were performed in water bath at 65^{0}C. The amplified products were then examined by using 2% agarose gel electrophoresis. Presence of *Mec* A gene was confirmed with the appearance of 6-10 bands in UV transilluminator. Visualisation of the amplified LAMP products was attempted with the addition of a visual dye, hydroxy naphthol blue (HNB) to the amplification system. Within 1 hour of time, *Mec* A gene was amplified successfully by LAMP with the isothermal conditions at 65^{0}C. Better amplification of desired gene was observed when optimized concentrations of (4mM) $MgSO_4$, (3%) glycerol & (0.1 molar) betaine were added to reaction mixture. Colour difference between amplified LAMP product and non template control was observed by adding (100µM) of HNB in the reaction mixture. The LAMP assay was performed for 54 *S. spp* isolates positive for *Mec* A gene in PCR. Then, an isothermal amplification based identification system was developed for *Staphylococcus spp* bacteria.

सार

वर्तमान अध्ययन का उद्देश्य रैपिड डिटेक्शन प्लेटफॉर्म द्वारा मल्टी ड्रग प्रतिरोधी स्टैफिलोकोकस प्रजातियों के विशिष्ट जीन की पहचान करना है। स्टैफिलोकोकस प्रजातियों की पहचान के लिए कई संस्कृति आधारित दृष्टिकोण विकसित किए गए थे, लेकिन इसमें बैक्टीरिया के विकास, एंटीबायोटिक हस्तक्षेप और अन्य सूक्ष्म जीवों के विकास की सीमाएं हैं। इस तरह की कमियों को दूर करने के लिए पिछले तीन दशकों से कई डीएनए आधारित तरीके विकसित किए गए थे। ज्यादातर पीसीआर 16 एस आरआरएनए जीन के लिए किया गया था जो स्टैफिलोकोकस प्रजातियों का पता लगाने के लिए सोने की मानक विधि साबित होती है, लेकिन उन्हें विशिष्टता, संवेदनशीलता और सादगी के मामले में और सुधारने की आवश्यकता है। वायरलेंस (सीओए, एचएलजी और पीवीएस) और रोगाणुरोधी प्रतिरोध (एमईसी ए) पैदा करने के लिए जिम्मेदार चार एसएसपी विशिष्ट जीनों का पीसीआर में 2 % agarose जेल की कल्पना करके विश्लेषण किया गया था। प्राप्त परिणाम बेहतर थे क्योंकि विशिष्ट जीनों को लक्षित किया गया था, लेकिन यह चक्र प्रवर्धन द्वारा एक दोहरीकरण द्वारा सीमित है और तापमान के विभिन्न ग्रेडिएंट्स की आवश्यकता होती है जो केवल थर्मल साइक्लर द्वारा प्रदान किए जा सकते हैं। LAMP एक इज़ोटेर्मल न्यूक्लिक एसिड आधारित प्रवर्धन विधि है जो पीसीआर द्वारा दर्शाई गई सीमाओं को पार करता है। यह 60-65°C के निरंतर तापमान पर प्रवर्धित उत्पादों के आधार पर तेजी से और आसान पहचान प्रदान करता है। LAMP प्राइमरों को ऑनलाइन बायो-सॉफ्टवेयर का उपयोग करके डिजाइन किया गया था और 65°C पर पानी के स्नान में प्रवर्धन अभिक्रियाएं की गई थीं। प्रवर्धित उत्पादों को 2% agarose जेल वैद्युतकणसंचलन का उपयोग करके जांच की गई थी। यूवी ट्रांसिल्यूमिनेटर में 6-10 बैंड की उपस्थिति के साथ मैक्स ए जीन की उपस्थिति की पुष्टि की गई थी। प्रवर्धित एलएएमपी उत्पादों के विज़ुअलाइज़ेशन को प्रवर्धन प्रणाली के लिए एक दृश्य डाई, हाइड्रॉक्सी नेफथोल ब्लू (एचएनबी) के अतिरिक्त के साथ लेने का प्रयास किया गया था। समय के 1 घंटे के भीतर, 650 A पर इस्मोथर्मल स्थितियों के साथ LAMP द्वारा मेक ए जीन को सफलतापूर्वक प्रवर्धित किया गया। वांछित जीन का बेहतर प्रवर्धन तब देखा गया था जब (4mM) MgSO4, (3%) ग्लिसरॉल और (0.1 मोलर) बीटालाइन की सांद्रता को प्रतिक्रिया मिश्रण में जोड़ा गया था। प्रवर्धित LAMP उत्पाद और गैर टेम्पलेट नियंत्रण के बीच रंग अंतर को प्रतिक्रिया मिश्रण में HNB के (100μM) जोड़कर देखा गया। LMP परख 54 एस के लिए किया गया था। एसपीआर ने पीसीआर में मेक ए जीन के लिए सकारात्मक को अलग कर दिया। फिर, स्टैफिलोकोकस एसपीपी बैक्टीरिया के लिए एक आइसोथर्मल प्रवर्धन आधारित पहचान प्रणाली विकसित की गई।

CHAPTER –1

Introduction

INTRODUCTION

Resistance is the ability of not being affected by something, is a bane for humans and livestock in terms of infection caused by deadly pathogens. Pathogens turn deadliest when they become resistance to multiple drugs and are referred as 'Multiple Drug Resistance'. Treatments for infections are compromised by the ability of bacteria to resist antibiotics. Mechanisms defined by MDR for antimicrobial resistance are acquired and natural or intrinsic defense mechanism. Acquired defensive mechanism to such antibiotics is associated with extra chromosomal elements or mobile elements like plasmids, transposons and integrons. Microorganisms adapt to antimicrobial resistance through several other mechanisms like reduction in drug permeability, decreased antibiotic activity via biofilm production, radiation and changes in light or pH (Junaid Ali *et al.,* 2018). Intrinsic resistance of destroying drugs by utilizing enzymes is naturally possessed within bacteria. Recent studies suggest Multidrug efflux pumps (EPs) are recognized contributors for antimicrobial resistance by expelling antibiotics in non specific manner (Varsha *et al*., 2018). Persistence to antibiotics and genetic gain of resistance is linked pleiotropically with successive mutation rates that create a kind of stress response to evade antibiotics (Etthel Martha *et al.,* 2019). MDR resistant bacteria transfer copies of DNA that codes mechanism of resistance to antibiotics in recipient cells by an evolutionary mechanism of transformation called as Horizontal gene transfer (HGT) (Dongchang Sun 2018).. *S. aureus* being gram positive cocci shows resistance to vancomycin, fluroquinolones and other beta-lactams by four mechanisms. Antimicrobial resistance to reduce antibiotic uptake is seen in vancomycin resistant intermediate *S. aureus* by producing a peptidoglycan layer, which generates thickened cell wall with mere irregularity making it difficult for vancomycin to reach cell membrane even in continuous exposure to vancomycin. Second kind of resistance is through active efflux of antibiotics by Efflux Pumps (EPs) resulted from chromosomal mutations or by Horizontal Gene Transfer (HGT) mechanisms. Fluroquinolones resistance to S. aureus is encoded by chromosomally mutated *nor A* gene which increases efflux of fluroquinolones by efflux pumps. Third mechanism is target modification shown by Methicillin Resistant *Staphylococcus aureus* (MRSA) encoding for methicillin resistance *mec A gene.* The last mechanism for antimicrobial resistance is altering antibiotics to have reduced effect on cell. Altering the antibiotics is done by bla Z, beta-lactamase and

aminoglycoside modifying enzymes that changes antibiotic structure and prevents antibiotic target interaction resulting in antimicrobial resistance.

Staphylococcus aureus is the major multidrug resistant, gram positive cocci colonizes glandular portions of skin and mucus membranes, which becomes a passage for their causative infection to humans and livestock. As a potent commensal of human skin, it is responsible for sepsis and bacteremia (Dedent *et al*., 2017). First *S. aureus* genome to be sequenced was N315 and MU50. Staphylococcus aureus dealt with a deadliest disease termed as mastitis in animals, a severe condition resulting in inflammation of udder and tits. Prevalence of Methicillin resistant *Staphylococcus aureus* (MRSA) is also found in poultry and poultry meats (Claudia *et al*., 2018). Bovine mastitis is the major concern in dairy farms associated with huge economic losses in world wide. Based on grade of infection it is categorized into clinical and sub clinical mastitis. Clinical mastitis is the highest degree of infection in bovine in relative to subclinical. Acute clinical mastitis characterized by swelling of udder, formation of watery milk, reduced yield of milk and hardening of quarters is the severe form in consideration to wild and moderate infections. Clots and flakes provide the best way for detection of clinical mastitis by stripping milk in strip cups. No visible changes in udder or appearance of milk is found in sub clinical mastitis, which makes no possible symptoms to diagnose the onset of infection leading in most severe form of acute clinical mastitis. Several approaches have been put forward for easy diagnosis of mastitis by California mastitis test (CMT), Somatic cell count (SCC) and monitoring through electronic testing device.

S.aureus being a facultative anaerobe and a non spore forming bacteria can withstand osmotic stress. Temperature, pH and salt concentration influence the growth of S. aureus to an optimum level. It is able to withstand high salt concentrations upto 15% Nacl and shows normal growth rate at 10% Nacl with an optimum temperature of 30-37 degrees and pH about 7.0-7.5. This makes them a good colonizer of skin and a major concern for food industries. Bacterial isolates are grown in selective media, which differentiates and promotes selective growth at an optimum range of temperature and pH by not allowing other isolates to be grown. Selective media used for growing *S. aureus* isolates are Mannitol salt agar media and Biard Parker agar media. Mannitol salt agar media is a selective and differential media differentiates isolates of *S. aureus* with respect to coagulase negative isolates based on colony morphology. Yellow colonies are produced by *S. aureus* with yellow zones and pink colonies are shown by *S. epidermidis* and

isolates of coagulase negative *staphylococci*. Characteristic yellow color is formed by the fermentation of Mannitol that leads to by product formation that turns phenol red to yellow. Biard Parker agar is a selective agar for isolation of gram positive *staphylococcus species*. It is composed of lithium chloride and tellurite that inhibits growth of other microbes. Black colonies are formed by *S. aureus* isolates in Biard Parker agar media. Glycine and pyruvate are promoting growth factors for gram positive *S. aureus*. Isolates grown in blood agar plates and trypticase soya agar shows a smooth and translucent colony growth. Staphyloxanthin production and Beta heamolysis are identification markers for *S. aureus* isolates. Staphyloxanthin is a carotenoid pigment helps in detoxification of reactive oxygen species and is responsible for golden color appearance of *S. aureus*. Various biochemical tests are being used for identification of *S. aureus*. Catalase test and coagulase test differentiates *Staphylococcus aureus* from *Streptococcus* and *Micrococcus*. Catalase enzyme in microorganisms detoxifies hydrogen peroxide in water and oxygen that results in bubble formation is a key identification for aerobes and facultative anaerobes. Coagulase test is confirmatory test to differentiate between *Staphylococcus aureus* from Coagulase Negative *Staphylococcus* (CONS). Coagulase found in *S. aureus* converts soluble fibrinogen to insoluble fibrin in plasma. Two types of coagulase are formed by *Staphylococcus aureus*, bound and free coagulase. Detection of bound and free coagulase is done by two tests namely slide and tube coagulase test. Slide coagulase test is used to determine clumping factor (bound coagulase) whereas tube coagulase test detects the free form of coagulase. Agglutination indicates s a positive test for *Staphylococcus aureus*. No clump formation determines CONS in slide test. All negative slides of coagulase negative must be confirmed by tube coagulase test for definitive *Staphylococcus aureus*.

Culture based approaches can be used for the identification of *Staphylococcus aureus* but it has limitations for bacterial growth, antibiotic interference and overgrowth of other bacteria. To overcome such limitations conventional and molecular techniques are used for effective screening and detection of multidrug resistant pathogens. These methods involve PCR and microarray assay, DNA sequencing and isothermal amplification techniques. PCR methods like Real time PCR, Multiplex PCR and qRT PCR show accurate results but are not applicable during outbreaks. Whereas isothermal amplification shows better results as it is rapid, less time consuming and resistant to PCR inhibitors. It has high specificity and sensitivity for identification of multidrug pathogens

in contrast to polymerase chain reaction. Isothermal amplification methods involve Loop mediated isothermal amplification (LAMP), Helicase dependent amplification (HDA), Recombinase polymerase amplification (RPA). Isothermal amplification is easy to operate and requires less instrument requirement than PCR. LAMP is a single tube technique for the identification of specific DNA. The target sequence is amplified at a constant rate of 60-65 degrees. LAMP uses four primers to identify distinct regions on target genes and produces higher amount of DNA with respect to PCR. Photometry detection of amplified product caused by increased amount of magnesium pyrophosphate precipitate as a byproduct of isothermal amplification, allows visualisation of larger samples by the naked eye and simpler detection techniques for smaller reaction volumes. Turbidity formed in solutions can be measured by real time or by fluorescence using SYTO 9 interchelating dye. Calorimetric indicator is used to determine LAMP product by the use of calcein with manganous ions to form green color. SYBR green and hydroxyl naphthol blue (HNB) is used without the need for expensive instrumentation to visualize the colour change with naked eye. In developing country like India, LAMP should be validated for the identification of *Staphylococcal* infection. In India, many scientists have worked in this interested area. Therefore the present work is designed to use LAMP for identifying positive genes in *Staphylococcus spp* with the following objectives:

- **Amplification of *Staphylococcus spp* DNA by specific PCR**
- **Isothermal amplification of *S. aureus* drug resistant gene**
- **Detection of amplified DNA by conventional/point of care device components**

CHAPTER –2

Review of Literature

REVIEW OF LITERATURE

Antibiotic resistance is associated with causes of global resistome like increased antibiotics usage for simple treatments in clinics and animal production, problems related to sanitary and sewage disposals due to overpopulation and enhanced global migration resulting in wildlife spread (Aslam *et al*., 2018). These resistome factors cause genetic selection pressure for emergence of MDR bacteria referred as "superbugs". By the year 2030, estimated usage of antibiotics will be increased to 67% in populated countries. Major concern of antibiotic resistance can be minimized by understanding the defense mechanisms like Horizontal gene transfer, modification of target sites, active efflux of antibiotics by Efflux pumps and altering antibiotics (Jiang S *et al*., 2018). Detection of genes encoding for antimicrobial resistance is necessary for diagnosis and treatment of infections caused by multidrug resistant bacteria. Loop mediated isothermal amplification detects these antimicrobial genes with ease in comparison to conventional methods that are time consuming and grueling (Li B *et al*., 2018). Methicillin resistant *Staphylococcus aureus* (MRSA) is a superbug defined by a single determinant Penicillin binding protein 2a (PBP2a) pose a serious global threat for its deadly infections. Diagnosis and detection of MRSA can be done by the prevalence of *mec A* gene on chromosome cassette of some variants (Alex van Belkum and Olivier Rochas., 2018). Isothermal amplification techniques provide better results in terms of specificity and sensitivity.

2.1 Antimicrobial resistance and defense mechanisms

Antimicrobial resistance is the ability of microorganisms to evade from the effects of antibiotics to which they were susceptible. Antibiotic resistant genes are considered as important reservoirs for antimicrobial resistance. Expression of resistant gene in determinants by its mobilization to integrons and plasmids pose a serious threat to public health by causing infections (Elizabeth Peterson and Parjit Kaur., 2018). Human activities have further resulted in enrichment of antibiotic resistant genes in such determinants brought about by selection pressure. Thus it is essential to understand the distribution of resistant genes in determinants of antimicrobial resistant bacteria to determine its resistant mechanisms and factors that promotes dissemination.

Antibiotic resistant bacteria pose infections to host by their ability to resist the activity of antibiotics by the use of defense mechanisms. Defense ability shown by bacteria to antibiotics is in two defined ways, natural defense and acquired defense mechanism. Intrinsic or natural defense mechanism is confined within the genes of bacteria before their exposure to antibiotics. Bacteria utilize the enzymatic action machinery for intrinsic defense mechanism against multidrug. These enzymes act upon the drugs by destroying them through hydrolysis. Continuous exposure of bacteria to antibiotics, gains an acquired or artificial kind of defense mechanism to that particular antibiotics to which it was susceptible. In general antimicrobial resistant organism shows four ways of defense mechanisms like formation of peptidoglycan layer to reduce the intensity of antibiotic uptake, active efflux of antibiotics by Efflux Pumps, modification of targets to which antibiotics bind and show its activity and alterations of antibiotics by multidrug organisms to have reduced effect on cell. Active efflux of antimicrobials by Efflux Pumps (EPs) has grabbed attention of researches for its potent and key involvement in providing antibiotic resistance against most of the antibiotics. Example for an Efflux Pump defense mechanism is fluroquinolones resistance to *S. aureus* because of chromosomally mutated *nor A* gene that results in increased efflux of fluroquinolones (ciprofloxacin, levofloxacin, ofloxacin etc). Antibiotic sequestration by special proteins and antibiotic target bypass are key defensive mechanisms employed by some bacteria for resistance against antibiotics. Bacteria can survive even in the presence of antibiotics by releasing substances in extracellular space that helps in drug sequestration and prevents drug from reaching its target (Akshay Sabnis *et al*., 2018). "Antibiotic interceptors" are safe spaces for bacteria, functioning as decoys by mimicking target and functions as structural component of biofilm, thereby making bacterial communities tolerant for antimicrobials. Many bacteria attain antibiotic resistance by bypassing the membrane and travelling through porins (Baker *et al*., 2018).

