Project ID: 443

Competitive Research Grant

Sub-Project Completion Report

on

Development of Effective Vaccine against Bovine Mastitis

Project Duration

July 2017 to September 2018

Department of Microbiology, Hazee Mohammad Danesh Science and Technology University, Dinajpur

Submitted to

Project Implementation Unit-BARC, NATP 2
Bangladesh Agricultural Research Council
Farmgate, Dhaka-1215

September 2018

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Citation

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Project Implementation Unit
National Agricultural Technology Program-Phase II Project (NATP-2)
Bangladesh Agricultural Research Council (BARC)
New Airport Road, Farmgate, Dhaka – 1215
Bangladesh

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Project Implementation Unit
National Agricultural Technology Program-Phase II Project (NATP-2)
Bangladesh Agricultural Research Council (BARC)
New Airport Road, Farmgate, Dhaka – 1215
Bangladesh

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Acronyms

-	:	Negative
#	:	Identifying number
%	:	Percentage
@	:	At the rate of
+	:	Positive
µg	:	Microgram
µl	:	Microlitre
^{0}C	:	Degree of Celsius
Ag	:	Antigen
Assist	:	Assistant
BA	:	Blood Agar
BD	:	Bangladesh
BGA	:	Brilliant Green Agar
EMB	:	Eosin Methylene Blue
ER	:	Erythromycin
et al.	:	Associated
etc	:	Etcetera
FAO	:	Food and Agricultural Organization
Gm	:	Gram
H.S	:	Haemorrhagic septicemia
H_2O_2	:	Hydrogen peroxide
H_2S	:	Hydrogen sulphide
HSTU	:	Hajee Mohammad Danesh Science and Technology University
i.e.	:	That is
Ltd	:	Limited
M.S	:	Master of Science
MC	:	MacConkey Agar
MI	:	Milliliter
MIU	:	Motility Indole Urease
MR	:	Methyl Red
NA	:	Nutrient Agar
NB	:	Nutrient Broth
No.	:	Number
PBS	:	Phosphate Buffered Saline
PM	:	Post Mortem
Prof.	:	Professor
PSS	:	Physiological Saline Solution
RPM	:	Rotation Per Minute
SC	:	Subcutaneous
SE	:	Standard Error
SL	:	Serial number
Sp	:	Species
SSA	:	Salmonella Shigella Agar
v/v	:	Volume by volume
VP	:	Voges-Proskauer
w/v	:	Weight by volume

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Executive Summary

Mastitis is the ultimate threat to the dairy industry throughout the world. It is one of the major causes of economic loss in a dairy farm. A good number of pathogenic organisms including *Streptococcus agalactiae, Staphylococcus aureus, E. coli, Klebsiella pneaumoniae* and other *Streptococcus* sp. are responsible for this disease. Indigenous as well as crossbreed cows are frequently affected by mastitis. The principle objective of the project was thus to isolate and identify the major pathogenic organisms from mastitis milk and development of vaccine against mastitis using local isolates. All the mastitis milk samples were tested by California Mastitis Test (CMT) for confirmation of mastitis in the respective cows. Organisms were isolated and identified by the cultural and biochemical tests. For confirmation, molecular identification of the isolates was done by PCR and RT-PCR. Gene amplification of the organism was done using 16S rRNA and 23S rRNA with the reference primers that also helped in further confirmation of the causal agents of mastitis. On the other hand, through phylogenetic analysis the isolated organisms were precisely specified. Mastitis causing most important organisms those were isolated and characterized using molecular techniques through this study included *E. coli, Klebsiella pneaumoniae*, *Streptococcus agalactiae, Staphylococcus aureus,* and other *Streptococcus* sp. Out of the total of 48 samples tested *E. coli* and *Staphylococcus* sp. was present in all the samples with 100% occurrence. On the other hand, the rate of occurrence of *Streptococcus* sp. and *Klebsiella* sp. was 28% and 30% respectively. All the four genus of the locally isolated organisms at a concentration of 1×10^{10} cfu/ml for *Staphylococcus aureus,* 4×10^{9} cfu/ml for each of the *Streptococcus agalactiae* and *Streptococcus* sp. along with 1×10^{9} cfu/ml for each of the *E. coli* and *Klebsiella Pneumonia* were then inactivated using formalin (0.4% V/V). After confirmation of inactivation the organisms were used in the production of Polyvalent Formalin Inactivated Vaccine (PFIV). Montanide @ ISA 206 was used as an adjuvant in the vaccine. Due to time constraints efficacy and potency test of the experimental vaccine could not be done. Further research therefore is needed before declaring that the vaccine is safe and effective against mastitis for using in the dairy cows in Bangladesh.

CRG Sub-Project Completion Report (PCR)

A. Sub-project Description

1. **Title of the CRG sub-project**:
   Development of Effective Vaccine Against Bovine Mastitis

2. **Implementing organization:**
   Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur.

3. **Name and full address with phone, cell and E-mail of PI/Co-PI (s):**

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   Associate Professor
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   Phone: 0531-61347, Cell: 01706-877533.

4. **Sub-project budget (Tk):**
   4.1 Total: Tk. 22,00,000.00
   4.2 Revised (if any):

5. **Duration of the sub-project:**
   5.1 Start date (based on LoA signed): July, 2017
   5.2 End date : 30 September, 2018

6. **Justification of undertaking the sub-project**:

Dairy industry is one of the important sub-sectors of livestock. It provides milk, which is the important source of nutrition for human. But one of the major problems of dairy industry is mastitis. Mastitis is defined as an inflammation of the mammary gland resulting from the infection with various different microorganisms. Mastitis is perhaps the costliest disease of dairy cattle because it causes major economic losses through reduction in milk yield, culling of high vigor breeds and losses on account of veterinary medical expenses. Globally, the economic loss due to mastitis is about $533 billion and in Bangladesh, it is $ 2.11 million per year (Shaheen *et al.,* 2 016). The prevention and treatment of mastitis present a serious hurdle to producers and it is always the primary concern of the dairy industry. In Bangladesh an imported vaccine-Mastivac is used to control mastitis. The vaccine is manufactured by Ovejero laboratories, Spain. The vaccine contains *Streptococcus agalactiae, S. dysgalactiae, S. uberis, S. pyogenes, Staphylococcus aureus, Escherichia coli* (strains Bov-13, Bov-14, Bov-15, Suis-21 and J5) *and Arcanobacterium pyogenes*. However, the vaccine was reported not to be effective to

control mastitis in Bangladesh (personal communication). This may be due to some additional causal agents that are not incorporated in the imported vaccine or due to differences of the antigenic properties of the causal agents. Therefore, the project was undertaken with the objectives to isolate and identify the causal agents of bovine mastitis in the country and preparation of effective vaccine using the local isolates.

7. Sub-project goal:

To reduce the economic losses caused by bovine mastitis in Bangladesh.

8. Sub-project objective (s):

i) To isolate and identify the microbial agents causing mastitis in cattle
ii) To characterize the bacterial isolates using molecular identification methods
iii) To develop vaccine against bovine mastitis from the selected bacterial isolates

9. Implementing location (s):

Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200.

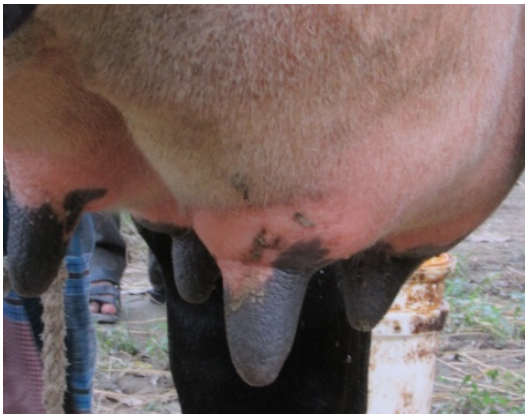
10. Methodology in brief:

The research work was conducted during the period from July 2017 to December 2018 at the bacteriology and Molecular Biology laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur.