2.2 Antibiotic resistance in *Staphylococcus aureus*

Staphylococcus aureus being an asymptomatic colonizer of human skin is responsible for causing infections in human. In spite of being not harmful to humans as a 30% colonizer, repetitive exposure to antibiotics turns it resistant to broad spectrum of antibiotics. Formation of biofilm on both biotic and abiotic state makes bacteria able to survive in presence of antibiotics, as free form of bacteria is culminated by phagocytosis in innate defense mechanism and killed by the use of antibiotics in acquired host defense system.

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been considered as one of twelve priority pathogens by World Health Organization (WHO) posing threat to human health (Kelly M. Craft *et al*., 2019). MRSA is responsible for both community and hospital associated infections. Many defense mechanisms are shown by *S. aureus* in response to antimicrobials either by innate or acquired defense mechanism or by the production of antimicrobial peptides (AMP) that leads to interception of antibiotics from reaching the defined targets. Antibiotic defense mechanism in *Staphylococcus aureus* is due to the acquisition of mobile genetic elements like plasmids and transposons, another means is through gene transferring mechanism. Gene transferring mechanism is categorized as vertical and horizontal gene transfer (HGT) mechanism. HGT is considered as the most reliable mechanism for antimicrobial resistant gene transfer to an independent organism. *Staphylococcus aureus* is resistant to beta-lactam group of antibiotics like penicillin and tetracycline due to the presence of *blaZ* gene located chromosomally on plasmids. In Coagulase negative *Staphylococcus aureus* (CONS) tetrac*y*cline resistance is mediated by *tet*M gene or *tet*K encoded efflux protein. MRSA is resistance to multi drugs and mainly to methicillin due to the presence of *mec*A gene. Genes like *erm*A, *erm*B and *erm*C in *S. aureus* and CONS provides ribosomal mediated resistance to macrolides, amyloides, lincosamides and type B streptogramin (Achek *et al*., 2018). For aminoglycosides like gentamicin, amikacin, kanamycin and tobramycin, *aac*A-*aph*D genes provides cross resistance to *S. aureus*. Various diagnostic methodologies are implied for detection of infectious agents and their diseases. *In silico* methods assessing susceptibility of host to colonization and infection (Suh *et al*., 2018) and molecular methods for direct and indirect identification of pathogens invitro in laboratory paves way for easy detection and identification of MDR *S. aureus.* Other than laboratory based test, simple approaches like point-of-care (POC) devices can be used for detection of MDR pathogens in open environment. Popular diagnostic tools range from culture based detection of pathogens to molecular methods which are rapid to perform and are specific and highly sensitive in contrast to culture based methods. Molecular tools involve nucleic acid amplification technologies (NAATs) (Alex Van Belkum and Olivier Rochas 2018). Prevention of cross infection and curing patients requires post amplification processing like DNA sequencing and DNA fragment analysis. For antimicrobial resistance detection there is an increase in use of antimicrobial susceptibility test (AST) (Kelly *et al*., 2016). Detection of species specific genes is a point of interest for identification of disease. For example, *CAT* gene is a

phenotypic gene present in *Staphylococcus aureus* and some gram positive bacteria, it is responsible for the production of catalase enzyme that breaks hydrogen peroxide to water and free oxygen.

2.2.1 Resistance to beta-lactam class of antibiotics

β-Lactam antibiotics are a class of antibiotics that contain β-lactam ring in their molecular structure. This includes derivatives of penicillin like methicillin and oxacillin, monobactams, cephalosporins and carbapenems. They function by inhibiting the biosynthesis of cell wall. Bacteria contain β-lactamase, an enzyme that acts upon β-lactam ring and provides resistance to β-lactam antibiotics. S. aureus resistant to penicillin was emerged soon after the introduction of antibiotics in early 1940s (Lowy 2003; Peacock and Paterson 2015; Walsh 2016). Substitution of aminoadipoyl chain of penicillin with bulkier moieties leads to introduction of methicillin (Timothy. J Foster; 2017). With the course of time bacteria have developed resistance to methicillin and led to the discovery of new strain termed as Methicillin resistance *Staphylococcus aureus* (MRSA).

Methicillin resistance in *S. aureus* is conferred by presence of staphylococcal cassette chromosome *mec* (SCC *mec)*.SCC *mec* cassette includes *mecA* gene that codes for penicillin binding protein (PBP)2a. (PBP)2a shows low affinity for β-lactam class of antibiotics and allows the cell to produce functional cell wall even in presence of antibiotics. SCC mec cassette is classified based on *mec* class and cassette chromosome recombinase (*crr*). *Mec* class is related to β-lactam resistance and *crr* is responsible for the mobility of genetic element. Non essential junkyard regions are also included in *mec* typing (Kondo *et al*., 2007).

Prevalence of MRSA in causing infections was classified as hospital and community associated infections. CA-MRSA infections differ genetically from HA-MRSA infections as they carry SCC *mec* type Ⅳ & Ⅴ and harbor panton valentine–leukocidin gene (*Pvl)*. Third emerging branch of MRSA was livestock associated MRSA found in animal species truly believe to be transmitted from humans to animals.

2.2.2 Mechanism and expression of methicillin resistance

Staphylococcus aureus resistance to methicillin is through acquisition of gene that encodes for penicillin binding protein 2a (PBP)2a an homologue to naturally occurring (PBP)2 and is not susceptible to drug action. This is because methicillin is prevented

from access to active site serine of the transpeptidase domain of PBP2a (Lim and Strynadka, 2002). MRSA strain grown in presence of β-lactams leads to biosynthesis of peptidoglycan (PG) which is poorly cross linked. These consequences result in stronger proinflammatory effects that contribute to pathology during infection (Muller *et al*., 2015). Altered peptidoglycan biosynthesis also accounts inability of accessory gene regulator (Agr) to be induced in MRSA strains.

Expression of *mec*A gene in prototype MRSA strain is induced only during exposure to drugs. *Mec*IR regulatory proteins control the expression of *mec*A gene which is homologous to the *Bla*IR protein that regulates *Bla*Z expression (Peacock and Paterson 2015, Fisher and Mobashery 2016). Beta-lactamase regulators like *Bla*I and *Bla*R repress *mec*A expression. Thus expression of penicillin binding protein 2a depends on the expression of *mec* and *Bla* regulators. Most MRSA isolates accounts for heterogeneous resistance against antibiotics means low level of resistance. Conversion of heterogeneous to homogeneous resistance accounts for increased resistance of MRSA against antibiotics and increases the expression of PBP2a. Mutation in *rel*A gene in MRSA strain resulted in increased resistance against broad range of antibiotics (Kim *et al*, 2013). Mutations in several different genes like *rpo*B, *erm*A, *erm*B and *erm*C also accounts for homogenous resistance which encode β-subunit of RNA polymerase.

2.3. Culture based detection:

Culture based methods involves colonization of *Staphylococcal* species by using high culture media and are coupled with specific enrichment media favorable for the growth of specific bacteria. For instance, growth of *S. aureus* in specific media like Mannitol salt agar (MSA) and Biard parker agar (BPA) requires high salt concentrations. Chromogenic components like phenol red add color to the colony and based on its morphology, detection of *S. aureus* is done with ease. Golden yellow colored colonies shows the presence of *S. aureus* and pink colored colonies determine *S. epidermidis*. Further techniques used for identification of MDR *S. aureus* is by agglutination test or coagulase test assay and catalase test. Coagulase enzyme is a protein that clots plasma by converting fibrinogen to fibrin. Golden colored colonies were selected for gram staining procedure, purple colored colonies determine the presence of gram positive bacteria and pink colored colonies determine gram negative bacteria. S. aureus shows purple colour in the form of grape like clusters when observed under microscope. Colonies were sub cultured and Glycerol stock is made with this sub cultured *Staphylococcus aureus.*

Antimicrobial susceptibility or sensitivity test determines the susceptibility pattern of bacteria to a wide range of antibiotics by Kirby-Bauer disc diffusion test (Thongchai Taechowisan *et al*., 2018). Zone of inhibition is formed around the antibiotic disk if the bacteria are susceptible to that particular antibiotic. If zone of inhibition is not formed it determines that particular bacteria are resistant to antibiotic disk used for test. Culture based detection though determines the morphology of *Staphylococcus aureus* for its detection prior to infection, it is time consuming and not specific than molecular methods.

2.4. Molecular methods based detection:

Molecular techniques are popular diagnostic tools for identification of multidrug resistant *Staphylococcus aureus,* superior to classical or culture based detection in terms of speed, specificity and sensitivity. Molecular techniques are categorized into 16S rRNA detection, PCR, microarray assay; DNA sequencing and isothermal techniques of target amplification like Loop mediated isothermal amplification (LAMP), Helicase dependent amplification (HDA), rolling circle amplification, NASBA and Recombinase polymerase amplification. Amplification of phenotypic genes specific to *S. aureus* helps to diagnose it before causing infections. Phenotypic genes specific to S. aureus are *mec*A gene located on mobile genetic element called *Staphylococcal Cassette* chromosome mec types. Resistance to methicillin is provided by *mec*A gene which encodes PBP2a. *S. aureus* is known for its ability to cause infections in host by its virulence factors like leukocidins, heamolysins, exotoxins, super antigen and enzymes allows protecting itself from host defense mechanism (Hamid Motamedi *et al*., 2018). *Alpha* heamolysin gene is encoded in the genome whereas Panton-Valentine leukocidin gene (PVL) is encoded in mobile genetic elements like prophage. Heamolysin gene results in lysis of red blood cells mediated by *S. aureus.* A variety of heamolysin gene, heamolysin *beta* (*hlb)* causes infection to bovine by increasing its adherence ability to bovine mammary epithelial cells (Giada Magro *et al*., 2017). Major virulent factor present in *S. aureus* is coagulase gene which causes clotting of plasma in the host. Fibrinogen is converted to fibrin, and it shields *Staphylococcus* from phagocytosis. *S. aureus* is characterized as coagulase-positive and is mainly causative pathogen for sub-clinical mastitis. Pathogen without *coagulase* gene are considered as CONS. Polymerase chain reactions uses a set of primers and *Taq* polymerase for the amplification of species specific genes that makes detection of *S. aureus* more accurate and rapid. Identification of phenotypic genes by

using PCR with relative to restriction fragment length (RFLP) is best way for disease diagnosis.

2.4.1. PCR based detection of phenotypic genes in *Staphylococcus aureus*:

Identification of causative pathogen for bacterial infection is necessary for effective treatment. PCR and oligonucleotide microarray are used for the detection and identification of *S.aureus* based on conserved regions of gene like *mec*A, *hlg, pvl and coa* gene. PCR is a molecular based approach discovered by Kary Mullis and Michael Smith in 1983 (Mullis *et al*., 1987). This technique produces billion copies of small to large targeted segments of DNA. Reaction occurs in a single tube with a principle based on thermal cycling involving repeated heating and cooling.

Basic requirement of PCR involves DNA sequence of known target region, primers prepared using DNA synthesizer, polymerase for amplification that can withstand high degrees of temperature like *Taq* polymerase and a thermal cycler. Three steps in PCR include denaturation of ds DNA template, annealing of primers and extension of ds DNA molecules. Denaturation separates the double stranded DNA into single stranded DNA at 95^0C, followed by annealing of primers at 50-65^0C. The last step in PCR is elongation of templates which occurs at 70-74^0C. Advantage of PCR over older culturing techniques is its high sensitivity i.e., very small amounts of DNA segments can be used in this technique. Disease diagnosis and identification of genetic makeup differences among different individuals makes it an efficient tool. It has limitations in terms of cost and maintenance. As PCR requires thermal cycler for amplification at various degrees of temperature, it is not suitable for point of care device. To overcome this problem, isothermal based amplification has been proved better in terms of amplifying DNA at constant temperature.

2.4.2. Isothermal Nucleic Acid Amplification:

Isothermal nucleic acid amplification is cost effective amplification of DNA fragments at constant temperature. Molecular approaches are preferred when the sample amount is less. Isothermal amplification method can be done in places with less financial aid. The enzyme used in isothermal amplification performs for a longer time without any disruption. It yields better amplification in terms of specificity and sensitivity.

Based on factors of complexity, sensitivity and specificity several techniques have been developed that performs isothermal nucleic acid amplification.

The major isothermal based amplification methods used are:

- Helicase Dependent Amplification
- Rolling Circle Amplification
- Recombinase Polymerase Amplification
- Nucleic Acid Sequence Based Amplification
- Loop Mediated Isothermal Amplification.

2.4.2.1. Helicase Dependent Amplification (HDA):

Amplification in invivo condition is promoted by DNA polymerase and DNA Helicase that unwinds double stranded DNA to single stranded DNA (Kornberg & Baker). Helicase dependent amplification uses the same mode of amplification in invitro mimicking the DNA Helicase present in invivo condition. It generates single stranded DNA for extension of primers by DNA polymerase. HDA assay is carried out at 60^0C with 10^6 fold DNA amplification. It involves the use of two additional proteins, MutL protein that stimulates helicase to unwind the DNA and SSB (single stranded binding protein) that prevents rewinding of DNA. A helicase-polymerase fusion complex was developed that was able to amplify 1.5kb DNA target (Lei Yan *et al*., 2014).

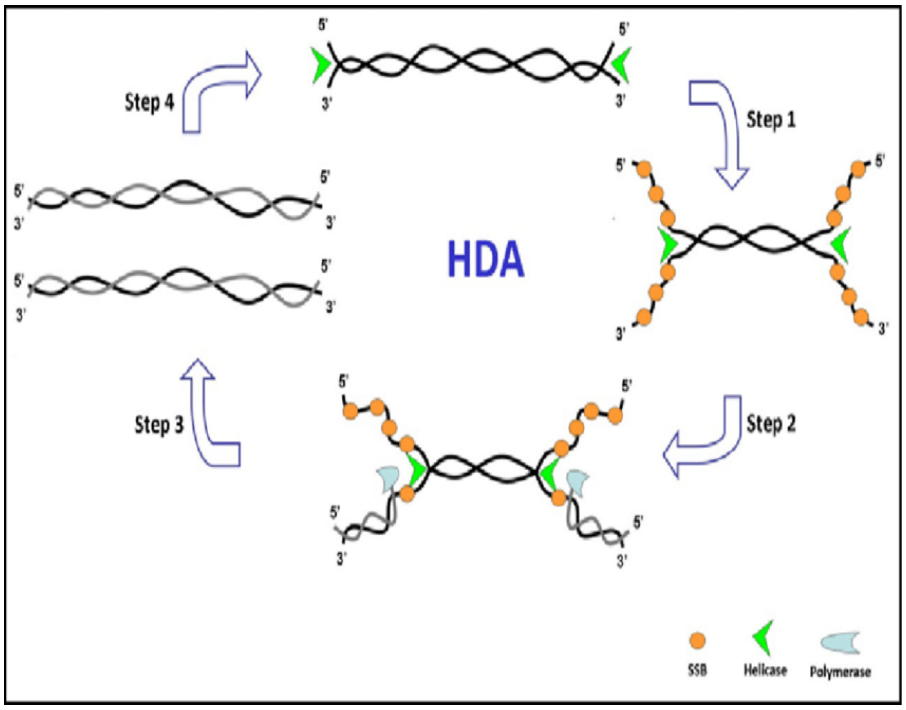


Figure 1. Schematic representation of helicase dependent amplification process

A combination of Helicase dependent amplification with "self avoiding molecular recognition system" (SAMRS) gave clear amplicon with the removal of artifacts like primer dimmers and off targeting. Its ability to support multiplexing of DNA at lower levels with HDA makes it a potent point of care isothermal amplification technique in resource limited places (Dr. Zunyi Yang *et al*., 2015). A real time electrochemical method for HDA was designed by Kivlehan *et al*., (2011) that use monitoring of intercalating redox probes. These probes bind to DNA and provide less electrochemical detectability than probe free technique.

2.4.2.2. Rolling circle amplification (RCA)

Rolling circle amplification (RCA) is an efficient isothermal enzymatic process that uses nucleases to generate single stranded DNA or RNA. It uses a single DNA primer and a padlock probe. Functional nucleic acid unit can be amplified number of times with limit of detection (LOD) when combined with surface plasma resonance, enzymatic reactions and bio-sensors. RCA uses DNA or RNA polymerase (Φ29 DNA polymerase) to produce ssDNA (Lide Gu *et al*., 2018). A padlock probe is designed in such a manner that it has two portions complementary to target at 5' and 3' ends that are connected by another sequence. RCA uses the ability of strand displacement activity to synthesize long DNA strands by displacing old strands.

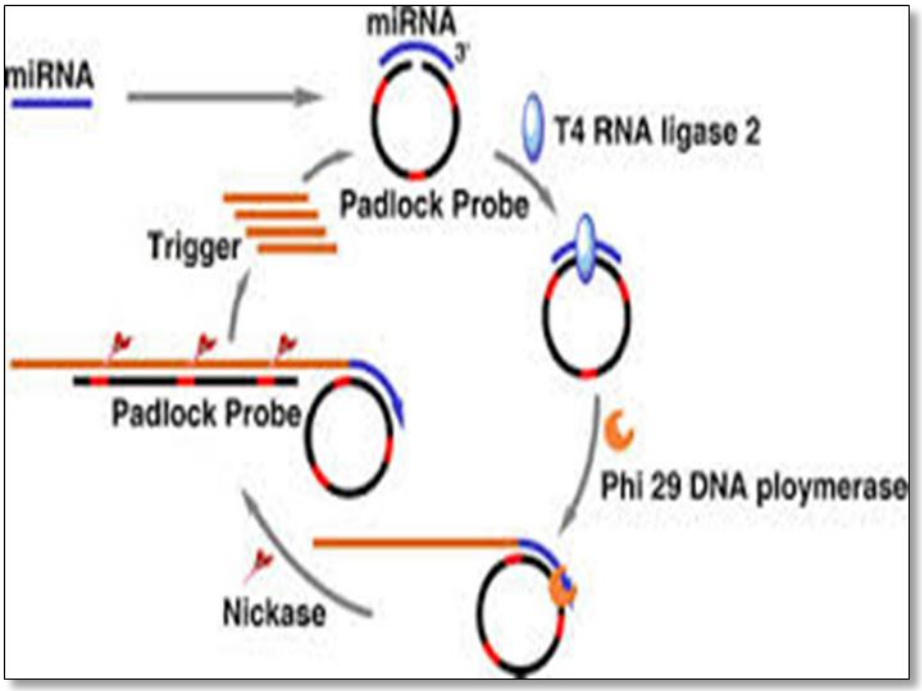


Figure 2. Padlock probes and rolling circle amplification. (Aiming Sun & Kaijian Liu).