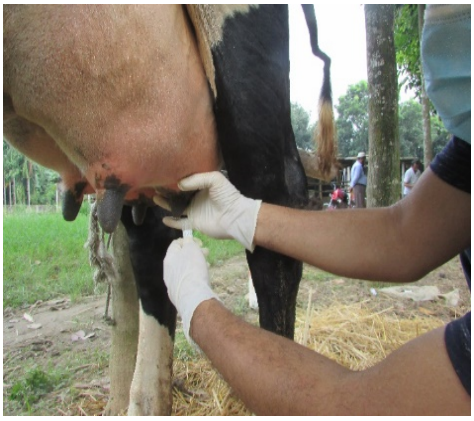
10.1. Isolation and identification of the causal agents of mastitis in cows

Sample collection

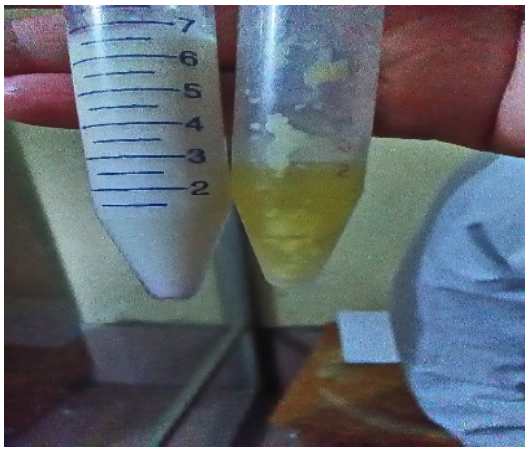
A Total of 48 clinical mastitis milk samples were randomly collected from Rangpur division including Dinajpur, Rangpur, Thakurgaon and Gaibandha districts. First the teats were soaked with 70% ethanol then after drying off by tissue paper, one to two drops of milk was discarded and then 10 ml of milk were taken from infected udder and teat. The milk samples were collected with the help of pre-sterilized corkscrew tube and immediately transferred into pre-sterilized container. After collection of milk samples California Mastitis Test (CMT) was performed for confirmation of the disease. The samples were then transferred, as soon as possible, to the ice box containing plenty of ice cubes which were then brought to the department of microbiology for laboratory analysis. Figure 1 shows the photos of infected udder, collection of sample from the infected udder, CMT test using specific kit and preserving the samples in the ice box before transporting to the laboratory.



Infected udder and teat



Collection of infected milk sample



Samples in sterilized tubes

CMT test kit

CMT positive jelly like appearance

Samples in ice box

Figure 1: Sample collection, preparation and CMT test

Preparation of samples and culture media

In the laboratory, mastitis milk samples were stored at 4°C until the preparation of inoculation media. All the samples were inoculated within the clean bench by maintaining aseptic procedure. All the work, from collection to inoculation, was done within 24 hours. All the media, broth and reagents used in this experiment were prepared according to instruction of the manufacturer (H1-MEDIA, India).

Morphological characterization, isolation and identification of bacteria

Gram's staining, Biochemical examination (Catalase test, Indole test, MR Test, Voges-proskauer test, Simmons citrate test, Triple sugar iron agar test, Motility-Indole-Urease test), Haemolytic activity and Isolation of bacteria in pure culture was done using the standard methods described by different workers for the specific tests (Hans Christian Joachim Gram, 1884; James G. Cuppuccion and Natalie Sherman, 1996; Cheesbrough, 1985; Cowan and Steel, 1979; Carter, 1986; Poindexter, 1971).

10.2. Characterization of isolated bacteria using molecular technique

PCR amplification, sequencing and phylogenetic analysis of isolated organisms

Bacterial genomic DNA extraction (Gram positive and Gram negative)

Bacterial genomic DNA was extracted using the basic protocol described briefly as follows:

1. Bacteria were cultured overnight in Luria Bertani Broth.

2. A 1 ml volume of overnight culture was taken to a 1.5 ml microcentrifuge tube and centrifuged at 13,000–16,000 × g for 2 minutes to pellet the cells and then the supernatant was removed. For Gram Positive Bacteria, all the steps described below were followed. However, for Gram Negative Bacteria the steps 3, 4 and 5 were skipped.

3. Resuspended the cells thoroughly in 480 µl of 50 mM EDTA.

4. Added the appropriate lytic enzyme(s) to the resuspended cell pellet in a total volume of 120 µl, and gently pipetted to mix.

Note: For certain Staphylococcus species, a mixture of 60µl of 10 mg/ml lysozyme and 60 µl of 10 mg/ml lysostaphin was required for efficient lysis. However, many Gram Positive Bacterial Strains (e.g., Bacillus subtilis, Micrococcus luteus, Nocardia otitidiscaviarum, Rhodococcus rhodochrous, and Brevibacterium albidium) were lysed efficiently using lysozyme alone.

5. Incubated the sample at 37°C for 30–60 minutes followed by centrifugation for 2 minutes at 13,000–16,000 × g and removal of the supernatant.

6. Then 600 µl of Nuclei Lysis Solution was added to the pellet and gently pipetted until the cells were resuspended.

7. Incubation was done at 80°C for 5 minutes to lyse the cells followed by cooling to room temperature.

8. A 3 µl volume of RNase solution was added to the cell lysate and mixed thoroughly by inverting the tube 2–5 times.

9. The cell lysate was then incubated at 37°C for 15–60 minutes followed by cooling the sample to room temperature.

10. A 200µl volume of Protein Precipitation Solution was then added to the RNase-treated cell lysate and vortexed vigorously at high speed for 20 seconds.

11. The sample was then incubated on ice for 5 minutes and centrifuge at 13,000–16,000 × g for 3 minutes.

12. Transferred the supernatant containing the DNA to a clean 1.5ml microcentrifuge tube containing 600 µl room temperature isopropanol; gently mixed by inversion until the thread-like strands of DNA formed a visible mass and then centrifuged at 13,000–16,000 × g for 2 minutes.

13. Carefully poured the supernatant off the tube which was then allowed to drain on a clean absorbent paper.

14. A 600µl of room temperature 70% ethanol was added and gently inverted the tube several times to wash the DNA pellet.

15. The tube containing the DNA was then centrifuged at 13,000–16,000 × g for 2 minutes followed by careful aspiration of the ethanol.

16. Drained the tube on clean absorbent paper and allowed the pellet to air-dry for 10–15 minutes.

17. A 100 µl volume of DNA Rehydration Solution was then added to the tube and rehydrated the DNA by incubating at 65°C for 1 hour with periodical mixing of the solution by gently tapping the tube. Alternatively, the DNA was rehydrated by incubating the solution overnight at room temperature or at 4°C.

18. Extracted DNA was stored at 2–8°C until use.

PCR amplification and sequencing

PCR was conducted for confirmation of different bacteria using primers specific for the organisms isolated. Details of the primers are given in Table 1. Description of Reaction mixture, Condition of PCR, Description of RT-PCR master mixture, RT-PCR condition and annealing temperature used for specific primers for conducting PCR are given in the Tables 2, 3, 4, 5 and 6 respectively. The PCR and RT-PCR products were analyzed by electrophoresis in TAE buffer on 1.7% agarose gel for 30-60 min at 100v. After agarose gel electrophoresis the bands were stained with ethidium bromide (0.5 µg/ml) for 10 min in a dark place, which were then visualized and examined under UV light using an ultraviolet light transilluminator. The sizes of the bands of test DNA were determined by comparing the distance migrated by samples of the DNA of known size.

Tabe 1. Details of the primers used for PCR/RT-PCR

Organisms	Primer Name	Sequence (5' – 3')	% GC Content	Amplicon Size (bp)	References
E. coli	Eco 223	F- ATC AAC CGA GAT TCC CCC AGT	52.4%	232	Riffon *et at.* (2001)
	Eco 455	R- TCA CTA TCG GTC AGT CAG GAG	52.4%		
Staphylococcus aureus	Sau 234	F- CGA TTC CCT TAG TAG CGG CG	60%	1267	Riffon *et at.* (2001)
	Sau 1501	R- CCA ATC GCA CGC TTC GCC TA	60%		
Streptococcus agalactiae	Sag 40	F- CGC TGA GGT TTG GTG TTT ACA	47.6%	405	Riffon *et at.* (2001)
	Sag 445	R- CAC TCC TAC CAA CGT TCT TC	50%		
Streptococcus sp.	Uni 1870	F- TGG AAG GTT AAG AGG AGT GG	50%	438	Riffon *et at.* (2001)
	Uni 2308	R- GCC TCC GTT ACC TTT TAG GA	50%		
Klebsiella sp.	27F	F- AGAGTTTGATCCTGGCTCAG	50%	1492	Lane *et al.* (1991)
	1492R	R- TACCTTGTTACGACTT	50%		

Table 2. PCR Reaction Mixture

PCR Master Mix	25 µl
Forward Primer	1.5 µl
Reverse Primer	1.5µl
Nano Pure Water	18 µl
DNA	4.0 µl
Final Volume	50 µl

DNA used as 35 X concentration, for PCR 20 pmol and for sequencing 10 pmol of primes were used

Table 3. Condition of PCR

Steps	Temperature	Duration	Cycle
Initial denaturation	94°C	2 min	01
Denaturation	94°C	45 Sec	35
Annealing	Mentioned in Table 6	1min	
Extension	72°C	2 min	
Final extension	72°C	10 min	01
Holding	4°C	hold	

Table 4. RT-PCR master mixture

qPCR Master Mix (Go taq probe)	10 µl
Forward Primer	1µl
Reverse Primer	1µl
Nano Pure Water	6 µl
DNA	2 µl
Final Volume	20 µl

Table 5. RT-PCR condition

Steps	Temperature	Duration	Cycle
Initial denaturation	95°C	2 min	01
Denaturation	95°C	15 Sec	40
Annealing and Extension	60°C	1min	

DNA used as 35 X concentration, primer for RT-PCR used 20 pmol.

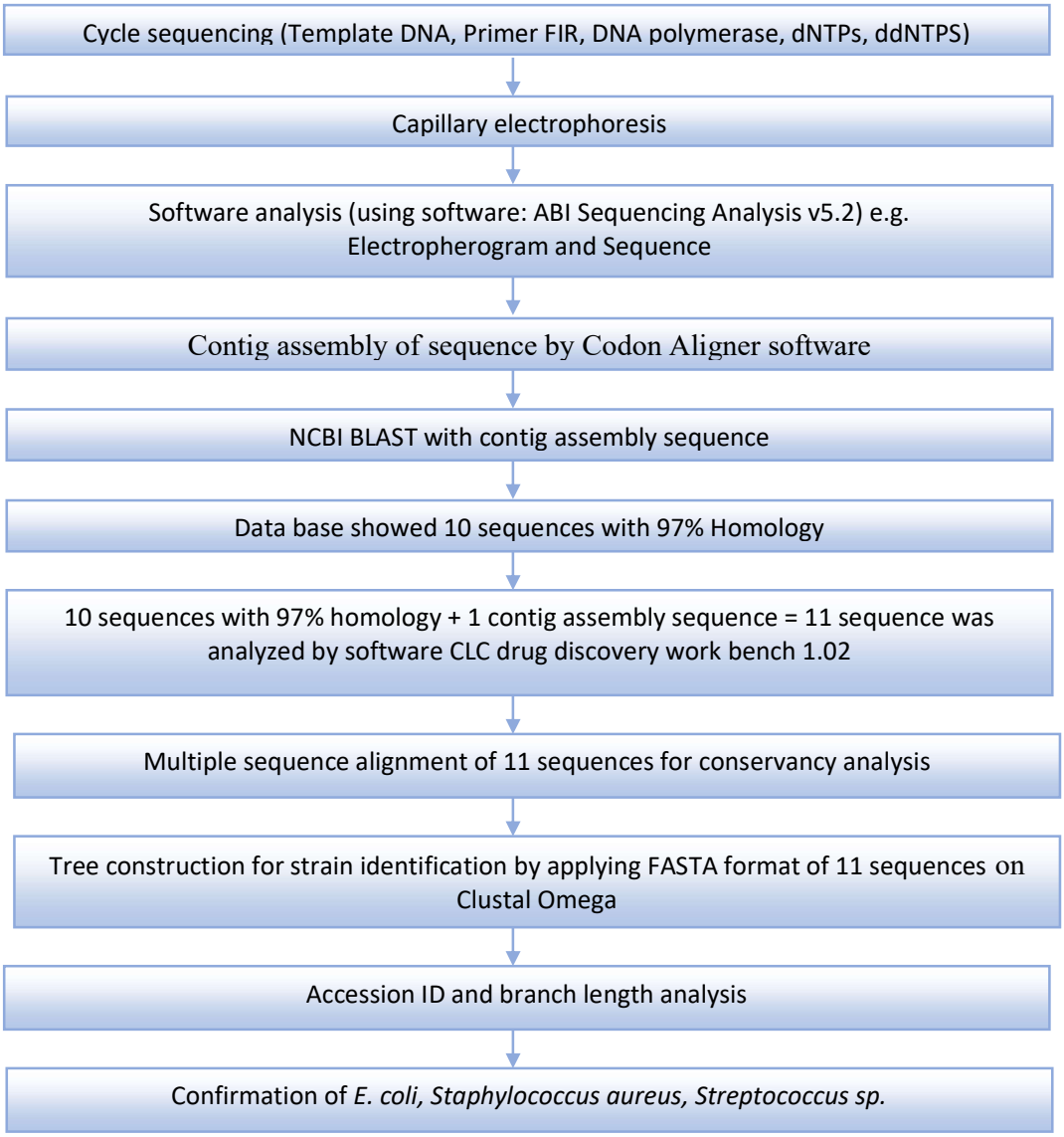
Table 6. Annealing temperature for specific primers

Organisms	Primer	Annealing Temperatures
E. coli	Eco 223 & Eco 455	64°C
Staphylococcus aureus	Sau 234 & Sau 1501	58°C, 60.5°C, 60.9°C, 61.3°C
Streptococcus agalactiae	Sag 40 & Sag 445	60°C, 61°C, 62.5°C, 63.2°C
Streptococcus sp.	Uni 1870 & Uni 2308	58°C, 59.1°C
Klebsiella sp.	27F & 1492R	62°C

Nucleotide sequence and BLAST analysis

The PCR products were sequenced using ABI3130 Genetic analyzer as stated below in Figure 2. The nucleotide sequence of 16S rRNA gene region data was submitted to NCBI nucleotide sequence database. Using BLAST tool, phylogenic tree, primer pairs were designed from NCBI database search tool.

Figure 2: Steps of Chain-termination methods (Sanger) of sequencing



10.3 Development of polyvalent vaccine

Maintenance of stock culture

The pure culture isolates were maintained as stock culture on Nutrient agar slants following the procedures of Choudhury *et al.,* (1987). After growth of the organisms in the slant, sterile mineral oil was overlaid and the culture was kept at room temperature for further use as seed. For longer period preservation of isolated *E. coil* equal volume of sterilized 80% glycerin and bacterial culture were mixed and sealed with paraffin wax and stored at 37°C.

Development of vaccine seed

After isolation, identification and molecular characterization of pathogenic bacteria, vaccine seed was developed according to Sayed *et al.,* 2015 and Giraudo *et al.,* 1997. Procedure is given bellow:

1. Organisms were grown in Brain Heart Infusion Broth (BHIB) at 37°C for 24 hours. However, for *E. coli* Tryptic Soy Broth Medium used.
2. A 5ml volume of each the samples were taken in a tube.
3. Cells were inactivated by 0.4% V/V formalin and centrifuged at 7000 rpm for 20 minutes at 4 °C
4. Suspended with normal saline (0.9% NaCl), pH 7.0
5. A 5 ml volume of each of the samples were taken in one tube having the bacterial concentration of 1×10^{10} cfu/ml for *Staphylococcus aureus,* 4×10^{9} cfu/ml for *Streptococcus agalactiae* and 1×10^{9} cfu/ml for each of the *E. coli* and *Klebsiella Pneumonia*. However, for *Streptococcus* sp. the concentration was 4×10^{9} cfu/ml.

Preparation of polyvalent formalin inactivated vaccine (PFIV)

Polyvalent formalin killed vaccine against mastitis was prepared by adding with the mixture of 4 formalin inactivated bacterial isolates (*Staphylococcus aureus*, *Streptococcus agalactiae*, *E. coli* and *Klebsiella Pneumonia*) of 20 ml volume (4 x 5 ml) as mentioned above, 20 ml of nontoxic adjuvant (Barnett *et al.,*1996) Montanide @ ISA 206 according to Acres *et al.* (1979).

Quality control testing of the experimental vaccine

Sterility test of the vaccine

Sterility test of the newly prepared PFIV for the foreign contaminants (aerobic and anaerobic bacteria and fungi) was carried out according to OIE (2013). Using a sterile inoculation loop the vaccine was streaked on to nutrient agar and observed for any growth at 37°C for 24 hours.

Safety test of the vaccine

Safety of the prepared vaccine was tested according to OIE (2013). A double dose of the newly prepared vaccine was inoculated intramuscularly in a pregnant cow which was kept under daily observation for 14 days for the appearance of any lesion on udder.

Efficacy and potency tests of the vaccine

The experiments for efficacy and potency tests of the vaccine could not be performed due to time constraints.

11. Results and discussion:

11. 1. Isolation and identification of the microbial agents causing mastitis in cattle:

Bacteria isolated from mastitis milk from different areas of Rangpur division

The results of frequency of different bacterial isolates are presented in Table 7. A total of 48 milk samples were examined for the isolation of bacteria. Here we mainly focused on 4 (four) types of bacteria. We also found *Corynebacterium* sp. and *Bacillus* sp. at very lower in number.