It has been used as straight forward signal amplification techniques for biodetection formats (Linck *et al*., 2012). Periodic DNA nanotemplates have been synthesized by rolling circle amplification (Beyer S *et al*., 2005). Many circular amplification variants have been developed with the aid of single primer RCA and dubbed primer variant (Murakami *et al*., 2005). RCA can be used in viral detection assay via microbead based assay (Schopf E *et al*., 2008).

Padlock probes has been used in detection of several disease related like cystic fibrosis conductance transmembrane regulator (CFTR) G542X mutation, influenza H1N1 and H3N2 mutations, Epstein Barr virus and Listeria monocytogens (Lei Yan *et al*., 2014).

2.4.2.3. Recombinase polymerase amplification (RPA)

Recombinase Polymerase amplification is a single tube isothermal amplification technique. Addition of reverse transcriptase enzyme to RPA reaction can be used to amplify RNA along with DNA without the requirement for cDNA preparation. It is carried out at 37–42^{0}C and is simpler to perform. In Rift valley fever virus, molecular detection was best performed by RPA at minimum concentration levels even smaller samples were lost by RT-LAMP and PCR.

RPA process comprises of three enzymes namely Recombinase, single strand DNA binding protein and single strand displacing polymerase. Role of Recombinase is to pair the oligonucleotide primer pair with homologous DNA sequences and strands are protected from being displaced by the single strand DNA binding protein. Finally polymerization is initiated by strand displacing polymerase. Based on the sequence complementarity of primers with target DNA sequence, the isothermal amplification of DNA takes place at a constant temperature. This process has further application by addition of two more enzymes like addition of exonuclease III and endonuclease I. It allows the use of exo probe and nfo probe for the real time fluorescence detection and lateral flow assay of strip detection of successful amplification respectively.

RPA is less established in primer and probe designing and leads to trial and error, standard PCR primers work well with RPA but has a limitation of producing low recombination rates becomes slow as the length of primers are short. Internal flurogenic probe can be complexed with forward and reverse primer for detection of isothermally amplified product. For effective recombinase action the amount of primers required are

about 30-38bp. It is better recommended in place of LAMP as it requires larger primer length than RPA and has a huge constrain in the design of primers.

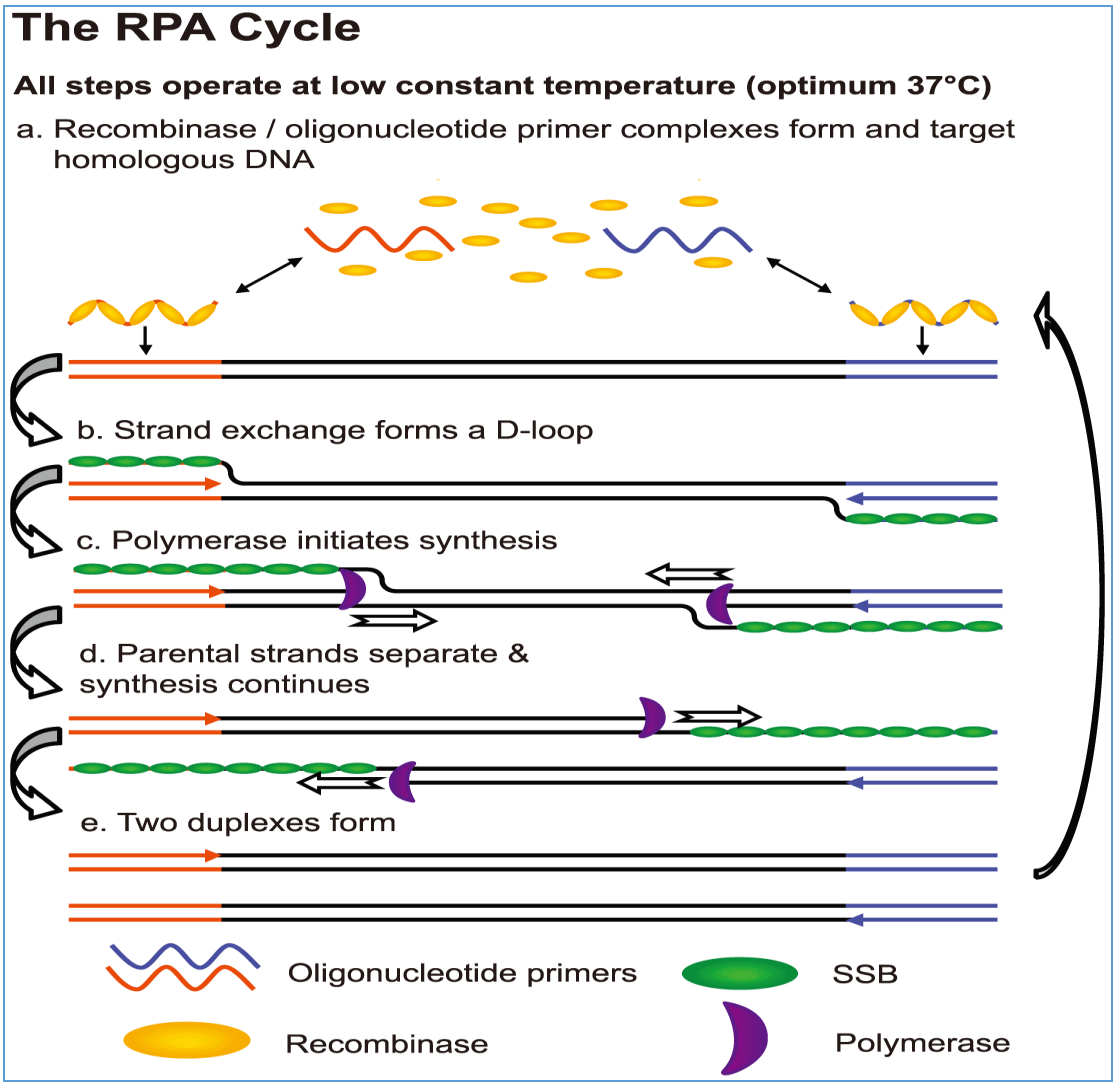


Figure 3. Schematic representation of RPA cycle (David S Boyle *et al*., 2014)

2.4.2.4. Nucleic acid sequence based amplification

NASBA is a RNA based amplification method developed by J Compton uses reverse transcriptase (RNA dependent DNA polymerase). It performs at a constant temperature of 41 °C.The enzymes used in NASBA are avian myeloblastosis reverse transcriptase T7 polymerase and Rnase H. The reaction in NASBA takes place in two phases termed as initial phase and cyclic phase. Initial phase involves denaturation and primer annealing at 65^0C. It requires single stranded RNA or single stranded DNA to synthesize DNA strands. In cyclic phase NASBA, it uses an optimum temperature of about 41^0C. Rnase H is provided to degrade RNA-DNA hybrid.

NASBA products can be quantified with the aid of molecular beacon probes that binds to amplified product and produce a fluorescent signal.

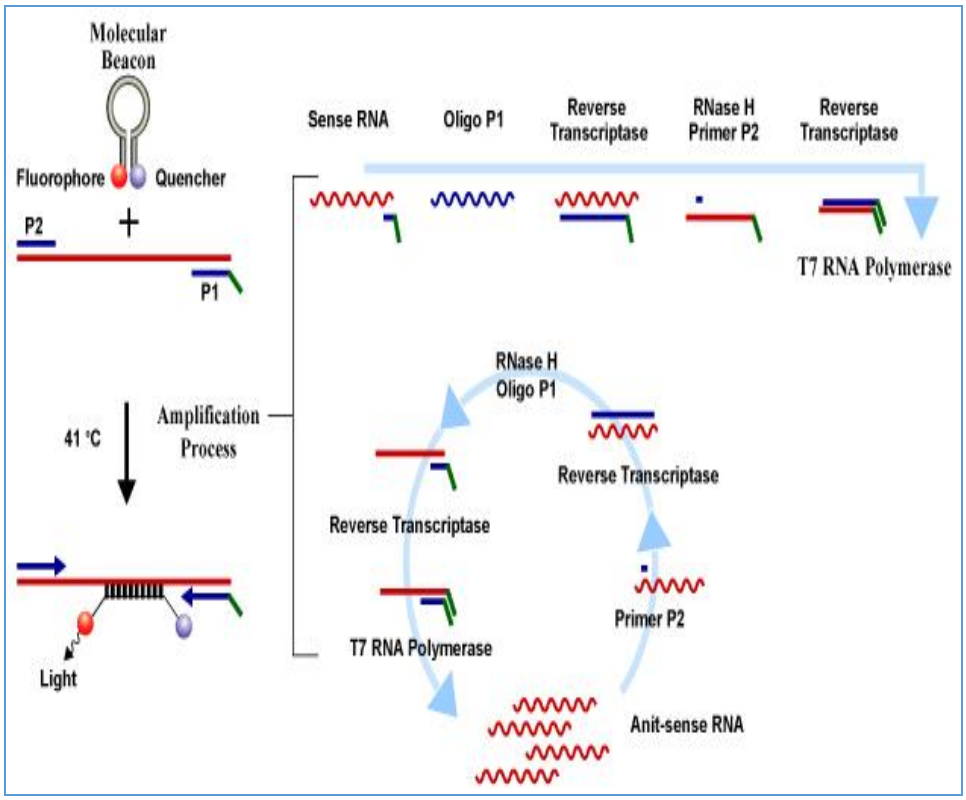


Figure 4. Overview of Nucleic acid sequence based amplification.

2.4.2.5. Loop mediated isothermal amplification (LAMP)

Loop mediated isothermal amplification is a prominent tool for its rapid amplification with high specificity and sensitivity. It is cost effective and is carried out at a constant temperature of 60-65^{0}C. It is used in detection or diagnosis of diseases due to simpler methodology. LAMP assay provides high specificity due to its provision of identifying 6 regions by 4 primers making it sensitive in contrast to PCR where a single band is formed generally. LAMP performs its activity based on the target region, either DNA or RNA. In case of RNA amplification, we prefer reverse-transcription (RT-LAMP) with use of an enzyme known as AMV reverse transcriptase.

2.5 LAMP principle

LAMP assay occur in two main steps: non-cyclic and cyclic step (K.L Bruce *et al*., 2014)

2.5.1 Non cyclic amplification

Non cyclic amplification begins with amplification of target DNA with reagents at 60-65^{0}C. This step is determined with the formation of stem loop in DNA confirmation that

is further required for amplification of DNA in cyclic (amplification) step. Initially at 65^0C ds (double stranded) DNA is in the confirmation state that represents that DNA polymerase is able to synthesize with strand displacement activity. Strand displacement activity displaces one strand from the double stranded DNA and releases the single stranded DNA. In PCR for denaturation we require incubation at 95^0C which is not necessary in case of LAMP due to strand displacement activity. In second stage a new DNA strand complementary to DNA template from the 3'end of F2 region of FIP is synthesized. Third stage involves DNA synthesis with strand displacement as F3c region of template DNA is annealed by F3 primer. This result in release of FIP- linked complementary strand. Next to this DNA strand is synthesized with F3 primer and DNA template leading in double stranded conformation. This leads to release of FIP-linked complementary strand which forms a stem loop confirmation at 5' end because of F1 and F1c regions in complementarity. FIP-linked acts as template in last stage for DNA synthesis by BIP and strand displacement performed by B3 primer. Initiation of DNA synthesis at 3' end is followed by annealing of BIP to FIP-linked strand. This results in reverted confirmation of FIP-linked DNA strand from loop to linear form. Next step is followed by strand displacement at 3' end by B3 primer. The newly synthesized DNA is formed with the release of single strand from BIP by strand displacement activity. Finally dumbbell like DNA structure is formed at each end of displaced BIP-linked complementary strand. This structure is required for initiation of cyclic (amplification) step of loop mediated isothermal amplification.

2.5.2 Cyclic amplification

Cyclic amplification is carried out for the synthesis of original stem loop DNA and new one synthesized from original stem loop twice its length. Initially FIP anneals to single stranded region in stem loop DNA generated in non cyclic (amplification) step. Followed with DNA synthesis, initiation of strand displacement DNA synthesis releases previously synthesized strand. Stem loop structure is formed at 3'end with release of single strand because of B1 and B1c. As DNA synthesis takes place from 3'end of B1 region FIP linked complementary strand is released. Dumbbell like structure is formed due to stem loop having complementary region (F1 & F1c, B1 & B1c).B1 primed DNA strand is released with complementarity of BIP primer with B2c region. Cyclic step results in amplification of 10^9 copies per hour

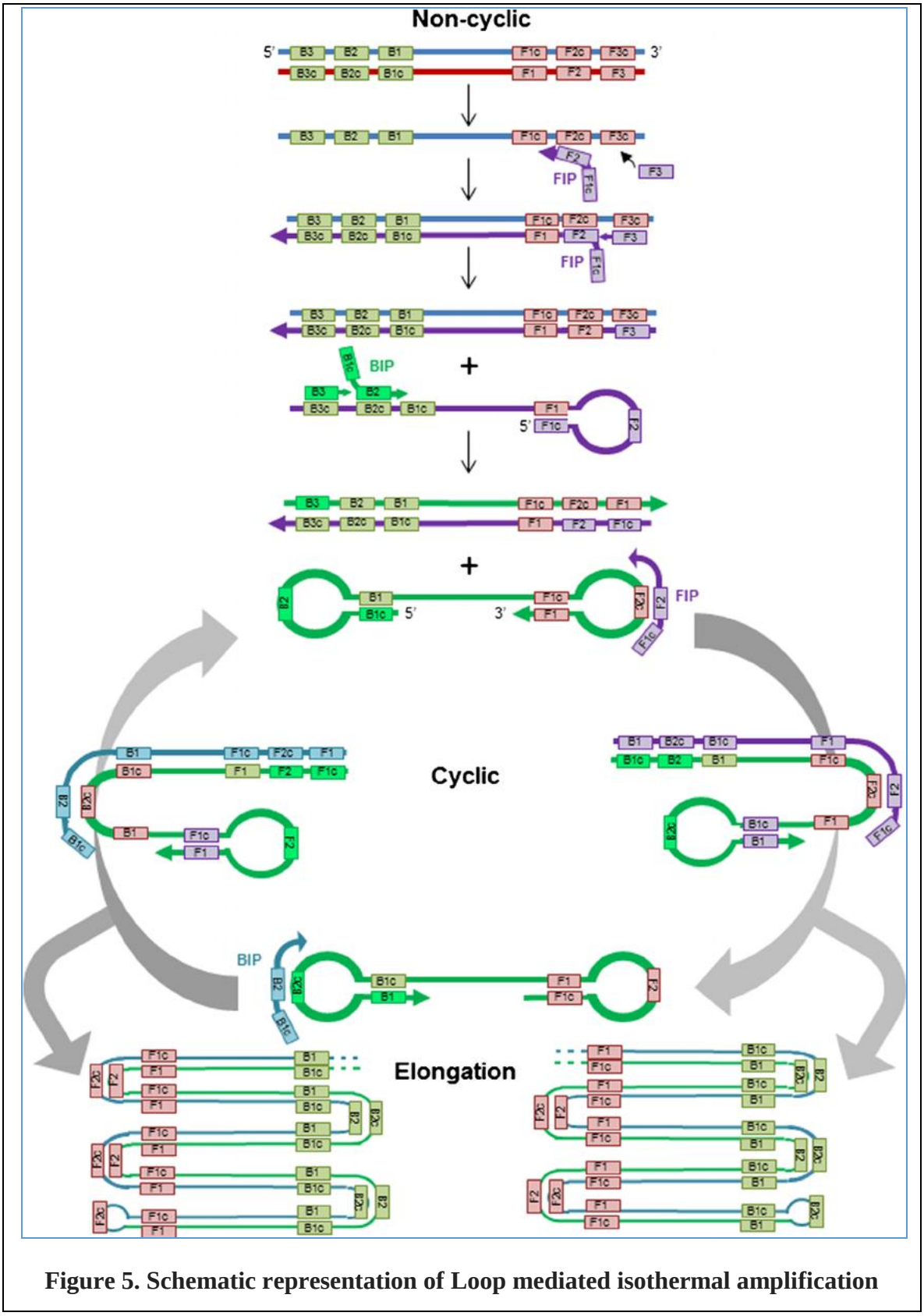


Figure 5. Schematic representation of Loop mediated isothermal amplification

2.5.3 Advantages of LAMP assay

1. LAMP based DNA amplification is highly efficient under constant temperature without the influence of non target amplification and doesn't require denaturation of ds DNA due to its strand displacement behavior. (Notomi *et al*., 2000).