Table 7. Frequency of occurrence of the isolates in milk samples

Sample collection Area	Number of samples	Positive for *E. coli* (%)	Positive for *Staphylococcus* spp (%)	Positive for *Streptococcus* spp (%)	Positive for *Klebsiella* spp (%)
Chirir bandar	12	100	100	58.34	50
Sadar Livestock office Dinajpur	8	100	100	62.5	62.5
Nandigram, Birganj	5	100	100	60	60
Birol, Dinajpur	10	100	100	70	60
Sadullahpur, Gaibandha	2	100	100	100	50
Kornai, Baserhat	2	100	100	-	100
Thakurgaon, Sadar	2	100	100	100	50
Thakurgaon, Haripur road	4	100	100	50	75
Birganj Livestock office	3	100	100	66.67	100
Total	48	48	48	28	30

Out of the total of 48 samples collected all were positive (100%) for *E. coli* and *Staphylococcus* sp. Zafolon *et al*. (2008) also found the presence of 100% *E. coli* in their work with mastitis milk samples. On the other hand, the rate of occurrence of *Streptococcus* sp. and *Klebsiella* sp. was 28% and 30% respectively.

Isolation and identification of the organisms by morphological and cultural characteristics

The morphological (Plates 2, 10, 11 and 12) and cultural characteristics of *Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia* and *Streptococcus* sp. including *Streptococcus agalactiae* on various selective media are presented in Table 8 and 9. All the isolates produced whitish, opaque, smooth or fuzzy colony on the nutrient agar (Plate 1).

On MSA *Staphylococcus aureus* produced golden yellowish pigment (Plate 3). *Escherichia coli* and *Klebsiella pneumoniae* produced metallic green sheen and dark pink colonies on eosin methylene blue agar respectively (Plates 4, 8). On the other hand, *Escherichia coli* and *Klebsiella pneumonia* produced pink colonies on MacConkey agar due to lactose fermentation (Plate 5). Whereas *Streptococcus* sp. produced pale yellow and sometimes red in color colonies. On Staphylococcus Agar No. 110 pale and opaque, small circular colony was appeared in case of *Staphylococcus aureus* (Plate 6). On Streptococcus agalactiae selective agar base media along with blood *Streptococcus agalactiae* when grown anaerobically produced dark brown colored colonies as well as black red colonies which were sticky to touch (Plate 7). On Sheep and Ox blood agar both the *Streptococcus agalactiae* and *Staphylococcus aureus* produced complete hemolysis (Plate 9).

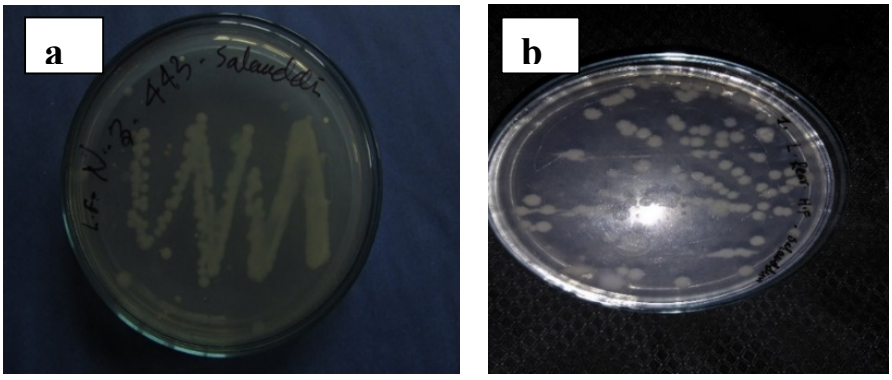


Plate 1: Bacterial colony on nutrient agar media
a) Direct smear on nutrient agar; b) Round circular cream colored colonies

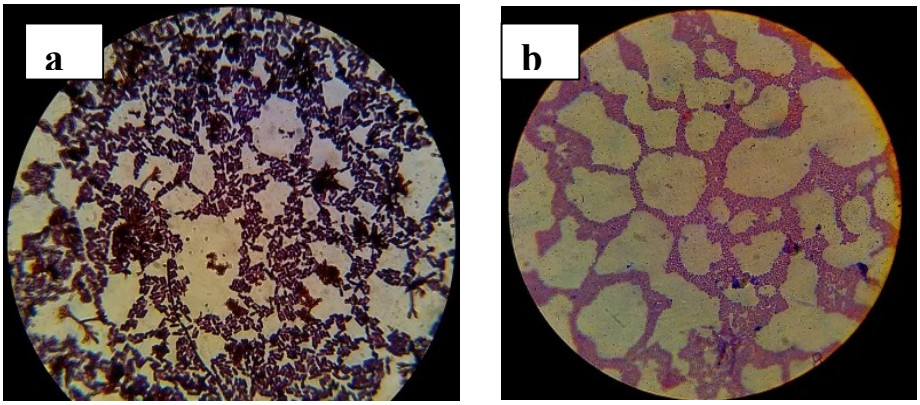
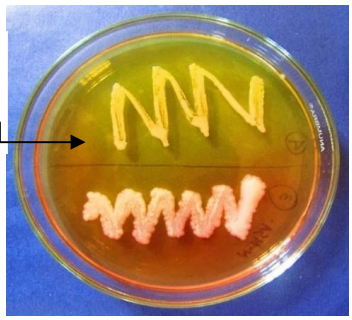
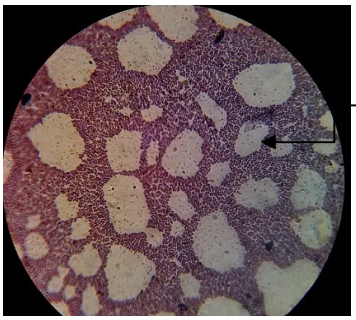


Plate 2 Microscopic images of Gram's stained bacteria
a) Gram positive spherical shaped organisms; b) Mixed clustered organisms

Golden yellow colored colony on MSA





Gram positive clusters of organisms

Plate 3: *Staphylococcus* sp. on MSA and Microscopic view

Metallic sheen colony on EMB *E. coli*

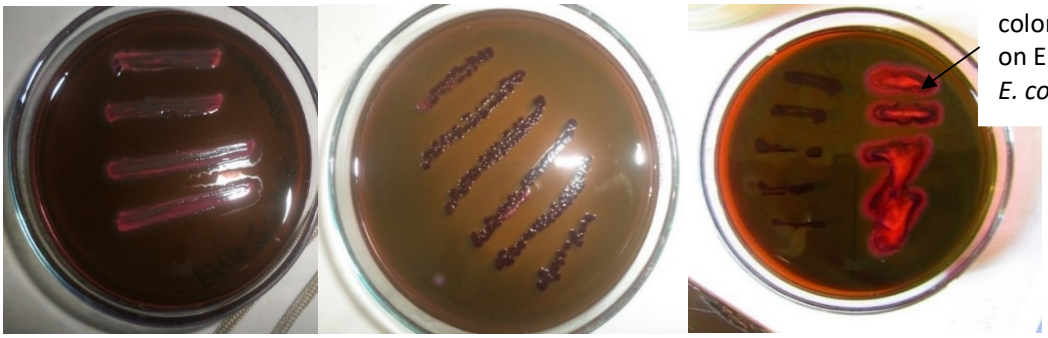
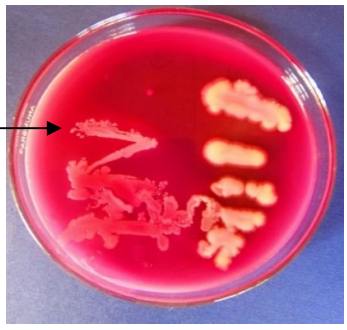
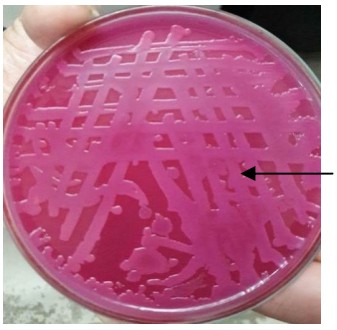


Plate 4: *Klebsiella* sp and *E. coli* on EMB agar media

Pink colony of Klebsiella sp. (Left), Blackish pink colony of E. coli (Middle), Mixed colony of Klebsiella sp.and E. coli (Right)

Rose pink colony on MacConkey agar

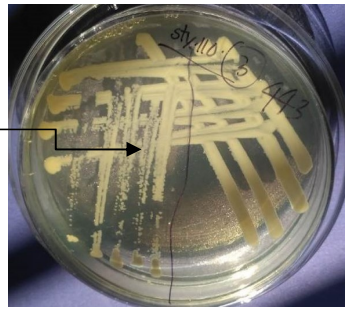


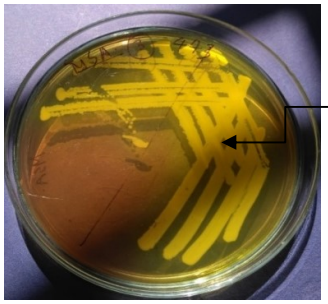


Rose pink glistening sticky colony on MacConkey *Klebsiella* sp.