2. Easy and simple molecular approach can be performed in places with less financial aid with proper primer preparation and DNA polymerase with a requirement of water bath.

3. Specificity and sensitivity of LAMP in contrast to PCR is efficiently high due to its specifically binding of 4 primers to recognize 6 distinct regions in template DNA.

4. LAMP when combined with reverse transcription is referred as RT-LAMP and amplifies RNA sequence with high efficacy.

5. Multiplex loop mediated isothermal amplification (M-LAMP) assay results in detection and diagnosis of diseases with single genome copy sensitivity. (James B. Mahony *et al*., 2013

2.6 Design of primers

Primers for LAMP are design based on six distinct regions: F3c, F2c and F1c at 3' end and B1, B2 and B3 at 5' end.

FIP: Forward Inner Primer consists of F2 region complementary to F2c at 3'end and F1 complementary to F1c at 5' end.

F3 primer: F3 is forward outer primer with F3 region complementary to F3c region.

BIP: Backward inner primer consists of B2 region complementary to B2c at 3' end and B1 region complementary to B1c at 5' end.

B3 primer: B3 is Backward outer primer with B3 region complementary to B3c region of target.

LAMP primers are designed with the use of software program and bioinformatics tools. "Primer Explore" is the software used in support for LAMP primers design. GC content should be maintained at 60%. Distance between region of primers must be featured and Tm value of about 60-65^{0}C for GC rich and 55-60^{0}C for AT rich is identified.

2.7 Different methods for LAMP detection

Amplified DNA products formed by LAMP are detected by various detection methods.

2.7.1 Naked eye monitoring by observing turbidity

Turbidity represents the amount of white precipitate formed by Bst polymerase formed in a LAMP reaction. It is due to the release of pyrophosphates from the dNTPs (deoxyribonucleotide triphosphate) as a byproduct (Mori *et al.,* 2001). These released pyrophosphates react with magnesium ions present in reaction buffer to form a visible white precipitate. Turbidity appearance allows easy distinction of nucleic acid amplification as an end point measurement in LAMP reaction in comparison to non turbid formation in non template solution (Kubota *et al*., 2008). Risk of contamination is less in amplicons, as not performed in open atmosphere but need to be monitored as soon as experiment is performed because of its less stability (Almasi *et al*., 2013).

2.7.2 Visualisation with the aid of DNA binding dyes

DNA binding dyes causes a visible colour change when bound to double-stranded DNA by forming dye-ds DNA complex. In contrast to measuring of nucleic acid amplification by turbidity, DNA binding dyes has higher sensitivity of detection (Le *et al*., 2012). Several fluorescent dyes exhibit visible colour change in LAMP reaction when bound to double-stranded DNA. SYBR Green 1 changes from orange to green when bound to DNA and is apparent under natural light and UV light (Zhang *et al*., 2012 and Le *et al*., 2012). The risk of contamination is higher than simple visualisation with turbidity because the amplicons need to be opened in open atmosphere for addition of fluorescent dyes. This is minimized with the use of tin foil that drops dye directly into reaction tube after centrifugation reducing the chance of open air contamination (Hong *et al*., 2012).

Quant-iT PicoGreen is another DNA binding dye forms a luminescent complex characteristically similar to SYBR Green 1. Wasting et al. reported that binding of PicoGreen to DNA emits fluorescence of high level. Based on DNA assays, sensitivity was shown at 1 pg/ml concentration. GeneFinder and ethidium bromide (Almasi *et al*., 2010, Zhang *et al*., 2009, 2011, Wasting *et al*., 2010) also visualize the change in colour with a high level of sensitivity.

Polyethylenimine based detection of detecting LAMP products is an another fluorescent probe label detection method in which LAMP products are allowed to bind with DNA labeled probes. White precipitate with a clear appearance is seen with DNA labeled dye.

Mori et al. determined that use of Polyethylenimine can lead to inhibition of LAMP reaction. Ethidium bromide used in LAMP visualisation may be carcinogenic or mutagenic as posted by Tomita *et al*., (2008).

2.7.3 Amplicons visualisation with calorimetric indicators

Direct visualisation of LAMP products can be done by visualisation with calorimetric indicators. These indicators are directly added during LAMP reaction mixture. Risk of contamination is low when compared to two step visualisation with fluorescent labeled dyes as visualisation is done at one step reaction (Tomita *et al*., 2008: Parida *et al*., 2008).

Calcein is an example of calorimetric indicator used for indirect visualisation of LAMP reaction. Before amplification the LAMP reaction solution appears orange due to calcein molecules quenching with manganous ions as the reaction proceeds the orange colour disappears with the appearance of green colour in the presence of target DNA. After amplification calcein gives out manganous ions that result in green colour appearance. Positive results can be seen by change in colour (Hong *et al*., 2012; Tomita *et al*., 2008). Detection limit was more than 100 copies when compared to fluorescent probe detection with DNA binding dyes.

Hydroxy naphthol blue (HNB) is another calorimetric indicating dye that develops purple colour in presence of magnesium ions. As the reaction proceeds significant amount of magnesium ions are replaced by formation of magnesium pyrophosphate that replace purple colour with blue colour and shows no inhibition of LAMP reaction as seen in case of PicoGreen (Goto *et al*., 2009). Detection limit of 30 CFU/ml or limit of 60 copies (Ma *et al*., 2010) can be obtained using HNB.

2.8. LAMP Reagents

Reagents used in LAMP vary with techniques being implemented and uses a special kind of DNA polymerase at 65^0C termed as *Bst* polymerase obtained from *Bacillus steanothermophillus. Bst* polymerase shows 5'- 3' polymerase activity but lacks exonuclease activity from 5'- 3'.Amplification at optimum condition provides proper primer annealing and increase tolerance capacity to inhibitors. DNA polymerase is supplied with Thermopol buffer (20 mM Tris-HCL, 10 mM $(NH_4)_2SO_4$, 10 mM KCL, 2mM $MgSO_4$, 0.1% Triton-X-100, pH 8.8 at 25^0C).

DNTPs used in LAMP reaction mixture provide desired results when mixed in reaction buffer. In LAMP assay dNTPs concentration used is very high (1.4mM). Presence of $MgSO_4$ in lamp reaction provides better polymerase activity, strand displacement DNA synthesis with primer annealing, resulting in high sensitivity and specificity. Optimum amount of $MgSO_4$ added should contain 0.5 to 2.5 mM. Ravindran et al reported an optimum concentration of 2.5 mM is sufficient for a LAMP reaction. As described by Zhang *et al*., 2009 and Tomita *et al*., 2008 magnesium ions are used for visual detection of DNA amplification with aid of DNA binding dyes and calorimetric indicators.

Enhancers are applied to reaction mixture to prevent non specific priming activity such as use of betaine and glycerol. Jensen *et al*., (2010) demonstrated that use of betaine improves specificity in GC rich regions with an increase in high yield. Glycerol increases the efficiency of primer annealing to target DNA by inhibiting non specific template binding. Studies suggest that 3% glycerol accounts for better use as enhancer in LAMP assay.

2.9 LAMP assay applications

Loop mediated isothermal amplification is a single tube DNA amplification technique combine with reverse transcriptase for RNA amplification is rapid, highly sensitive and simple gene amplification technique. Due to its high specificity in binding specific region of DNA it is implied as a quantification tool in fields of microbiology and disease diagnosis. LAMP is robust in nature and is cost effective. Easy visualisation of amplicon makes LAMP as a potent point of care device used by clinicians for detection and diagnosis of diseases at outbreaks.

LAMP provides an ease in detection of diseases caused by virus with the help of RT-LAMP. RT- LAMP uses reverse transcriptase for conversion of RNA to DNA and kits have been developed for some viruses like norovirus (Fukuda *et al*., 2006). RT-LAMP has been used for disease diagnosis caused by H5N1 virus, SARS, HIV and H1N1 swine flu. Robust nature of LAMP makes it a powerful tool in detection of bacteria like food borne bacteria. Multiplex LAMP approaches have been applied for detection of food related toxins caused by botulin toxins. Amplicons are visualized by interchelating fluorescent probes that bind to grooves of DNA mainly SYBR Green and SYTO 9.

CHAPTER –3

Materials & Methods

3. Microbiological Methods

3.1 Collection of mastitis infected milk samples

Mastitis milk samples (226) were obtained from regional centre (LUVAS) at Uchani, Karnal for isolation and identification of *Staphylococcus aureus.* Milk samples were collected from infected animals in sterile containers and transferred aseptically. Samples were summarized based on laboratory number and sample codes were assigned for samples collected on that particular date. Milk samples were immediately stored in ice and transferred to the laboratory.

Table 1 Collection schema for mastitis infected milk samples

S. No.	Sample Code	Isolate Numbers	Date of collection	Animals infected with mastitis	Lactation period
1	Sample B	B6159-B6320	10/01/2019	Cattle and Buffalo	L1-L4
2	Sample C	C6333-C6485	18/01/2019	Cattle and Buffalo	L1-L7
3	Sample D	D6496-D6808	01/02/2019	Cattle and Buffalo	L1-L5
4	Sample E	E6891-E7202	20/02/2019	Cattle and Buffalo	L1-L5
5	Sample F	F7177-7365	01/03/2019	Cattle and Buffalo	L1-L4

3.1.2 Sample processing

Mastitis milk samples (100µl) were transferred aseptically in sterile nutrient broth for enrichment of pathogenic bacteria for 6-8 hours at 37^0C. Characteristic turbid formation has been observed in tubes due to growth of bacteria. Samples were spread on Mannitol salt agar media and cultures were incubated at 37^0C for 4-6 hours. Characteristic growth of bacterial colonies such as colour (yellow-pink), round in shape and size less than 1mm in diameter were selected as characteristic of *S. aureus* culture.

3.1.2.1 Antimicrobial susceptibility test

Single colony of each bacterial isolate was inoculated in freshly prepared Nutrient broth and left for overnight incubation at 37^0C.Then enriched samples enriched in nutrient broth were spread in Nutrient agar media and antibiotic discs were placed for antimicrobial susceptibility test. Plates were incubated overnight at 37^0C and were screened for antibiotic susceptibility pattern and zone of inhibition. Different classes of antibiotics discs were chosen for determining susceptibility pattern of different bacterial cultures with defined doses.

Table 2 Class of antibiotics used for antimicrobial susceptibility test

S. No.	Class of Antibiotics	Antibiotic discs used for AST	Concentration of antibiotic discs (µg)
1	Aminoglycosides	Gentamicin (HLG)	120µg
2	β-Lactams	Ceftazidime (CAZ)	30µg
3	Aminoglycosides	Amikacin (AK)	30µg
4	Quinolones	Ciprofloxacin (CIP)	05µg
5	Macrolides	Azithromycin (AZM)	15µg

Bacterial isolates that showed resistance to different antibiotics were again cultured by streaking them on the Mannitol salt agar plates and kept for overnight incubation at 37^0C**.** Golden yellow colonies were now subjected to identification by biochemical test.

3.2 Biochemical test

A number of biochemical tests were performed to detect positive isolates of *S. aureus* with the help of catalase test, coagulase test, methyl red test and gram staining. Catalase test was performed by taking a single colony with the help of sterile inoculation loop and was spread on the glass slide. A drop of hydrogen peroxide was added on to this colony and was checked for bubble formation. 194 *S. aureus* isolates were found positive for catalase. Coagulase test was performed by taking 1 ml of plasma in a sterile tube. Plasma was diluted in 1:6 ratio by using normal saline containing 0.85%NaCl. To this plasma isolated colonies of *S. aureus* were emulsified to give a milky appearance inside the tube. The tubes were incubated at 35^0C for duration of 4hours in a water bath. Tubes

containing *S. aureus* isolates were examined at different time intervals for the formation of clots by tilting it through 90^0C. 40 number of *S. aureus* isolates gave positive results for coagulase test. Further, these isolates were detected morphologically by performing Gram staining method.

3.3 Gram staining method

Gram staining was performed for all bacterial isolates (194) found positive for catalase and coagulase test. A single colony from the nutrient plate was mixed with a sterile drop of peptone and mixed on the glass slide. The bacterial smear was then heat-fixed to affix the bacteria on the slide, so that they do not get rinsed out during staining.

Process: Primary stain (Crystal violet) was applied to the heat-fixed bacterial smear and allowed to dry for few minutes. Stain was rinsed out with water and trapping agent (iodine) was added. Rapid decolorization was performed with alcohol. Counter staining was performed by adding a drop of safranin. The smear was rinsed out properly and air dried for 10-15 min. Glass slides were visualized under compound microscope with a magnification either of 40X or 100X. Purple colored crystal violet colonies represent Gram positive bacteria and pink colored colonies represent Gram negative bacteria. Grape like clusters with round cocci confirmed colonies of *S. aureus* isolates that showed positive for catalase test and stained purple with round colonies were preserved in nutrient broth containing 50 % glycerol and stored at -80^0C for future purposes.

3.4 Molecular methods

3.4.1 Genomic DNA isolation from *S. aureus*

1. Frozen isolates (100) were thawed and revived by taking a loop full of glycerol stock and streaking was done on selective MSA plates and incubated at 37^0C for overnight.
2. Colonies appearing golden yellow colour were selected for genomic DNA isolation by inoculating in five milliliter nutrient broth and incubation at 37^0C for 6-8 hrs. Genomic DNA isolation was performed in the bacterial log phase.
3. To the 5 ml of log phase culture, 5 µl of ampicillin solution (50 mg/ml) was added and incubated at 37^0C for 1 hour.

4. Post incubation, cells were centrifuged at 5000 rpm for 5 min at 4^0C. Supernatant was decanted and pellet was washed with 1 ml of Nacl-EDTA (30 mM Nacl, 2 mM EDTA, pH 8.0). This washing step was repeated for two more times.

5. The washed pellet was resuspended in 100 µl of Nacl-EDTA (30 mM Nacl, 2 mM EDTA, pH 8.0). Two hundred microlitre of freshly prepared lysozyme solution (10 mg/ml in Nacl-EDTA) was added to pellet and mixed properly.

6. The mixture was incubated in dry bath at 55^0C for 1-2hrs till a clear, transparent solution appears and cellular debris settles at the bottom of tube.

7. The volume of the mixture was made upto 1 ml with additional Nacl-EDTA and 50 µl of 10 % SDS followed by 20 µl of proteinase K solution (20 mg/ml). The contents were mixed thoroughly and incubated at 55^0C for 3-4 hours.

8. After incubation, 800 µl of Tris-saturated phenol (pH 8.0) was added and mixed thoroughly. The mixture was centrifuged at 5000 rpm at 26^0C for 10 min.

9. The upper aqueous phase was separated from the lower organic phase containing cell debris and an interphase containing denatured protein layer. This step was repeated once with the use of Phenol-Chloroform mixture (1:1) and supernatant was collected in sterile 2 ml micro centrifuge tube. To this aqueous phase equal amounts of Chloroform-Isoamyl alcohol (24:1) was added and supernatant was collected in a fresh tube.

10. DNA was precipitated out by adding equal volume of Isopropanol in presence of 5M sodium acetate (pH-5.3)

11. The precipitated DNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4^0C. The supernatant was discarded and pellet form of DNA was washed twice with 70 % ethanol and air dried.

12. The pellet was dissolved in 50 µl Tris-EDTA (10:1, pH 8.00) and stored at -20^0C.

3.4.2 Qualitative and Quantitative Assessment of DNA

Concentration of DNA samples were analyzed by recording its absorbance at 260 nm using a nanodrop spectrophotometer (Bio-Tek instrument, inc.). Purity of DNA was determined by A_{260}/A_{280} ratio. Quality assurance of DNA was also checked by running electrophoretically in 1% agarose gel. The type of band pattern formed indicates the quality of DNA.

Concentration was calculated by: OD_{260} × dilution factor

Table 3: Yields of DNA isolated from *S. aureus* isolates by the Ampicillin Lysozyme tandem lysis method

	Concentration of DNA (ng/ µl)	**Absorbance at 260 nm (A_{260})**	**Absorbance at 280 nm (A_{280})**	**Purity [A_{260}/A_{280}]**
Average	137.58	2.05	1.10	1.86
Maximum	426.99	4.76	2.53	1.88
Minimum	8.20	0.41	0.22	1.86

3.4.3 Polymerase chain reaction using isolated DNA

Stock DNA solutions were added uniformly to get a working solution. Approximately 100 ng of DNA sample was used as a template for amplification of 16S rRNA gene and specific bacterial genes from *Staphylococcus aureus* genome.

3.4.3.1 16S rRNA amplification of *S. aureus* isolates

16S rRNA specific PCR assay was used in our study for the detection of 16S rRNA gene sequence specific for *S. aureus.* Primer sequences used for 16S rRNA PCR amplification were 16S rRNA forward 5'CTTTTTATGGAGAGTTTGATCCTGGCTCAG3' and reverse 5'TCCCTACGGTTACCTTGTTACGACTT3'. PCR was performed using a Veriti ® 96 Well® Thermal Cycler (Applied Biosystems®). Expected size of the PCR amplified DNA fragment was 1.5 kb. The following components added sequentially for the preparation of master mix were as follows: 2.5 µl of 10X reaction buffer (15 mM $MgCl_2$), PCR primers (final concentration, 0.2 µM each) and deoxynucleoside triphosphate (final concentration, 0.2 mM). The master mix was made to 25 µl with the addition of nuclease free water. Approximately 100 ng of isolated S. aureus DNA was added to 25 µl of reaction mixture for PCR amplification with 0.05 U *Taq* DNA polymerase. The reaction mixture was subjected to initial denaturation at 95^0C for 3 min followed with final denaturation at 30 sec, annealing temperature was maintained for desired amplification at 55^0C for 20 sec and extension at 1 min. Cycle was repeated for 30 times. Final extension was performed for 4 min. The amplified product of 16S rRNA PCR was visualized in 2% agarose gel.