Plate 5: *Klebsiella* sp. on MacConkey agar media

Cream colored colony of *S. aureus* on SAN 110





S. aureus on MSA with golden yellow colony

Plate 6: *Staphylococcus* sp. on Staphylococcus agar No. 110 (Left) and *Staphylococcus* sp.in MSA (Right)

Anaerobic jar containing *S. agalactiae*

Plate 7: Pure culture of *Streptococcus agalactiae* on *Streptococcus agalactiae* selective Agar Base (anaerobic)

E coli on EMB

Plate 8: *E coli* showing metallic sheen colored colony on EMB

Plate 9: *Streptococcus sp.* and *Staphylococcus* sp on sheep and ox blood agar
Sheep blood agar (Left) complete hemolysis Streptococcus sp. (Streptococcus agalactiae); Ox blood agar Staphylococcus sp. (Right)

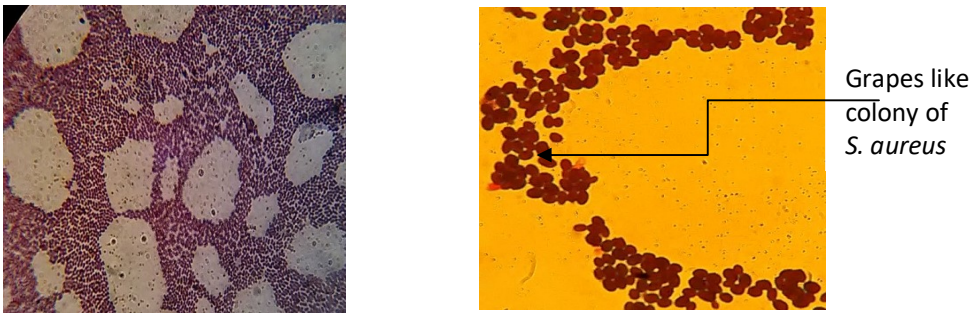


Plate 10: Microscopic view of gram positive *Staphylococcus* sp.
From MSA agar media (Left); From Staphylococcus agar No. 110 (Right)

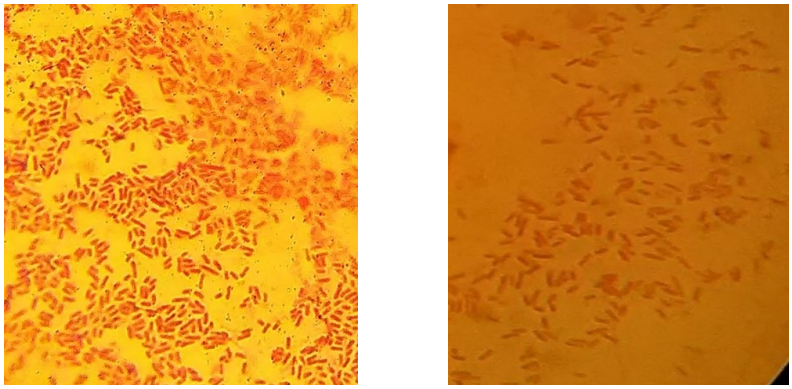


Plate 11: Microscopic view of *Klebsiella* sp. and *E. coli*
Gram negative, short rod with capsul showing Klebsiella sp. from MacConkey agar media (Left); Gram negative short rod, E. coli from EMB (Right)

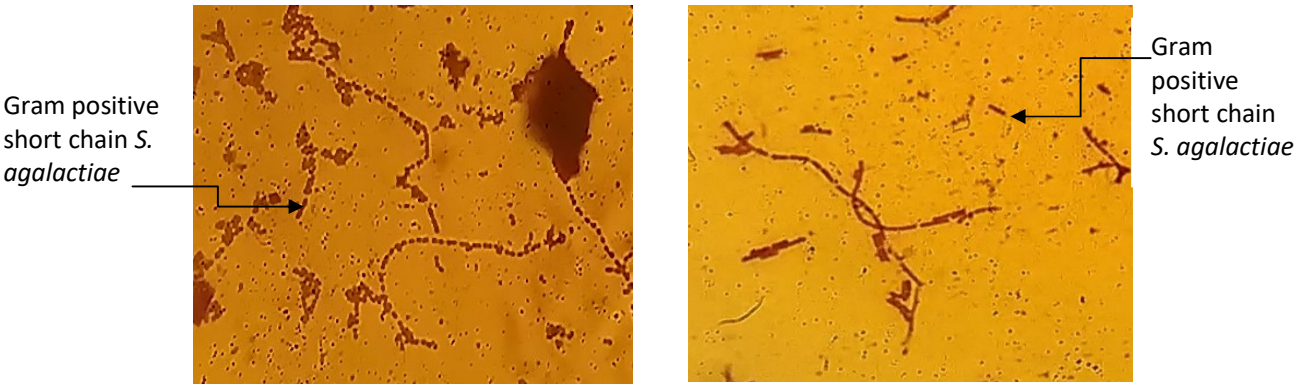


Plate 12: Microscopic view, *Streptococcus* sp. and *Streptococcus agalactiae* on *Streptococcus agalactiae* Selective Agar Base (Anaerobic)

Table 8. Identification of isolated organisms by using morphological characteristics

Staining and morphological characteristics	Name of the organisms
Gram negative, pink colored, small rod shaped organisms that were arranged in single, pairs or short chain.	*Escherichia coli*
Gram negative, pink colored, small rod shaped organisms arranged in single, pairs or short chain.	*Klebsiella pneumoniae*
Gram positive, Violet colored, cocci shaped organisms arranged in clusters. Sometimes they also appeared is single, paired and short chains of three or four bacteria.	*Staphylococcus aureus*
Gram positive, violet colored, cocci arranged in short chain. They also appeared in long chain when grown in liquid medium.	*Streptococcus agalactiae*

Table 9. Isolation and identification of bacteria by using cultural characteristics

Name of the culture media	Name of the organisms with the observed colony characteristics			
	Escherichia coli	***Staphylococcus aureus***	***Klebsiella pneumoniae***	***Streptococcus* sp. and *Streptococcus agalactiae***
Nutrient agar	Large, circular, low convex, grayish, white, moist colonies.	Circular, small, smooth, convex and gray-white colonies.	Large, circular, smooth, convex, colonies	Circular, small smooth, convex, and golden, yellowish colonies
MacConkey agar	Bright, pink colored, smooth colonies.	—	Round, pink, slightly raised translucent and mucoid colonies.	—
EMB agar	Smooth and green Metallic sheen colonies.	—	Round, pink, slightly raised translucent and mucoid colonies.	—
MSA	—	Golden yellow colored pigment and sometimes pale yellowish colonies.	—	Pale yellowish colonies.
Staphylococcus agar No. 110	——	Pale opaque in color colonies and sometimes golden yellowish colonies		Opaque in color colonies and sometimes red in color
Streptococccus agalactiae selective agar base	—	—	—	Dark brown in color colonies. Sometimes dove blue in color and blackish red colonies were also appeared
Blood agar	—	Complete hemolytic zone (β hemolysis) in Sheep and Ox blood agar. Sometimes partial hemolysis appeared.	—	Complete hemolytic zone (β hemolysis) in Sheep and Ox blood agar.

Characterization of the isolates by using different biochemical tests

Biochemical test results are shown in Table 10. Results of different biochemical tests are also shown in Plates 13, 14, 15 for *Streptococcus* sp.; Plates 16, 17 for *Staphylococcus* sp.; Plate 18 for *Klebsiella* sp. and Plate 19 for *E. coli*.

Table 10. Biochemical properties of the isolated organisms

Organisms	SC	IT	TSI	MR	VP	C
E. coli	-	+	S- Yellowish with colony, B- No change, Gas +, H_2S -	+	-	-
Klebsiella sp.	+	-	S- Yellowish with colony, B- No change, Gas +, H_2S -	-	+	+
Staphylococcus sp.	-	-	S- Yellowish with colony, B- No change, Gas -, H_2S -	+	+	+
Streptococcus sp.	-	-	S- Pinkish with colony, B- No change, Gas -, H_2S -	-	-	-

S= Slant, B= Butt, SC= Simon Citrate test, IT= Indole test, TSI= Triple sugar iron test, MR= Methyl-Red test, VP= Voges-Proskauer test, C= Catalase test, + = Positive reaction, - = Negative reaction.

Plate 13: Biochemical test of *Streptococcus* sp. (Left to Right) Indole (negative), Simmon's citrate (negative), TSI and MR (negative)

Plate 14: Catalase test, *Streptococcus* sp. showing no bubble (left)

Plate 15: Voges-Proskauer reaction, *Streptococcus* sp. showing negative reaction (left)

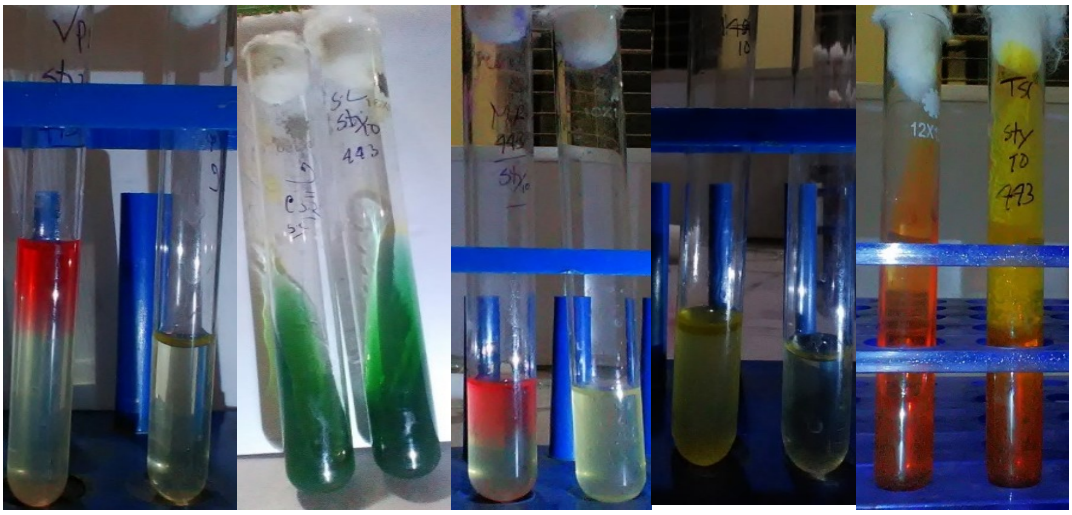


Plate 16: Biochemical test of *Staphylococcus* sp. (Left to Right) VP (positive), Simon citrate (negative), MR (positive), Indole (negative), TSI test

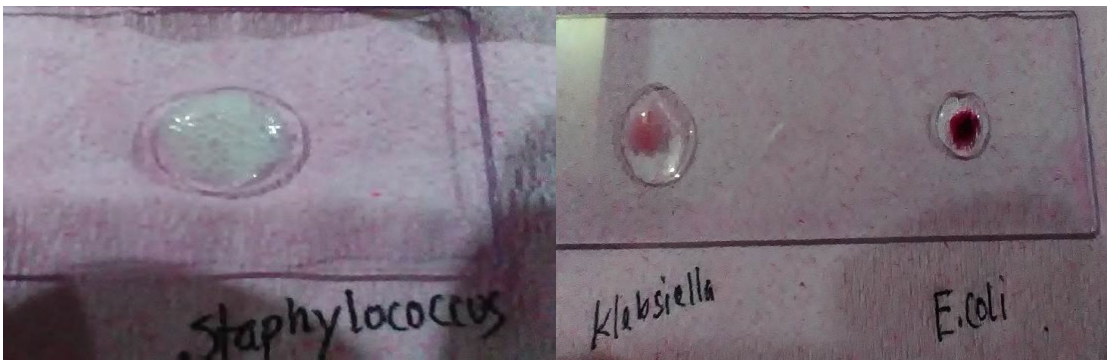


Plate 17: Catalase test, *Staphylococcus* sp. show bubble (Left); *Klebsiella* sp. give positive result and *E. coli* show no bubble (Right)

Plate 18: Biochemical test of *Klebsiella* sp. (Left to Right) Indole (negative), VP (positive), Simon citrate (positive), TSI, MR (negative)

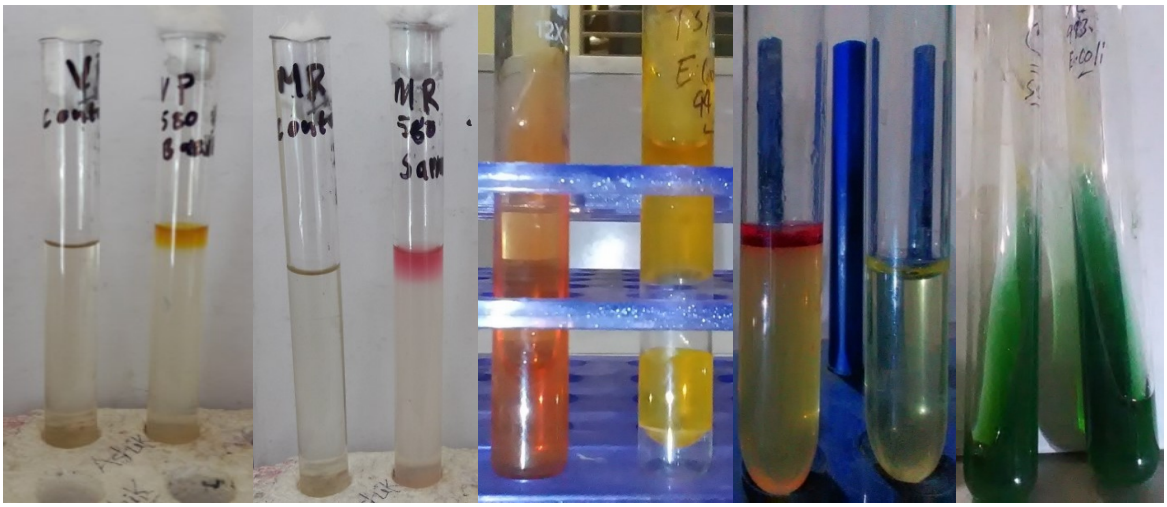


Plate 19: Biochemical test of *E. coli* (Left to Right) VP (negative), MR (positive), TSI, Indole (positive), Simon citrate (negative)

Catalase test was performed by placing a drop of hydrogen peroxide on slides and mixing the colony of the bacteria to be tested thoroughly. Presence of bubbles indicated the positive result. Indole test was performed by inoculating the buffered peptone water broth with the bacteria to be tested, followed by incubation for 24 hours at 37°C. The next day tubes were observed after adding Kovac's reagent. Formation of cherry red colored ring on the surface of the broth medium indicated positive result. Methyl red test was performed by inoculating the target organisms into MR medium and incubating for 48 hours at 37°C. Formation of red colored ring on the surface of the broth medium indicated positive result. Voges-Proskauer test was performed by inoculating the target organisms into VP medium and incubating for 72 hours at 37°C. Formation of red colored ring on the surface of the broth medium indicated positive result. The organisms to be tested were inoculated onto Simmon's citrate agar medium slant and incubated for 24 hours at 37°C. The turning of the media slant into Prussian blue color indicated the positive result. TSI agar slant was inoculated by the organisms to be tested and incubated for 24 hours at 37°C. Glucose fermentation was indicated by yellow butt, lactose fermentation by yellow slant and H_2S production by blackening of the medium and gas production was indicated by the presence of bubble or gas space.

Characterization of field isolates by using serological test

Staphylococcus showed positive and *Streptococcus* showed negative result with rabbit serum agglutination test (Figure 3).

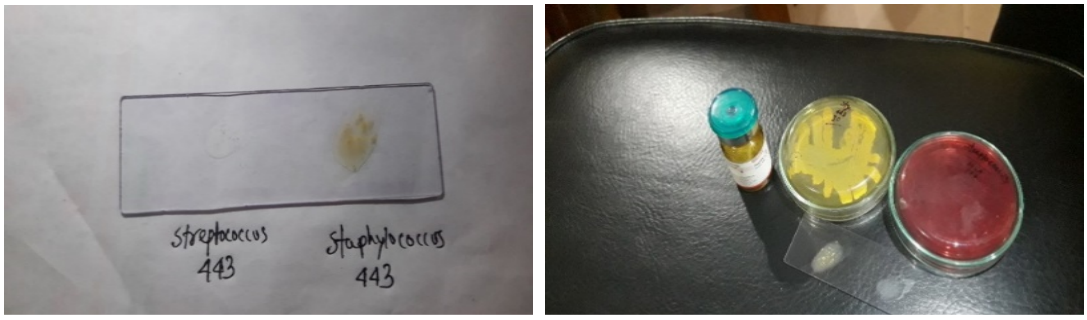


Figure 3: Serological test of *Streptococcus* sp. and *Staphylococcus* sp. by rabbit serum

11.2. Characterization of isolated bacteria using molecular technique

Identification of *E. coli* by PCR amplification of 23S rRNA gene Presence of *E. coli* in the samples was confirmed by PCR amplification of 23S rRNA gene region with the Primer sets of Eco 223 (F) and Eco 445 (R). The positive samples produced bands at 232 bp position as seen after electrophoresis on 1.7% agarose gel (Figure 4).