3.4.3.2 Detection of *S. aureus* specific genes by PCR

The primer sequence used for the detection of *S. aureus* specific genes (*Coa* gene, *Mec* A gene, *Hlg* gene and *PVS* gene) was listed below.

Table 4: The selected primers for DNA amplification by PCR are listed in below table.

Primer name	Primer sequence	T_m	Product size
Coa Forward	5' GCGCTAGGCGCATTAGCAGTTGC 3'	61	173 bp
Coa Reverse	5' CGCTGGTTCTCTAGATTTTCAATTATTCCCC 3'	60	
Mec A Forward	5' CGCATCAATAGTTAGTTGAATATCTTTGCCATC 3'	59	232 bp
Mec A Reverse	5' GCGGTTATGTTGGTCCCATTAACTCTG 3'	60	
Hlg Forward	5' CGGTAATTTCCAATCAGCCCCATCACT 3'	60	296 bp
Hlg Reverse	5' GCGCGATAAATGAAGGGTTAAATCCACTTTG 3'	60	
PVS Forward	5' GTGTGACCCCCCACTTATCGCTAC 3'	61	126 bp
PVS Reverse	5' TAATCACTCCTATTGCTACTTCGTTTCATGAATC 3'	60	

3.4.3.2.1 PCR Reagents

All PCR ingredients except the primer required for PCR were procured from New England BioLabs. The amplification reaction was carried out in a final volume of 25 µl. The PCR reaction was performed in a Thermal Cycler (Veriti®96 Well, Biosystems®). The following components were added sequentially to a nuclease free eppendorf tube.

Table 5: PCR reagents for 25 µl reaction volume.

Components	Initial concentration (Stock solution)	Volume	Final concentration (Working solution)
Taq Buffer	10X(15 mM $MgCl_2$)	2.5 µl	1X(1.5 mM $MgCl_2$)
dNTP mix	10 Mm	0.5 µl	0.2 mM
Forward primer	10 pM	0.5 µl	0.2 µM
Reverse primer	10 pM	0.5 µl	0.2 µM
Taq polymerase	5U/µl	0.25 µl	0.05U/µl
Template	~100 ng DNA	1.0 µl	4.0 ng DNA
Nuclease free water		To 25 µl	
Total reaction volume		25 µl	

3.4.3.2.2 PCR parameters for amplification of *S. aureus* DNA

The amplification parameters for PCR were optimized keeping the initial denaturation temperature at 95^0C for 3 minutes followed with final denaturation for 15 seconds at 95^0C. Annealing temperature maintained for desired amplification was 55^0C for 15 seconds. Final extension was performed for 5 minutes at 72^0C after initial extension of 15 seconds. The amplified product was visualized in 2 % agarose gel.

3.5 Agarose gel electrophoresis

Two percent of Molecular biology grade (low electro endosmosis, EEO) agarose in 1X TAE buffer was used for separation of PCR amplified DNA product. Ethidium bromide (10mg/ml) stock was added directly to molten agarose at the rate of 0.5 µg/ml in the final concentration before casting the gel in cassette, the comb was placed in the gel cassette for making wells to load amplified product of PCR. After the gel solidifies, remove the comb carefully and the gel casting tray was placed in electrophoresis tanks containing 1X TAE (pH 8.0) buffer. The PCR product was mixed with 1 µl of gel loading buffer (6X, 30% glycerol, 0.025% Xylene cyanol, 0.025% bromophenol blue). It was loaded onto the wells submerged in the buffer using a (10 µl) micro pipette. Electrophoresis was carried out at 8 Volts/cm depending on the product size of gene amplified.

After completion of electrophoresis, the gel was visualized in a UV transilluminator and photographed using Molecular Imager Gel Doc ^{tm} XR (Bio-Rad Laboratories, Inc).

Primer design

Lamp primer design

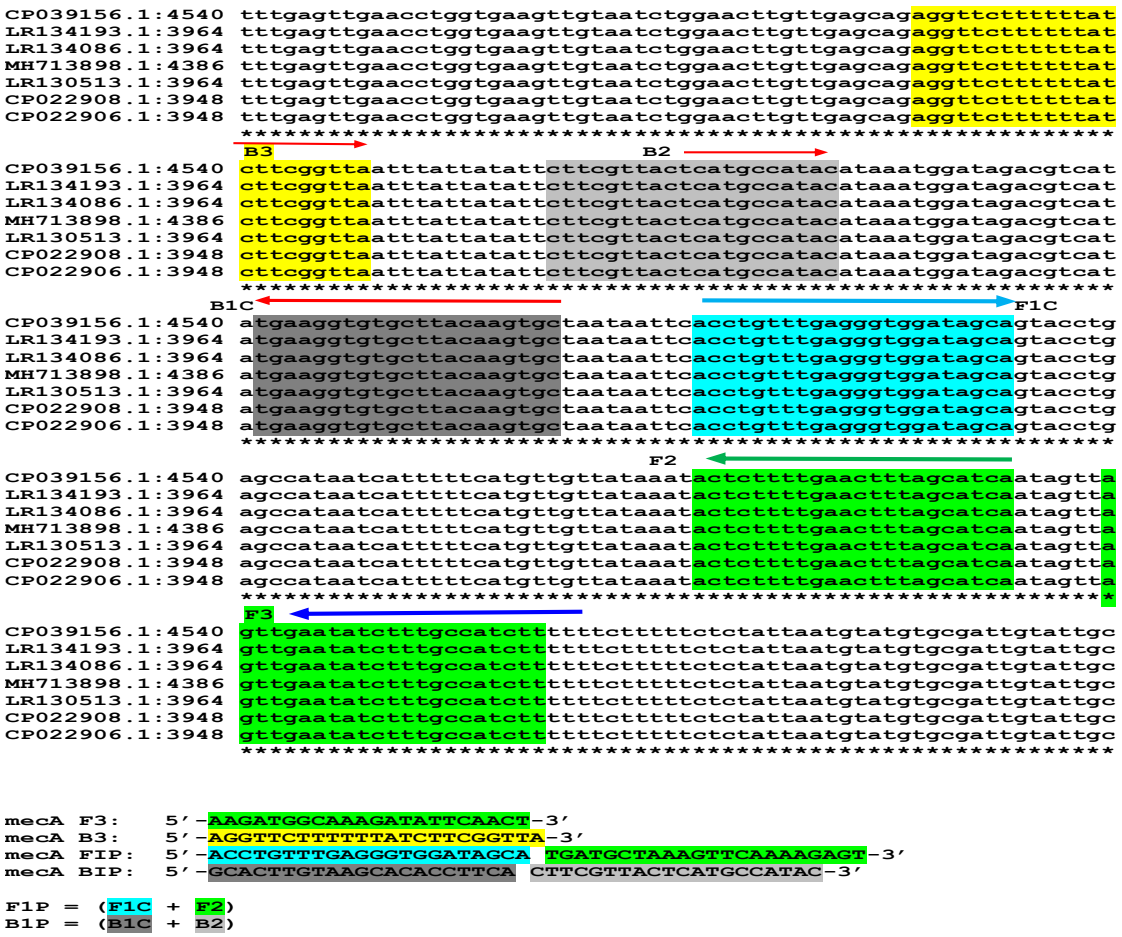


Figure 6: Multiple sequence alignment of *S. aureus* Mec A gene. The colour boxed sequences indicate the region where the specifically designed LAMP sequences are found.

3.5.1 Lamp assay and primers specific for *Mec* A gene

Four primers (F3, B3, FIP and BIP) were used for LAMP assay. FIP primer contains F1C and F2 portion and BIP primers have B1C and B2.

Table 6 Primers used for LAMP assay

Primer name	Primer sequence	T_m	Length
Mec AF3	5’ AAGATGGCAAAGATATTCAACT 3’	47	22
Mec AB3	5’ AGGTTCTTTTTTATCTTCGGTTA 3’	48	23
Mec AFIP	5’ACCTGTTTGAGGGTGGATAGCATGATGCTAAGT T CAAAAGAGT 3’	66	44
Mec ABIP	5’ GCACTTGTAAGCACACCTTCACTTCGTTACTCAT GCCATAC 3’	68	41

LAMP reaction was performed for 25 µl in PCR tubes. Master mix was prepared with following LAMP reagents: polymerase buffer, Bst polymerase 3.0 (M0374S, New England Bio labs), four primers and dNTPs. Additives ($MgSO_4$, betaine & glycerol) were added and performed at 65^0C for 1 hour.

Table 7: Optimized LAMP protocol

Components	Initial concentration	Volume	Final concentration
Thermopol Buffer	10X	2.5 µl	1X (2 mM MgSO4)
dNTP mix1	10 mM	2.5 µl	1 mM
F3/B3 primers	10 pM	0.5 µl	0.2 µM each
FIP/BIP primers	10 pM	1.0 µl	0.4 µM each
Bst DNA polymerase	8U/µl	0.25 µl	2U/µl
$MgSO_4$	100 Mm	1 µl	4 mM
Glycerol	100%	0.75 µl	2%
Betaine	5M	0.5 µl	0.1 M
Template	~100ng	1 µl	4ng
Nuclease free water		To 25 µl	
Total reaction volume		25 µl	

3.5.2 *Bst* polymerase

Bst polymerase is a DNA polymerase obtained from a thermophillic, gram positive rod shaped bacterium *Bacillus stearothermophilus*. Bst polymerase has helicase like activity that unwinds DNA at an optimum temperature between 60 and 65^0C. These features make it useful in loop mediated isothermal amplification. In addition to DNA polymerase activity, *Bst* polymerase exhibits 5'-3' exonuclease activity but lacks 3'-5' exonuclease (proof reading) activity. Large fragment lacks 5'-3- exonuclease activity. It requires four primers to specifically recognize six target regions (Notomi *et al*., 2000). The enzyme is a genetic fusion of *Bst* DNA polymerase large fragment and maltose binding protein (MBP). MBP has an advantage for easy purification of the enzyme by affinity chromatography.

*Bs*t polymerase efficiently displaces the double stranded DNA by its strand displacement activity. High amount of molecular weight DNA is produced within a short period of time and it mainly depends on specificity shown by four primers by targeting six distinct regions. Two loop primers can be added optionally to increase specificity of *Bst* polymerase.

3.5.3 Water bath or heat block

A water bath is laboratory equipment made from a container filled with heated water. Samples are incubated in water at a constant temperature over a long period of time. It comprises a digital or analogue interface for setting a desired temperature by user. Utilisation involves incubation of cell cultures, warming of reagents or melting of substrates. LAMP is performed in water bath as it maintains a single constant temperature. It is cheaper and best in comparison to thermal cycler for performing LAMP reaction.

3.6 Visualisation of LAMP product

3.6.1 Detection by observing turbidity

DNA polymerization reaction mediated by *Bst* polymerase in LAMP is visualized by the release of pyrophosphates from dNTPs as a byproduct (Mori *et al*., 2001). These pyrophosphate ions react with magnesium ions in the reaction buffer, yielding a white precipitate (Abdul-Ghani *et al*., 2012, Tanner and Evans, 2014, Mori *et al*., 2001). The presence or absence of the white precipitate as an end point measurement allows easy distinction of amplified nucleic acid in LAMP reaction.

No instrumental cost is required for measuring the turbidity, furthermore risk of contamination is prevented as tubes are not exposed to open atmosphere. Turbidity formed in the LAMP reaction is stable for a short period of time and monitoring should be performed as soon as possible (Almasi *et al*., 2013). This method of visualizing the turbidity is efficiently required mainly during point of care testing.

3.6.2 Detection by agarose gel electrophoresis

Agarose gel electrophoresis is used for the detection of LAMP products. The amplified DNA are stained with ethidium bromide and visualized on an ultraviolet illumination (265-310). The positive amplification of DNA is visualized by generated amplicons with

different sizes consisting of alternately inverted repeats of target sequence and show a ladder like pattern on agarose gel (Tsai *et al*., 2009).

3.6.3 Visual detection of LAMP product

Visualisation of LAMP products in the presence of DNA binding dyes and calorimetric indicators such as ethidium bromide, hydroxy naphthol blue, SYBR Green 1 and calcein can be monitored in UV lamp. For visualizing amplified DNA 1 µl of SYBR Green 1 is added to the reaction tubes at the end. Positive LAMP amplification will turn orange colour to green in the natural light or UV light. Orange colour remains the same in reaction tubes if the reaction turns to be negative. Amplicons after post amplification gets contaminated while adding SYBR Green 1 in reaction tubes as it requires to be opened.

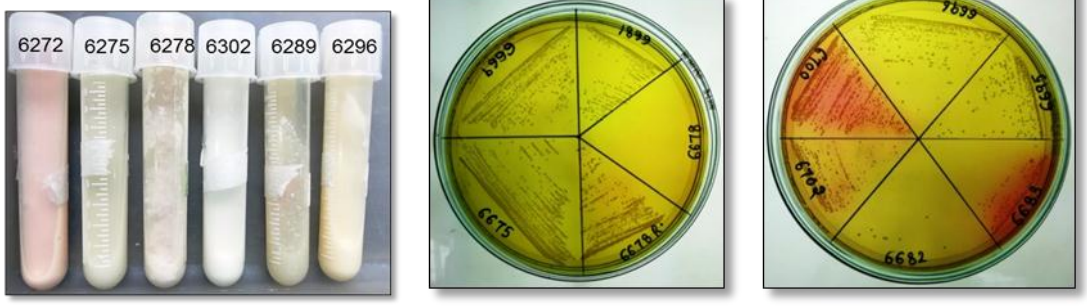
LAMP reaction results can be visualized indirectly with the aid of calcein for a single step assay. Cross contamination is much lower as tubes are not opened after the reaction (Parida *et al*., 2008). Before amplification calcein molecules quench with manganous ion to appear orange in colour. As the reaction is processed in the presence of target DNA, manganous ions get displaced from calcein resulting in change of fluorescence from orange to green determining the amount of DNA amplified. Liang et al determined that LAMP detection limit was more ≥100 copies. Another dye that was used in a similar way was HNB (Hydroxy naphthol blue). During the process of amplification significant amount of insoluble form of magnesium pyrophosphate is produced that reduces the concentration of magnesium ions in solution as reported by Goto *et al*., 2009. The decrease in magnesium ions results in change of color from purple to sky blue color determining the positive amplification of DNA (Wastling *et al*., 2010). In India, M chahar et al., 2019 reported the use of HNB (120 µM) in SNP-LAMP assay for detection of *Plasmodium falciparum.*

CHAPTER –4

Results and Discussion

4. Isolation of *Staphylococcus aureus* from mastitis milk samples

A total of 226 mastitis milk samples were collected from regional centre (LUVAS), Uchani, Karnal for isolation and identification of *Staphylococcus aureus*. Milk samples were collected from infected animals in sterile containers. The samples were stored in 4^0C (sample containers) before processing in the laboratory. The samples were inoculated in nutrient broth for incubation at 37^0C for 4-5 hours for enrichment of pathogenic bacteria. After incubation, a loop full of inoculum from nutrient broth tubes was streaked on MSA plates by following all aseptic standard operating procedures. Plates were kept for incubation at 37^0C. MSA plates were observed with small, circular, golden yellow colonies and dense pink colonies. This colony appearance was characteristic type for *Staphylococcus aureus.*



(A) (B) (C)

Figure 7: (A) Mastitis milk samples collected from infected animals. (B) Growth of *S. aureus* in MSA as round and golden yellow colonies. (C) Pink colonies in MSA determine growth of Coagulase negative species like *S. epidermidis.*

Out of 226 samples tested for detection of *S. aureus* on MSA plates, 194 (85.8%) mastitis samples were found positive for *Staphylococcus aureus* species. After detection of positive isolates of *S. aureus*, they were processed for antimicrobial susceptibility test.

4.1 Antimicrobial susceptibility test (AST)

AST was performed for 194 *S. aureus* isolates positive for culture test. Different class of antibiotics was used for determining the susceptibility and resistance patterns of bacteria. These antibiotics include Amikacin, Ceftazidime, Gentamicin, Ciprofloxacin and Azithromycin. Selection of antibiotics was based on their use in treatment of mastitis

cases.AST was performed for 45 *S. aureus* isolates in which five isolates were resistant to Ceftazidime and Azithromycin, two were resistant to Ciprofloxacin and one isolate showed resistance to gentamicin. In the following table the result of 10 random isolates were presented.

Table 8: List of 10 random *S. aureus* isolates susceptible and resistance to antibiotics

Isolate Numbers	Amikacin (AK)	Ceftazidime (CAZ)	Gentamicin (HLG)	Ciprofloxacin (CIP)	Azithromycin (AZM)
6088-C	-	-	-	+	-
6193-B	-	+	-	+	+
6191-C	-	-	+	-	-
6269-C	-	-	-	-	-
6279-B	-	-	-	-	+
6320-B	-	-	+	-	-
6278-C	-	-	-	-	-
6275-C	-	-	-	-	-
6296-C	-	-	-	-	-
6317-B	-	-	-	-	+

'+' sign represents *S. aureus* isolates resistance to the particular class of antibiotics and '-' sign represents susceptibility of bacteria to antibiotics. B represents as buffalo and C represents as cattle.