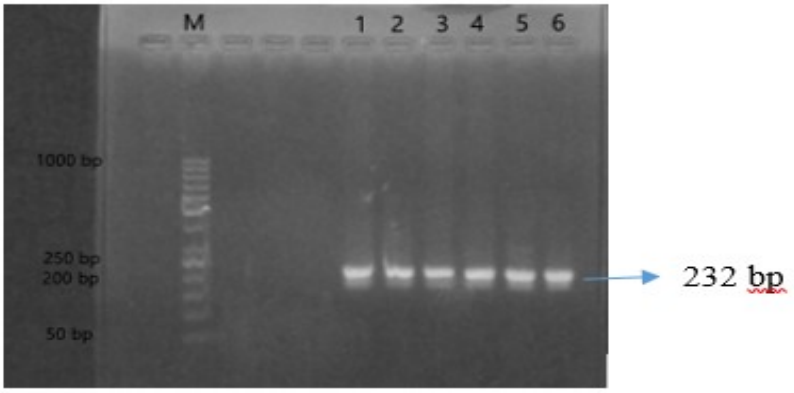


Figure 4: Electrophoresis (1.7% agarose gel) of PCR products containing *E. coli* specific primer *Lane M = 1 kb plus DNA ladder marker. Lanes 1–6 = positive samples (positive band at 232 bp).*

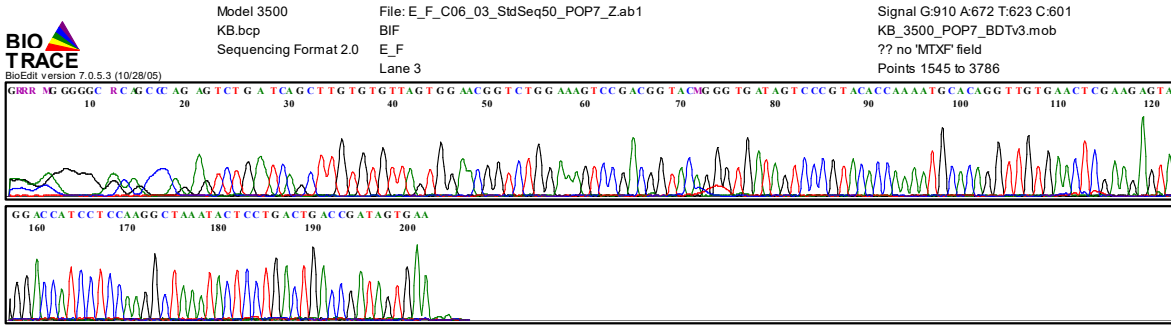
Sequence of *Escherichia coli* 23S rRNA gene region using the same Forward and Reverse Primers as for PCR

Forward Primer Sequence

>E_F (Underline gene are omitted during analysis)

GRRRMGGGGGCRCAGCCCAGAGTCTGATCAGCTTGTGTGTTAGTGGAACGGTCTGGAAAGTCCGACGG
TACMGGGTGATAGTCCCGTACACCAAAATGCACAGGTTGTGAACTCGAAGAGTAGGGCGGGACACGTG
GTATCCTGTCTGAATATGGGGGGACCATCCTCCAAGGCTAAATACTCCTGACTGACCGATAGTGAA

Chromatogram of Forward (F) primer

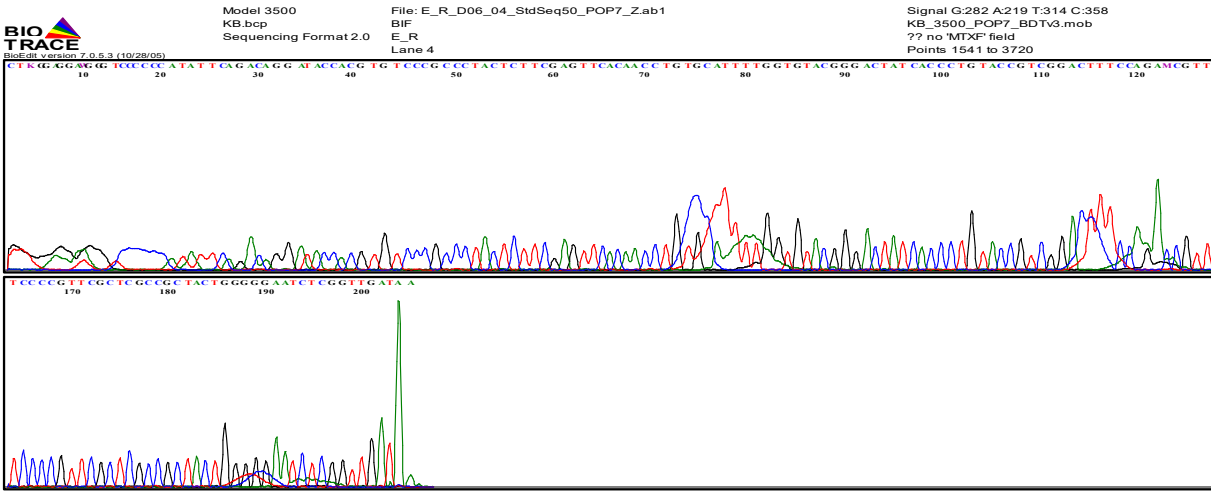


Reverse Primer Sequence

>E_R (Underline gene are omitted during analysis)

CTKGGAGGAWGGGTCCCCCCATATTCAGACAGGATACCACGTGTCCCGCCCTACTCTTCGAGTTCACAA
CCTGTGCATTTTGGTGTACGGGACTATCACCCTGTACCGTCGGACTTTCCAGAMCGTTCCACTAACACAC
AAGCTGATTCAGACTCTGGGCTGCTCCCCGTTCGCTCGCCGCTACTGGGGGAATCTCGGTTGATAA

Chromatogram of Reverse (R) primer



Identification of *Staphylococcus aureus* by PCR amplification and sequencing of 23S rRNA gene

Presence of *Staphylococcus aureus* in the samples was confirmed by PCR amplification of 23S rRNA gene region with the primer sets of Sau 234 (F) and Sau 1501 (R). The positive samples produced bands at 1267 bp positions on 1.7% agarose gel after electrophoresis (Figure 5).

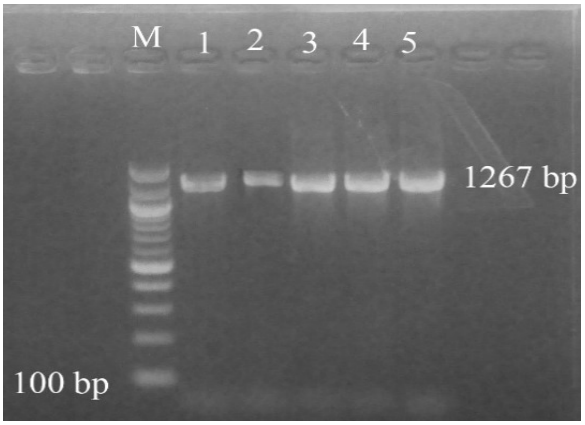


Figure 5: Electrophoresis (1.7% agarose gel) of PCR products containing *S. aureus* with specific primer. *Lane M= 1 kb plus DNA ladder marker. Lanes 1–5= positive samples (positive band at 1267 bp).*

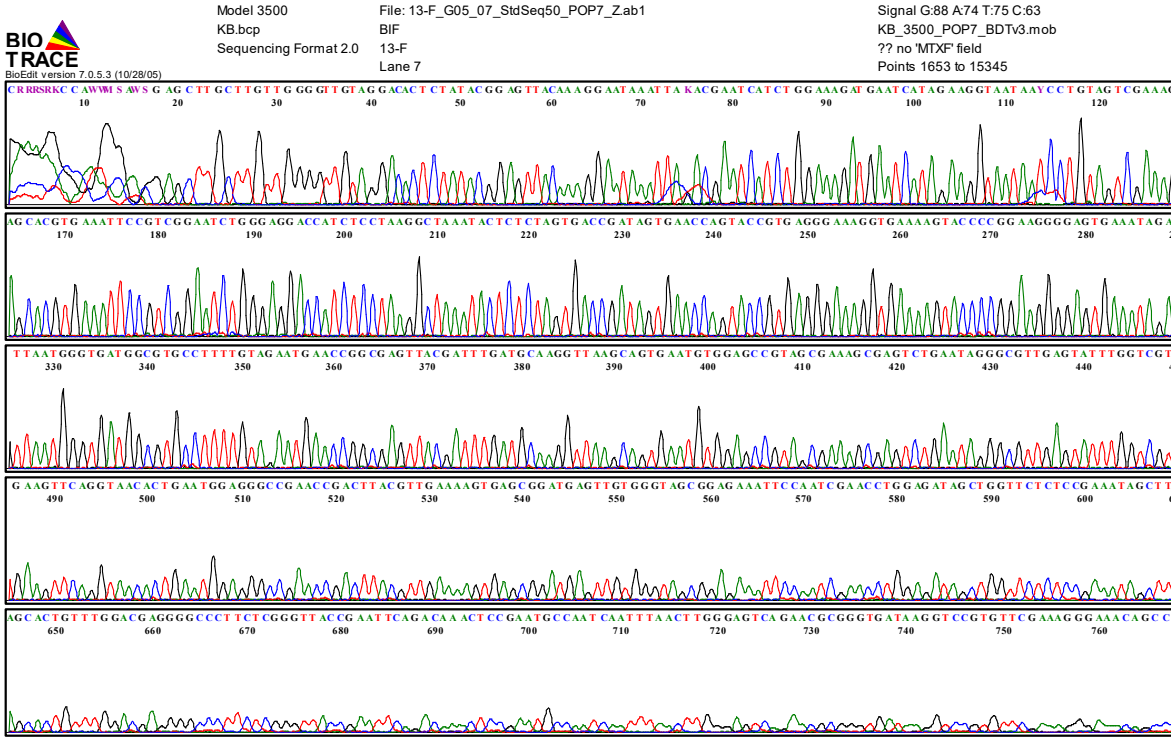
Sequence of *Staphylococccus aureus* 23S rRNA gene region using the same Forward and Reverse Primers as for PCR