Out of all 194 *S. aureus* isolates selected for AST, 38 (20%), 32 (17%), 77 (40%), 97 (50%) & 58 (30%) isolates were resistance to amikacin, ceftazidime, gentamicin, ciprofloxacin & azithromycin respectively .Out of ten random *S. aureus* isolates represented for antimicrobial susceptibility test, 6193-B isolate of *S. aureus* showed resistance to the three classes of antibiotics like Ceftazidime, Ciprofloxacin and Azithromycin. Three isolates (6278-C, 6275-C and 3296-C) did not show any kind of resistance to any class of antibiotics and were highly susceptible to antibiotics. Figure of Two isolates of *S. aureus* (6278 & 6193) susceptible or resistance to antibiotics were shown below. Selected *S. aureus* isolates were then streaked on Mannitol salt agar media and incubated for 4-5 hours at 37^0C. Yellow and round colonies of *S. aureus* from MSA plates were streaked for other microbiological tests which included catalase test, coagulase test, methyl red test and gram staining to confirm *Staphylococcus aureus* positive isolates.

(A) (B)

Figure 8: NAM plates showing antimicrobial susceptibility test for two S. aureus isolates. A) Isolate 6278-C is susceptible to all five classes of antibiotics. B) 6193 isolate shows resistance to ciprofloxacin, ceftazidime and azithromycin.

4.2 Biochemical identification of *S. aureus*

Three biochemical tests were performed for the identification of *S. aureus* isolates. In our study we performed catalase test, coagulase test and Gram staining method for *S. aureus* detection.

4.2.1 Identification of *S. aureus* by catalase test

Catalase enzyme is found in aerobic microorganisms that reduces toxins like hydrogen peroxides. It mediates the breakdown of hydrogen peroxide into water and oxygen.

$$2H_2O_2 \longrightarrow 2H_2O+O_2$$

S.aureus produces lot of virulent factors like toxins and enzymes during oxidative stress. To minimize the level of stress, *S. aureus* uses catalase enzyme to degrade harmful toxins like oxygen metabolites (B Park *et al*., 2008). When Catalase test was performed for all 194 isolates by adding hydrogen peroxide (H_2O_2) on the selected yellow colonies, formation of bubbles was observed due to the breakdown of hydrogen peroxide into water and oxygen. Catalase test is used to differentiate between *Staphylococci* and *Streptococci*.

All 194 isolates showed positive results for catalase test. Figure 9 shows the formation of bubble by the action of Hydrogen peroxide on yellow colonies.

4.2.2 Identification of *S. aureus* by coagulase test

All 194 isolates positive for catalase test were tested for coagulase test to differentiate Coagulase positive *S. aureus* from Coagulase negative *S. aureus* isolates. The production of coagulase enzyme by coagulase positive *S. aureus* correlates with high pathogenicity and CONS are determined as minor pathogens (Nikolina Rusenova and Anton Rusenova., 2016). Coagulase is an enzyme found in *S. aureus* that converts soluble fibrinogen to insoluble fibrin in plasma. Catalase positive isolates were inoculated in nutrient broth and kept for overnight incubation at 37^0C. After incubation the cells were pellet out by centrifugation and the supernatant was discarded. Freshly prepared PBS was added to the plasma followed with human plasma and was checked for clot formation at different intervals. 45 isolates were found positive for coagulase producing *S. aureus.* Figure given below shows the formation of clots in tubes formed by the action of coagulase on plasma.

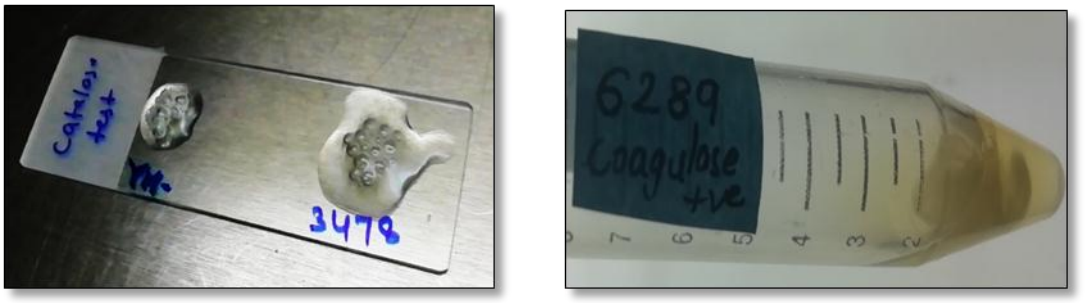


Figure 9: *S. aureus* isolates showing positive for catalase test and coagulase test

4.2.3 Identification of *S. aureus* by Gram staining

All isolates shown positive for catalase & coagulase test were subjected to Gram staining method. *E.coli* culture (XL-1 Blue MRF') was used as representative sample for Gram negative organism and stained with primary stain (crystal violet) on glass slide followed by trapping agent (iodine) and rinsed out under tap water. Decolorizing agent and counter stain (safranin) were added and rinsed out under tap water and kept for air drying. Results were visualized under 100X compound microscope. Purple colonies with grape like clusters and round shape determined the presence of *S. aureus* and pink colonies with rod shape determined the presence of *E. coli* as shown in the figure given below.

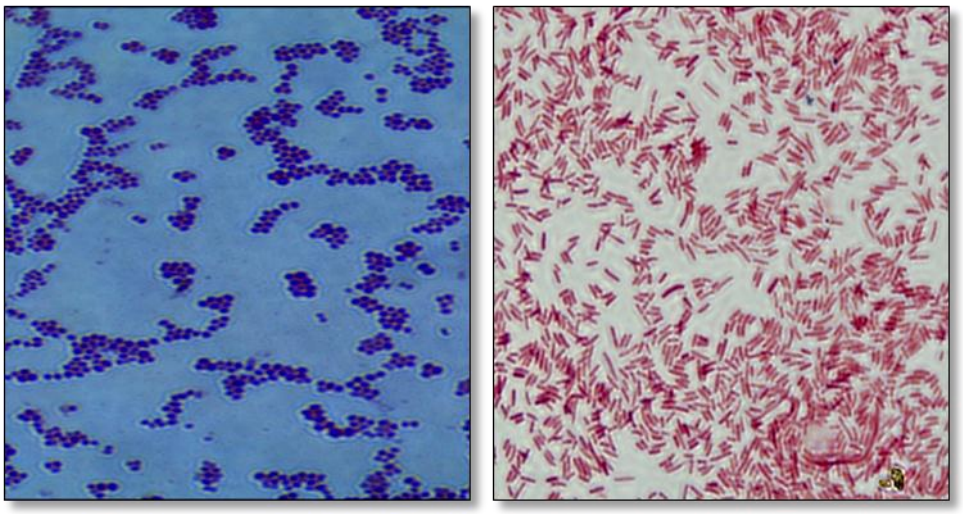


Figure 10: Microscopic view of *S. aureus* and *E. coli* under microscope (100X) after Gram staining. A) Purple colonies with grape like clusters determined S. aureus. B) Pink colonies with rod shaped determined *E. coli*.

194 *S. aureus* isolates that were determined as gram positive by staining method were cultured for 4-5 hours at 37^0C in 4 ml nutrient broth. After incubation the cultured isolates of *S. aureus* were preserved with 50% glycerol and stored in cryo vials at -80^0C for future use. Annette E. LaBauve and Matthew J. Wargo reported the use of 20% glycerol for the preservation of *P. aeruginosa*. Gram staining is a rapid and simple test to characterize organisms morphologically. Gram positive organisms retain purple colour due to the presence of peptidoglycan layer.

4.3 Molecular detection of *S. aureus* by PCR

One hundred isolates preserved in glycerol stock were randomly chosen from 194 S. aureus isolates and were revived by streaking them in MSA and grown for 7-8 hours incubation at 37^0C. Selective enrichment of *S. aureus* prevents the growth of other possible bacteria in Mannitol salt agar media, the colonies in MSA agar appeared as golden yellow in colour. Genomic DNA from these isolates was extracted by using tandem ampicillin-lysozyme, standard phenol-chloroform method (De *et al*., 2010). Concentration, optical density and quality of DNA was checked by measuring the absorbance at 260nm (A_{260}) and 280nm (A_{280}) in a spectrophotometer (Nanodrop, Thermo Fisher scientific inc. USA). Concentration of DNA obtained from spectrophotometer was within a range from 20 ng/µl-500 ng/µl. The difference in amount of DNA is due to simultaneous handling of large number of isolates obtained

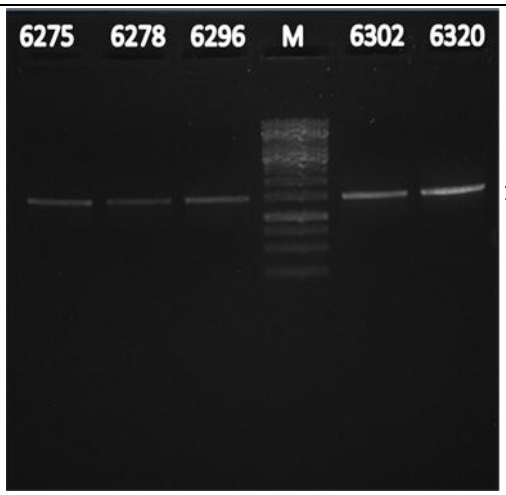
from different clinical samples, differential growth of individual bacteria and different stages of growth cycle and differential lysis of bacterial cells. For PCR reaction all isolated DNA was diluted to 100 ng/µl DNA. This was chosen as standard concentration for PCR amplification. Spectrophotometer reading of 10 random *S. aureus* isolates in terms of quantity and quality was shown in the figure below. Quality was necessary to check for any contamination that would interrupt in PCR reactions.

Table 9: List of 10 random isolates for determining quality and quantity of DNA

Isolate No	Concentration of DNA (ng/µl)	A_{260}	A_{280}	A_{260}/A_{280}	Amount of DNA(ng/5ml)
6272	426.99	10.189	5.177	1.87	21349.5
6275	317.94	6.359	3.100	1.87	15897
6278	287.23	5.745	2.850	1.85	14361.5
6289	359.50	7.190	3.518	1.88	17975
6302	241.12	4.822	2.397	1.86	12056
6320	265.28	5.306	2.661	1.88	13264
3756	186.71	3.734	1.799	1.87	9335.5
6296	272.11	5.422	2.699	1.87	13605.5
6783	133.31	2.666	1.291	1.86	6665.5
6827	24.04	0.481	0.348	1.85	1202

4.4. 16S rRNA specific PCR for *S. aureus* detection

The 16S rRNA gene is amplified for the identification of then isolated bacteria. The identities of organisms belonging to any genus could be assigned by sequencing the 16S rRNA gene region. A total of 100 *S. aureus* DNA isolates were being selected for detection of 16S rRNA gene. The amplicon size of the PCR product was 1.5 KB when visualized on 2% agarose gel and were positive for all 100 DNA isolates selected for PCR as shown in the figure given below. The purified 16S rRNA gene product was sent for capillary sequencing to confirm their identity as *Staphylococcus spp*. It is the gold standard method for identification of any unknown bacteria.



Buffer	**: 2.5 µl 1x Taq buffer**
dNTPs	**: 0.2mM each**
Fw primer	**: 0.2 µM each**
Rv primer	**: 0.2 µM each**
***Taq* pol**	**: 0.05U/µl**
DNA	**: 100 ng**
dH$_2$O	**: to 25 µl**

Figure 11: 16S rRNA based amplification of *S. aureus* purified DNA isolates when visualized in 2% agarose gel showed amplicon size at 1.5 kb

4.4.1 PCR amplification of *S. aureus* virulence and antimicrobial resistant genes

Staphylococcus aureus is considered as an opportunistic pathogen. *S. aureus* infections are highly prevalent among infants, elderly and immunocompromised patients (Foster *et al*., 2014). David and Daum, 2010 determined that *S. aureus* transmits from animals to humans. The pathogenicity of *S. aureus* depends on the presence of pathogenicity islands and antimicrobial genes (Dongli Rong et al., 2017). Virulence factors related to pathogenicity includes fibronectin-binding protein A and B, *S. aureus* enterotoxins (*SEs*), Panton-Valentine leukocidin (*PVL*) and hesmolyin g (*hlg*) (Puha *et al*., 2016). Antimicrobial resistant genes present in *S. aureus* are also responsible for increasing their pathogenicity towards host organism. The major class of antimicrobial resistance gene involved in providing resistance to antibiotics was *Mec* A gene. *Mec* A gene is found genetically in MRSA strains having a mutated version of penicillin binding protein 2a that provides resistance to methicillin.

PCR was performed for detection of *S. aureus* targeting *Coa* gene, *Mec* A gene, *Hlg* and *PVS* gene. In this PCR assay, the desired size of 173 bp, 232bp, 296bp and 126 bp was observed respectively for four genes when the PCR mixture contained 100ng/µl template DNA, 1X PCR buffer with 15mM $MgCl_2$, 10pM of each forward and reverse primer, 200 µM dNTPs and 0.5U of *Taq* DNA polymerase in a 25 µl volume of reaction mixture.

The cyclic conditions were standardized as follows: five minutes of initial denaturation at 95^0C, followed by 30 cycles of amplification and each cycle consist of denaturation

for 95^0C/15sec, annealing at 55^0C/15sec, elongation at 72^0C/15sec and final elongation for 72^0C/5min.

In the present study 100 catalase positive *S. aureus* from different sources were screened for the presence of following virulence genes of which 46(46%) isolates were *Coa* gene positive, 62(62%) and 18(18%) isolates were positive for *Hlg* and *PVS* gene respectively. The highest prevalence of *Hlg* (62%) gene was found among the rest of genes in 100 purified DNA isolates. Ten purified DNA isolates positive for *S. aureus* virulence genes were represented in the table given below.

Table 10: List of 10 purified DNA isolates positive for virulence genes.

DNA ISOLATES	***COA* GENE**	***HLG* GENE**	***PVS* GENE**
6272	-	+	-
6275	+	+	+
6278	+	+	+
6289	+	+	+
6302	+	+	+
6320	-	-	-
3756	+	+	+
6296	-	+	-
6208	+	+	+
6243	+	+	+

'+' & '-'sign represents the presence and absence of virulence genes in the respective DNA isolates.

Amplification of *Coa* gene has been an accurate method for *S. aureus* detection. It has a polymorphic region repeated that can be used for differentiating purified isolates of *S. aureus* (AA Gharib *et al.*, 2013). *Coa* gene is responsible for the formation of biofilms in *S. aureus* (Marta Zapotoczna et al., 2015). *S. aureus* isolates positive for coagulase test (45) gave similar result to PCR amplification of *Coa* gene. Heamolysin gene (*hlg*) is a pore forming toxin gene, creating pores on cytoplasmic membrane of erythrocytes,

leukocytes and platelets. Heamolysin gene in *S. aureus* secrets alpha monomers and produces a ring shaped complex. Resistance to antibiotics is provided by antimicrobial genes present in *S. aureus*. *Mec* A gene is an antimicrobial resistant gene provides resistance to β-lactam antibiotics. Most common carrier of *Mec* A gene in *S. aureus* is MRSA. It provides resistance to antibiotics due to its low affinity to penicillin and methicillin and enabling transpeptidase activity for bacterial wall synthesis (FJ Chenet al., 2014). Role of *Mec* A gene in oxacillin resistance was provided by the presence of penicillin binding protein 2a (DC Oliveria *et al*., 2011). Out of 100 catalase positive purified S. aureus isolates, 54 (54%) were found positive for *Mec* A gene by polymerase chain amplification. List of ten S. aureus isolates positive for *Mec* A gene were listed below.

Table 11: List of *S. aureus* isolates positive for *Mec* A gene

DNA ISOLATES	*Mec* A gene
6272	+
6275	+
6278	+
6289	+
6302	+
6320	+
3756	+
6296	+
6208	+
6243	+

⇨ '+' sign represents the presence of *Mec* A gene in the respective DNA isolates

Figures of PCR amplified products on 2% agarose gel were visualized for the presence of *S. aureus* virulence and antimicrobial genes were shown below.

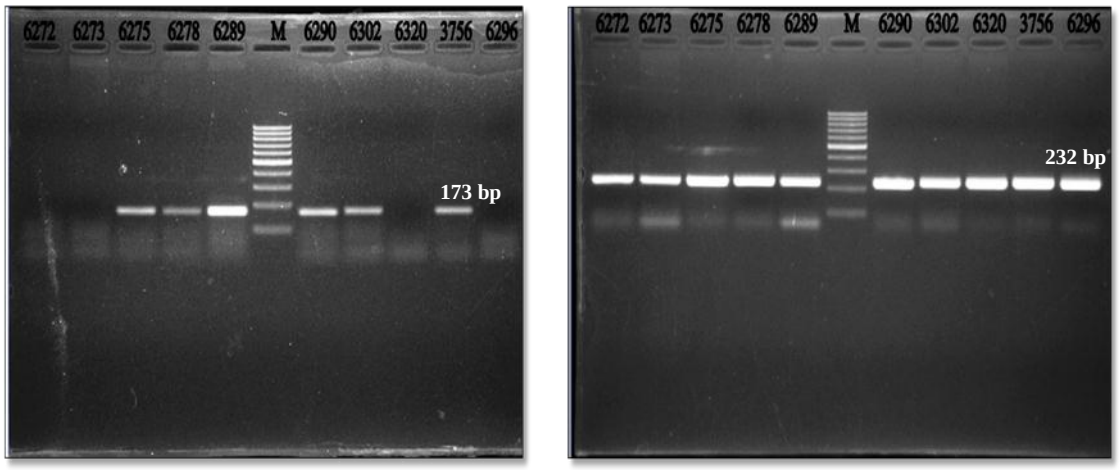


Figure 12: Purified DNA isolates positive for *Coa* and *Mec* A gene showed amplicon size at 173 and 232bp with respect to 100bp DNA ladder.

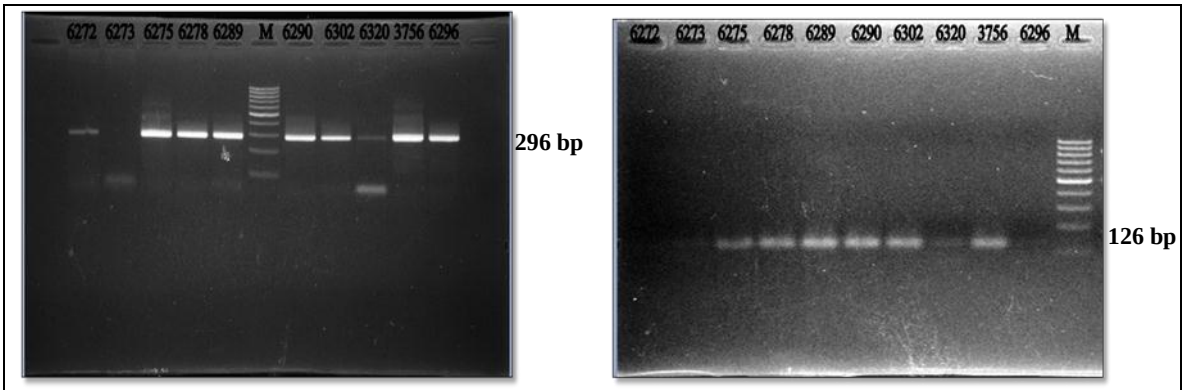


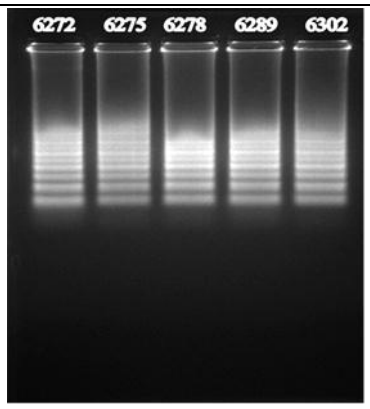
Figure 13: Purified DNA isolates positive for *Hlg* and *PVS* gene showed amplicon size at 296 bp and 126 bp with respect to 100bp DNA ladder

4.5 Isothermal amplification for *Mec* A gene positive DNA isolates

Isothermal amplification enables specific and accurate amplification of DNA at constant temperature (60-65^{0}C) (L M Zanoli *et al*., 2013). *Bst* polymerase is used in isothermal amplification with high strand displacement activity about 10^9 amplification factors comparable to PCR with 30 cycles. Isothermal amplification is resistant to inhibitors (blood, plant tissues) in complex samples that allow the detection of desired gene from minimally processed samples. Combination of isothermal amplification with portable devices or Microsystems improves nucleic acid based assays (Yongxi Zhao *et al*., 2015). Isothermal methods evaluated were helicase dependent amplification (HDA), rolling circle amplification (RCA), nucleic acid sequence based amplification (NASBA),

recombinase polymerase amplification (RPA) and loop mediated isothermal amplification (LAMP). Loop mediated isothermal amplification has been developed for its high specificity and simplicity in amplification of DNA isolates at a constant temperature of 65^0C (Notomi *et al*., 2015). LAMP provides high specificity due to its provision of four primers binding six distinct regions of the target gene. In case of RNA amplification RT-LAMP is used with the use of enzyme AMV reverse transcriptase. *Mec* A gene was selected in our study for the detection of methicillin resistant *Staphylococcus aureus*. It provides resistance to methicillin due to its low affinity PBP2a (S. Sudhaharan *et al*., 2015). DNA isolates positive for *Mec* A gene in PCR was selected for isothermal amplification with LAMP.

In order to use the initial LAMP process for identification of *Mec* A gene specific DNA isolates, the standard LAMP protocol (New England BioLab) was performed at 65^0C for an hour and results were visualized by agarose gel electrophoresis. Results were visualized with the appearance of six to 10bands targeting six distinct regions of template. The concentration of reagents and template DNA used in this experiment was more, so there was a need in optimization of both for better isothermal amplification



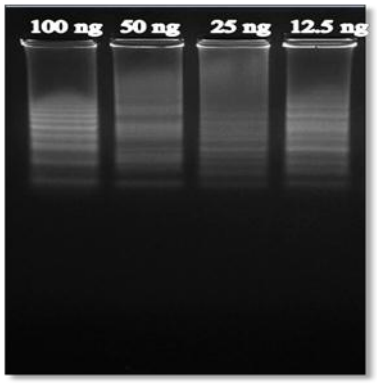
Buffer	**: 2.5 µl 1x(contains 2mM $MgSO_4$**
dNTPs	**: 1mM each**
FIP/BIP	**: 0.4 µM each**
F3/B3	**: 0.2 µM each**
DNA	**: (200ng)**
***Bst* pol**	**: 8U/25 µl**
dH_2O	**: to 25 µl**

Figure 14: LAMP product in (2%) agarose gel with different DNA isolates performed for LAMP assay with higher concentrations of reagents and without negative control.

4.5.1 Optimization of Template DNA

Experiment was setup to optimize the concentration of DNA isolates per 1µl of template used for LAMP assay. Different concentrations (100ng, 50ng, 25ng and 12.5ng) of a single isolate of *S. aureus* DNA were mixed to the reaction mixture comprising 8U/µl of DNA polymerase and 1mM of dNTPs. The reaction was made up to 25 µl reaction volume and performed at 65^0C for an hour. The results were visualized in 2% agarose gel using Molecular Imager Gel Doc. DNA isolate about 100ng/µl gave better

amplification in comparison to other concentrations. Ravindran *et al*., determined that concentration of about 0.01 ng-100 ng template gave better amplification in lamp assay. He used 50 ng/µl as the standard amount for amplification of loop mediated isothermal DNA.



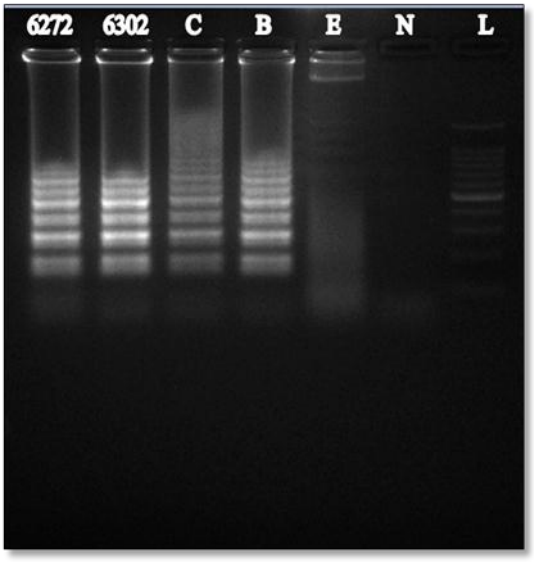
Buffer	**: 2.5 µl 1x(contains 2mM $MgSO_4$**
dNTPs	**: 1mM each**
FIP/BIP	**: 0.4 µM each**
F3/B3	**: 0.2 µM each**
DNA	**: (100ng, 50ng, 25ng, 12.5ng)**
dH_2O	**: to 25 µl**

(Bst DNA polym.erase with 8U/25 µl of reaction)

Figure 15: Agarose (2%) gel with different concentrations of template DNA of a single isolate tested for optimization in LAMP. 100ng template DNA of LAMP product gave better

4.5.2 LAMP assay with control

LAMP is an error prone amplification technique that gives false positive results , it needs to be performed along with proper control. LAMP assay for *Mec* A gene *S. aureus* DNA isolates was performed along with DNA of cattle, buffalo and E.coli (100ng/µl each). Negative control without template DNA was also used for this experiment. The amplification was observed for test DNA isolates along with non specific amplification of cattle, buffalo and *E.coli* DNA. No amplification was observed for negative control in 2% agarose gel when visualized in transilluminator. Non specific amplification should be prevented by optimizing concentration of *Bst* polymerase 3.0 enzyme and LAMP additives like $MgSO_4$, glycerol and betaine. Betaine reduces the formation of secondary structures in GC rich regions (Henke W *et al*., 1997). $MgSO_4$ increases annealing of primers and DNA polymerase activity (Hung Yeh *et al*., 2005) and glycerol inhibits non specific amplification of purified DNA isolates. The figure shown below represents two S. aureus DNA isolates (6272 and 6302) showing amplification in 8U/25 µl of reaction.



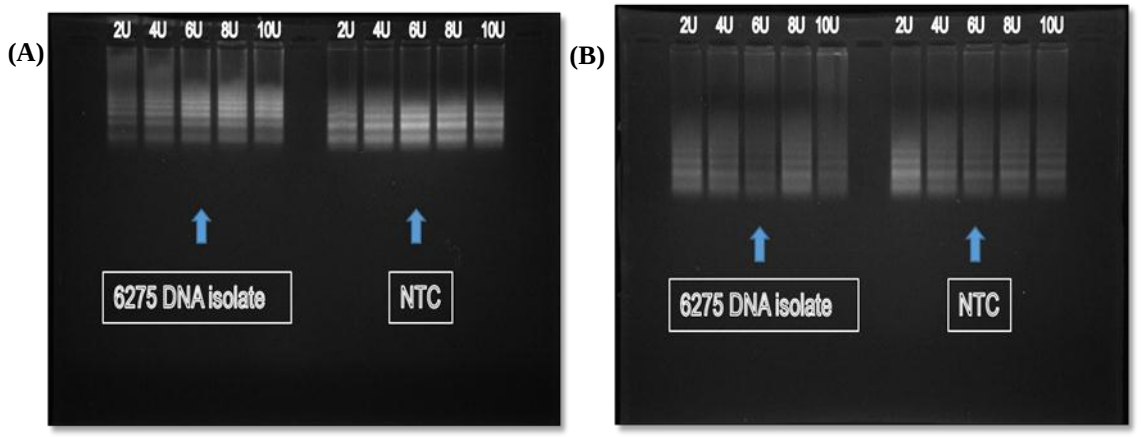
Buffer : 1X contains 2mM $MgSO_4$
dNTPs : 1mM
FIP/BIP : 0.4 µm each
F3/B3 : 0.2 µm each
***Bst* pol : 8U/25 µl**
DNA : 100ng
dH_2O : to 25 µl

(LAMP assay with negative control)

Figure 16: Agarose 2% gel with non specific amplification observed for cattle, buffalo and E.coli DNA at 100ng per 25 µl reaction. No amplification was observed in case of non template.

4.5.3 Enzyme titration for LAMP assay

Bst polymerase enzyme used in LAMP assay was optimized to desired concentration for better amplification of DNA. A single isolate (6275) of *S. aureus* DNA was used as template to optimize the amount of enzyme concentration. Enzyme in higher amounts leads to non specific amplification in negative control and shows false positive results for LAMP products. About (100ng/µl) amount of template DNA was used with graded concentrations of enzymes (2U, 4U, 6U, 8U and 10 U) per reaction mixture and the experiment was performed at 65^0C for 1 hour. Results were obtained better with 2U/µl of *Bst* polymerase 3.0 as shown in figure. Notomi *et al*., reported the use of 8U of *Bst* polymerase and 4mM of $MgSO_4$ for better amplification of DNA in his experiment at 63^0C for 1hour. 8U of Bst polymerase provides better amplification of DNA isolates reported by Arvind *et al*., Yanyong Cao et al., also reported the use of 8U *Bst* polymerase gave better amplification for purified DNA isolate in real time fluorescence. 3.2U of *Bst* polymerase 2.0 was used for loop mediated isothermal amplification of Staphylococcus aureus that gave better amplification results (Xiaolan Tian et al., 2018). Initially 2mM $MgSO_4$ was added for the above experiment and gave the same amplification which was obtained before without adding $MgSO_4$ as shown in the figure. Thus the amount of $MgSO_4$ required for LAMP assay should also be optimized for better DNA amplification.



(Different concentrations of *Bst* polymerase without $MgSO_4$)

(Different concentrations of *Bst* polymerase with (1 mM) $MgSO_4$)

Buffer : 1X contains 2mM $MgSO_4$

dNTPs : 1mM

FIP/BIP : 0.4 µm each

F3/B3 : 0.2 µm each

DNA : 100ng

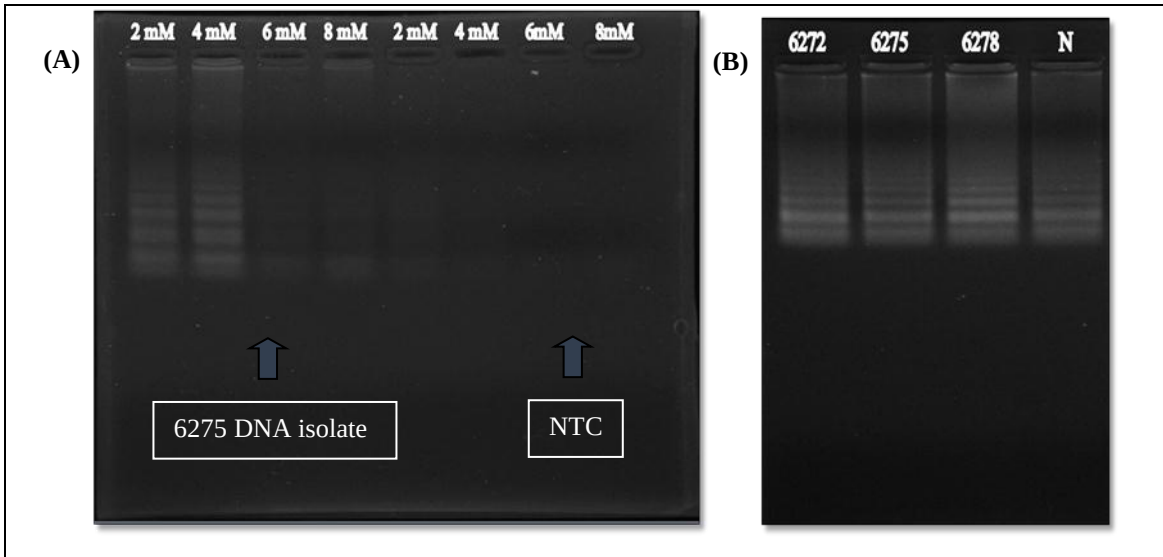
Figure 17: Agarose (2%) gel with different concentrations of *Bst* polymerase were optimized with single DNA isolate (6275) and Non template control (NTC). (A) Optimization of enzyme performed without $MgSO_4$. (B) Optimization of enzyme performed with 1mM $MgSO_4$

4.5.4 Optimization for $MgSO_4$

For specific amplification of DNA isolate by LAMP, non specific amplification of DNA should be minimized by optimizing the amount of Mg^{2+}. Magnesium amount is considered as one of the important factors that can affect the specificity as well as efficiency of the DNA polymerase activity. Higher amount of $MgSO_4$ concentration leads to enhanced activity of *Bst* polymerase by minimizing the non specific amplification, but it has a chance in reducing the DNA fidelity (higher amounts leads to no DNA amplification even in the presence of targeted gene). Low amount of $MgSO_4$ is preferred to reduce this error but may effect in polymerase activity. So there is a need to optimize the concentration of $MgSO_4$ to balance between fidelity and activity of enzyme. Zhou *et al* and Keizerweerd *et al* determined 4mM as optimum concentration for good amplification in sugarcane. Reza Ranjbar and Davoud Afshar, 2015 determined 4mM of $MgSO_4$ provided better amplification in *Yersinia enterocolitica*. Therefore it was important for optimizing $MgSO_4$ concentration for reducing non specific amplification.

Single bacterial isolate (6275) of 100ng was treated with different concentrations of $MgSO_4$ (4mM, 6mM, 7mM and 8mM) in 25 µl reaction mixture and LAMP assay was performed. Non template control was used in this assay. Products were visualized in 2% agarose gel in Gel Doc XR, it was found that 4mM concentration of $MgSO_4$ provided better amplification. Increase in $MgSO_4$ concentration also provided amplification of template but its fidelity has been reduced as shown in the figure given below. Deguo Wang performed real time LAMP detection of *Staphylococcus aureus* with 6mM $MgSO_4$ as optimum concentration for better amplification of *S. aureus* isolates. In LAMP $MgSO_4$ increases the annealing capacity of primers with target gene and prevents non specific amplification (Hung Yeh *et al*., 2005).

When this experiment setup was used for different *S. aureus* DNA isolates with a negative control with no DNA template by adding 4mM $MgSO_4$ and visualized in agarose gel. Results showed the non specific amplification in negative control amplified at 65^0C for 1 hour as shown in the figure below



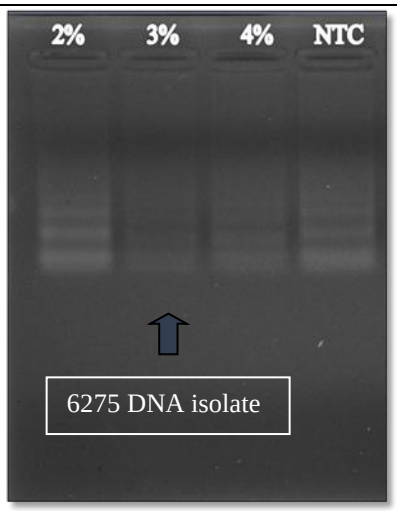
(**Optimization of $MgSO_4$ at different concentrations)** **(4mM concentration of $MgSO_4$/25 µl)**

Buffer : 1X contains 2mM $MgSO_4$
dNTPs : 1mM
FIP/BIP : 0.4 µm each
F3/B3 : 0.2 µm each
***Bst* pol : 2U/25 µl**
DNA : 100ng
dH_2O : to 25 µl

Figure 18: (A) Agarose (2%) gel with different concentrations of $MgSO_4$ were optimized with single DNA isolate (6275) & non template control.(B) Non specific amplification was seen in non template with 4mM.

4.5.5 Optimization of additive glycerol

Non specific amplification in LAMP assay was minimized with the addition of glycerol as an additive. Glycerol helps in enhancing the activity of DNA polymerase activity by preventing the formation of secondary structures in LAMP assay. 0.87 µM of glycerol was used for enhancing activity of *Bst* polymerase by Sayli S Modak *et al*., 2016 for the detection of malarial parasite using LAMP assay. In PCR addition of glycerol (15-25%) lowers the temperature for strand separation by increasing polymerase thermal stability as given by *Sigma-Aldrich* as PCR reagent. To check the amount of glycerol that could minimize the errors of non specific amplification, experiment was setup again with the same phenomenon of selecting a random single bacterial isolate that showed positive result for *Mec* A gene in PCR. Three different concentrations of glycerol (1%, 2% and 3%) were added to the purified DNA isolates (100ng) and non templates for a total of six reactions respectively. Reaction mixture that contained 3% of glycerol provided better amplification of template DNA. Glycerol acts as enhancer of Bst polymerase for better amplification at lower temperatures. Reaction mixture with non template as negative control with 3% glycerol did not show any kind of amplification, when visualized in 2% agarose gel after amplification at 65^0C for an hour as given in the figure below.



Buffer : 1X contains 2mM $MgSO_4$

dNTPs : 1mM

FIP/BIP : 0.4 µm each

F3/B3 : 0.2 µm each

***Bst* pol : 2U/25 µl**

DNA : 100ng

$MgSO_4$: 4mM

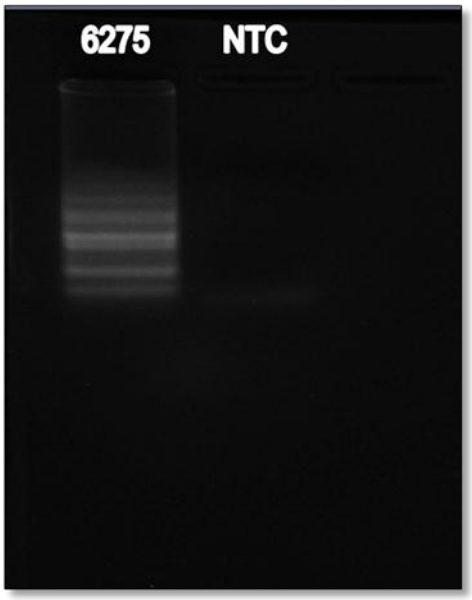
dH_2O : to 25 µl

(Optimization of glycerol at different concentrations)

Figure 19: Agarose 2% gel with different concentrations (2%, 3% and 4%) of glycerol was added to reaction mixture with 100ng of template DNA. NTC (non template control) was used and resulted in non specific amplification at 65^0C for 1 hour.

4.5.6 Optimization of LAMP with betaine

Betaine is an additive of PCR, improves amplification of DNA by reducing the formation of secondary structure in GC rich regions. It enhances the activity of polymerases by eliminating the base pair composition dependence of DNA melting as reported by Henke *et al*., "Betaine improves the PCR amplification of GC rich DNA sequence". Increase in concentration of betaine increased the LAMP products at 0.8M (Hung Yueh Yeh *et al*., 2005). Experiment was setup with a reaction mixture containing 2U of *Bst* polymerase, 1X Thermopol buffer, 1 mM concentration of dNTPs, 0.2 µm and 0.4µm of inner and outward primers, 4mM $MgSO_4$, 3%glycerol, 0.1 molar of 5M betaine and 100ng template DNA of a single bacterial isolate (6275), the total reaction volume was make up to 25 µl. Same reagents were added in negative control with no template DNA and LAMP assay was performed at 65^0C for 1 hour. The result obtained from gel electrophoresis showed no amplification in negative control followed with a clear amplification band pattern for template DNA. 0.1 molar of betaine proved to be efficient additive for DNA amplification as shown in the figure below.



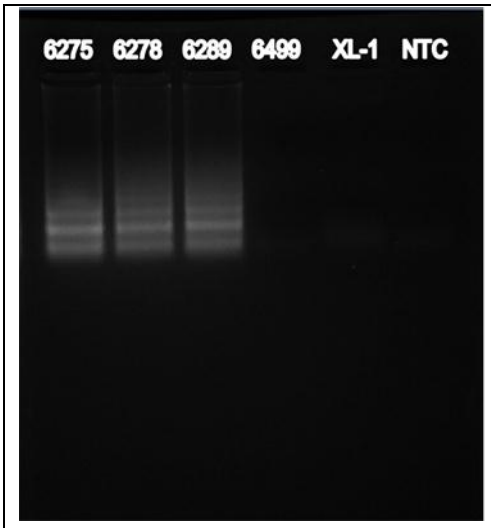
Buffer : 1X contains 2mM $MgSO_4$
dNTPs : 1mM
FIP/BIP : 0.4 µm each
F3/B3 : 0.2 µm each
***Bst* pol : 2U/25 µl**
DNA : 100ng
$MgSO_4$: 4mM
Glycerol : 3%
Betaine : 0.1 molar of 5M
dH_2O : to 25 µl

(Lamp optimization with 0.1 molar additive Betaine)

Figure 20: Agarose (2%) gel with 0.1 molar of 5M betaine was added to the raction mix containing 0.4mM $MgSO_4$ and 3% glycerol. Purified DNA isolate (6275) was amplified along with non template DNA. No amplification was observed in NTC after LAMP performed at 65^0C for 1hr

4.7 Specificity of LAMP assay

LAMP assay has also been used for the detection of specific species. Its high specific nature differentiated Enterococci species from non bacterial strains for the presence of 23S rRNA as reported by Roland Martzy *et al*., LAMP detection for specific species was also being implemented by the addition of loop primers for higher specificity as described by A. Yoshida in species P. gingivalis and T. forsythia in 2015. S. aureus enterotoxins genes were specifically determined by (M Goto *et al*., 2007). LAMP assay was also been tested for its specificity between *Mec* A gene positive DNA isolate and a non *Mec* A DNA isolate. Experiment was performed for 100ng of three different purified isolates of DNA positive for *Mec* A gene and a non *Mec* A gene DNA isolate (6499). XL-1 Blue (100ng) and Non template control were used as negative control for this experiment and performed at 65^0C for 1 hour. Results were viewed in 2% agarose gel in Molecular Gel Doc. Non specific amplification was clearly absent in the case of *Mec* A gene negative isolate followed with clear visualisation of bands for the three *Mec* A gene positive isolate. XL-1 Blue strain and non template control did not show any kind of amplification determining the LAMP specificity. 0.1 molar betaine followed with 4 mM $MgSO_4$ and 3% glycerol gave better amplification results as shown in the figure given below.



Buffer : 1X contains 2mM $MgSO_4$
dNTPs : 1mM
FIP/BIP : 0.4 µm each
F3/B3 : 0.2 µm each
***Bst* pol : 2U/25 µl**
DNA : 100ng
$MgSO_4$: 4mM
Glycerol : 3%
Betaine : 0.1 molar of 5M
dH_2O : to 25 µl

Figure 21: Agarose (2%) gel showed positive amplification for species specific *Mec A* gene performed at 65^0C for 1 hour. No amplification was observed in *Mec* A gene negative isolate (6499) and Xl-1 Blue followed with NTC.

4.8 Detection of amplified DNA by conventional point of care device

Point of care is a diagnostic device used to obtain results close to patient and provides quick feedback during outbreaks. Visualisation of LAMP products is simply detected with the release of pyrophosphate ions resulting in turbidity (Abdul Ghani et al., 2012; Tanner and Evans, 2014).

4.8.1 Types of detection

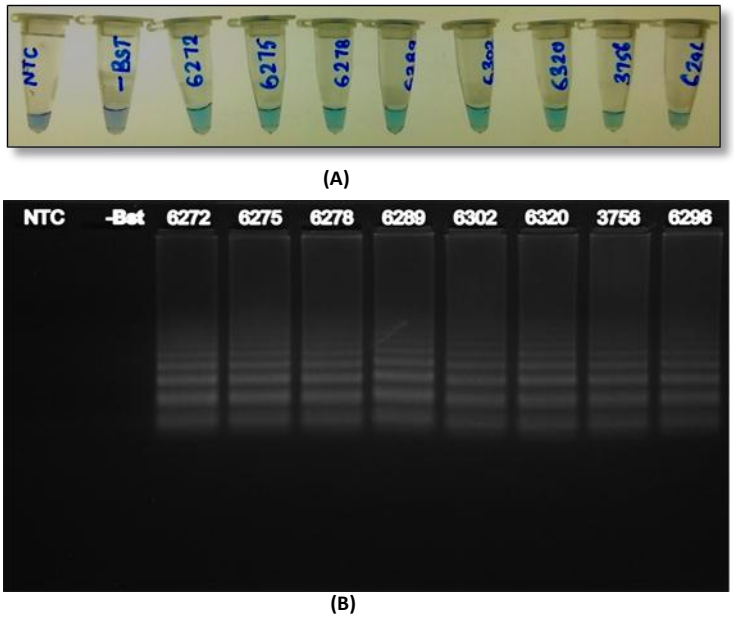
4.8.1.1 UV transilluminator based detection of LAMP product

SYBR Green 1 is the most common dye used for detection of color change from orange to green when the tube contains positive isolates of DNA (Zhang *et al*., 2012). Color change of amplified LAMP product was determined under UV light by Le *et al*., 2012. The use of SYBR Green 1 as a visualizing dye enhances sensitivity in comparison to normal visualisation with turbidity (Soli *et al*., 2013).

4.8.1.2 Colorimetric based detection of LAMP products

Calcein is a good calorimetric indicator for detection of amplified DNA. Before amplification calcein appears orange color in tube containing reaction mixture. Orange color is change to green with enhanced sensitivity have been observed after amplification and visualized with naked eye (le Roux et al., 2009).

Hydroxy Naphthol blue was used a new colorimetric detection of LAMP amplified products of purified DNA isolates in our study. LAMP products were visualized by the addition of 100 µm of diluted HNB solution from the stock (20 mM) before amplification was performed. Visualisation of LAMP products was carried out in single tube for each purified DNA isolates (Ten in number). Negative controls without Bst polymerase and template DNA were even used for LAMP detection visually. After amplification, LAMP products with template DNA showed color change from violet to sky blue, whereas in negative controls the violet color remained the same due to absence of *Bst* polymerase and template DNA when performed at 65^0C for 1 hour. The reduction in color change from purple to blue is due to change in concentration of Mg^{2+} (Goto *et al*., 2009, Wasting *et al*., 2010) .To check their amplification, samples were loaded in 2% agarose gel and were visualized in Gel Doc. Ten isolates of DNA that showed sky blue color showed amplification bands but it was clearly absent in negative controls as shown in figure below.



Buffer : 1X contains 2mM $MgSO_4$
dNTPs : 1mM
FIP/BIP : 0.4 µm each
F3/B3 : 0.2 µm each
***Bst* pol : 2U/25 µl**
DNA : 100ng
$MgSO_4$: 4mM
Glycerol : 3%
Betaine : 0.1 molar of 5M
dH_2O : to 25 µl

Figure 22: (A) Detection of ten LAMP products positive for Mec A gene was visualized using 100µM Hydroxy Naphthol Blue at 65^0C for 1 hour. (B) Agarose gel (2%) showed positive amplification for LAMP products that were visualized as positive isolates by HNB. Negative controls like NTC and without Bst tube showed no visualisation with HNB and in agarose gel electrophoresis.

Gel based method for visualisation of LAMP amplicons directly is a conventional method and also considered as a gold standard method of DNA visualisation (Abdul Ghani et al., 2012; Tanner and Evans, 2014). After staining with fluorescent labeled dyes, positive LAMP products produces different sizes in a ladder like pattern (Le *et al*., 2012). Chahar *et al*., 2019 determined the use of HNB (120 µM) as an optimized concentration for the detection of LAMP product by visualisation.

CHAPTER –5

Summary and Conclusions

SUMMARY AND CONCLUSIONS

In the past decade use of culture based methods to determine morphological characteristics of deadliest pathogens has been a time taking process. It has limitations in terms of bacterial growth phase, different antibiotics interfere with bacterial growth and growth of different microorganisms. Such limitations were improvised with the use of conventional methods like Polymerase chain reaction. PCR based studies are widely used by clinicians in the fields of disease diagnosis, cloning of genes followed with sequencing and for wide genomic studies. 16S rRNA PCR proves to be an efficient gold standard method for the detection of bacteria, but it cannot determine the drug resistant gene responsible for causing infections. To overcome this situation PCR was performed for virulence and antimicrobial genes specific for *Staphylococcus aureus.* Results proved to be better in contrast to culture and 16S rRNA based identification of bacteria. Though use of PCR has been proved as a valuable approach it has limitations in terms of sensitivity. Contamination of samples in tracer amounts could produce misleading results. In addition primers used in PCR anneals non specifically to the similar sequences but not to identical template DNA. So to improvise in terms of specificity and sensitivity in detection of deadliest pathogens we require a less versatile technique. Isothermal amplification methods prove to be efficient in detection of multidrug resistant *Staphylococcus species*. Many nucleic acid based isothermal amplification have been developed from which LAMP was used in our area of study. Loop mediated isothermal amplification performs amplification of desired DNA at 65^0C. *Bst 3.0* polymerase was used for isothermal amplification of *S. aureus* DNA by using strand displacement activity. Results showed non specific amplification in non template solution used as negative control. To minimize such non specific amplification optimization was done for enzyme concentration (2U/25µl) and template DNA (~100 ng) but it resulted in non specific amplification (nuclease free water was freshly used for each experiment performed). Additives for PCR like $MgSO_4$ (4mM), glycerol (3%) & betaine (0.1 M) were added to the LAMP reaction mixture and amplification process was performed for 1 hour at 65^0C. Results visualized in 2% agarose gel showed no non specific amplification in negative control (NTC). Specificity of LAMP assay was checked by performing LAMP process with a DNA sample negative for *Mec* A gene and results were visualized under UV transilluminator with no further amplification. Detection of

LAMP amplification was visualized by the use of point of care device component (HNB). Concentration of about 100 µm HNB was used to visualize the change in color from purple to sky blue if amplification of DNA was in positive sample.

The results concluded that;

1. Out of 226 mastitis milk samples collected in total, 194 isolates were positive for *Staphylococcus aureus.*
2. Out of 194 isolates selected for AST 38 (20%), 32 (17%), 77 (40%), 97 (50%) & 58 (30%) *S. aureus* isolates were resistance to amikacin, ceftazidime, gentamicin, ciprofloxacin & azithromycin.
3. All 194 isolates gave positive results for biochemical identification by catalase test and gram staining. 45 *S. aureus* isolates were positive for coagulase gene.
4. 100 *S. aureus* isolates selected from total 194 gave positive results with 16S rRNA PCR and three samples were pooled & send for sequencing.
5. Out of 100 *S. aureus* isolates screened for the presence of virulence and antimicrobial genes, 46 (46%) isolates were positive for coagulase gene, 62 (62%) & 18 (18%) isolates were positive for heamolysin and panton valentine leukocidin gene respectively. 54 (54%) isolates were found positive for methicillin resistant gene (*Mec* A)
6. Out of 54 isolates positive for *Mec* A gene in PCR. 50 isolates were shown positive for *Mec* A gene in Loop mediated isothermal amplification with the appearance of 6-10 bands.
7. **Visualisation by conventional/point of care device:**

   Detection by turbidity: Turbid formation was observed in all positive isolates for LAMP but not seen in case of non template control.

   Detection by dye method:

   HNB: Purple to sky blue color is formed in tubes positive for *Mec* A gene (50) and no color change is seen in case of non template control

   Visualisation with methyl red, m-cresol, p-cresol & neutral red did not give clear results due to improper standardization of buffer.

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Annexures

ANNEXURE

Composition of the solution and buffer used

R.B.C Lysis buffer (1X)	
NH_4Cl	8.29g (155mM)
$KHCO_3$	1.00g (10mM)
Na_2EDTA (0.5 mM) pH-8	400µl
Fill to 1000 ml with autoclaved distilled water	

DNA extraction buffer (1X)	
NaCl (5mM)	8 ml
Na_2EDTA (0.5 mM) pH-8	1 ml
Tris (1M) pH-8	1 ml
Fill to 100 ml with autoclaved distilled water	

Saturated phenol/chloroform/isoamylalcohol (25:24:1)	
Saturated phenol (pH-8)	25 ml
Chloroform	24 ml
Isoamylalcohol	1 ml

Chloroform/isoamylalcohol (24:1)	
Chloroform	24 ml
Isoamylalcohol	1 ml

SDS (10%) pH-7
100 g/L (heat to assist dissociation). Do not autoclave.
Proteinase K (20 mg/ml)
Dissolve 200 mg proteinase K in 10 ml TE (1:10, pH-8.00) for 30 min. at room temperature.

Sodium acetate	
3 M sodium acetate	246 g/L
Adjust to pH 5.2 with glacial acetic acid.	

1 X ThermoPol Reaction Buffer
20 mM Tris-HCl
10 mM $(NH_4)_2SO_4$
10 mM KCl
2 mM $MgSO_4$
0.1% Triton X-100
pH-8.8 @ 25°C