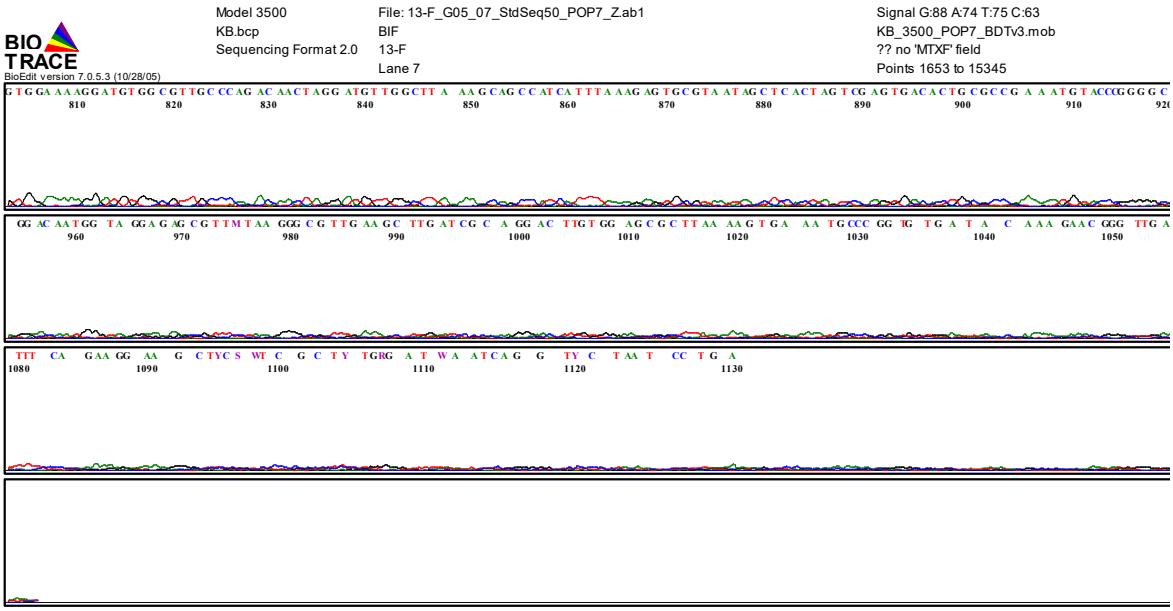
Forward Primer Sequence

>13-F (Underline genes are omitted during analysis)

CRRRSRKCCAWWMSAWSGAGCTTGCTTGTTGGGGTTGTAGGACACTCTATACGGAGTTACAAAGGAAT
AAATTAKACGAATCATCTGGAAAGATGAATCATAGAAGGTAATAAYCCTGTAGTCGAAAGTTTATTCTCT
CTTGAGTGGATCCTGAGTACGACGGAGCACGTGAAATTCCGTCGGAATCTGGGAGGACCATCTCCTAAG
GCTAAATACTCTCTAGTGACCGATAGTGAACCAGTACCGTGAGGGAAAGGTGAAAAGTACCCCGGAAG
GGGAGTGAAATAGAACTTGAAACCGTGTGCTTACAAGTAGTCAGAGCCCGTTAATGGGTGATGGCGTG
CCTTTTGTAGAATGAACCGGCGAGTTACGATTTGATGCAAGGTTAAGCAGTGAATGTGGAGCCGTAGCG
AAAGCGAGTCTGAATAGGGCGTTGAGTATTTGGTCGTAGACCCGAAACCAGGTGATCTACCCATGACCA
GGCTGAAGTTCAGGTAACACTGAATGGAGGGCCGAACCGACTTACGTTGAAAAGTGAGCGGATGAGTT
GTGGGTAGCGGAGAAATTCCAATCGAACCTGGAGATAGCTGGTTCTCTCCGAAATAGCTTTAGGGCTAG
CCTCAAGTGATGATTATTGGAGGTAGAGCACTGTTTGGACGAGGGGCCCTTCTCGGGTTACCGAATTCA
GACAAACTCCGAATGCCAATCAATTTAACTTGGGAGTCAGAACGCGGGTGATAAGGTCCGTGTTCGAAA
GGGAAACAGCCCAGACCACCAGCTAAGGTCCCAAAATATATGTTAAGTGGAAAAGGATGTGGCGTTGC
CCAGACAACTAGGATGTTGGCTTAAAGCAGCCATCATTTAAAGAGTGCGTAATAGCTCACTAGTCGAGT
GACACTGCGCCGAAATGTACCCGGGGCTAAAMATATTACCGAAGCTGTGGATTGTCCGTAGGACAATG
GTAGGAGAGCGTTMTAAGGGCGTTGAAGCTTGATCGCAGGACTTGTGGAGCGCTTAAAAGTGAAATGC
CCGGTGTGATACAAAGAACGGGTTGAAATCCCGTCCACCGAATGAYTAAGTTTCAGAAGGAAGCTYCSW
TCGCTYTGRGATWAATCAGGTYCTAATCCTGA

Chromatogram of Forward (F) primer



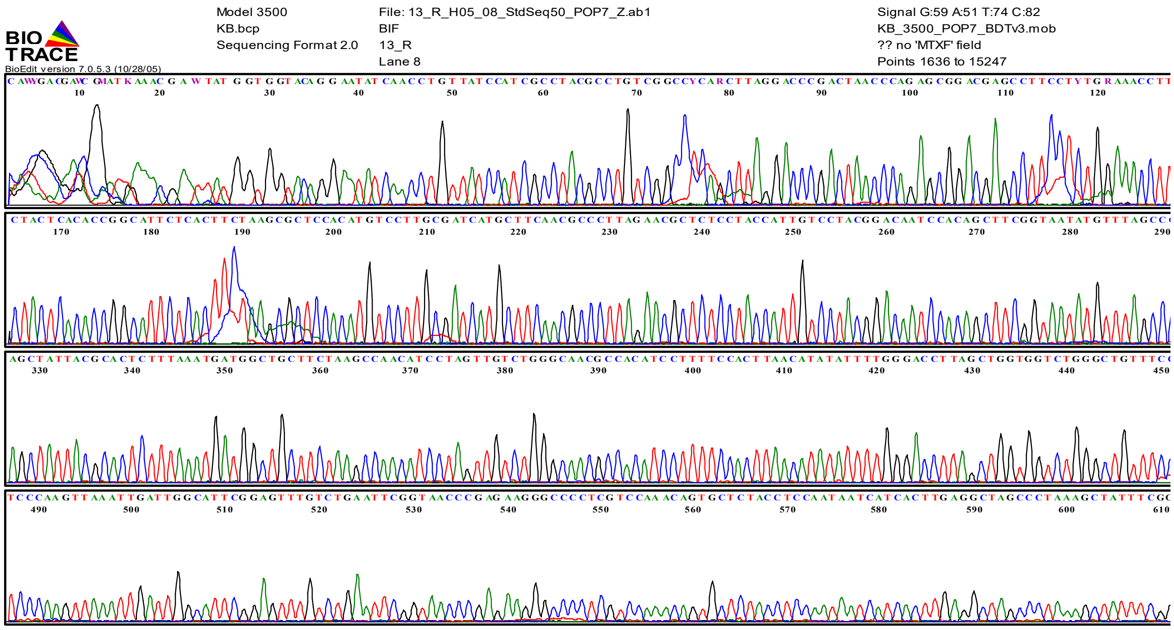


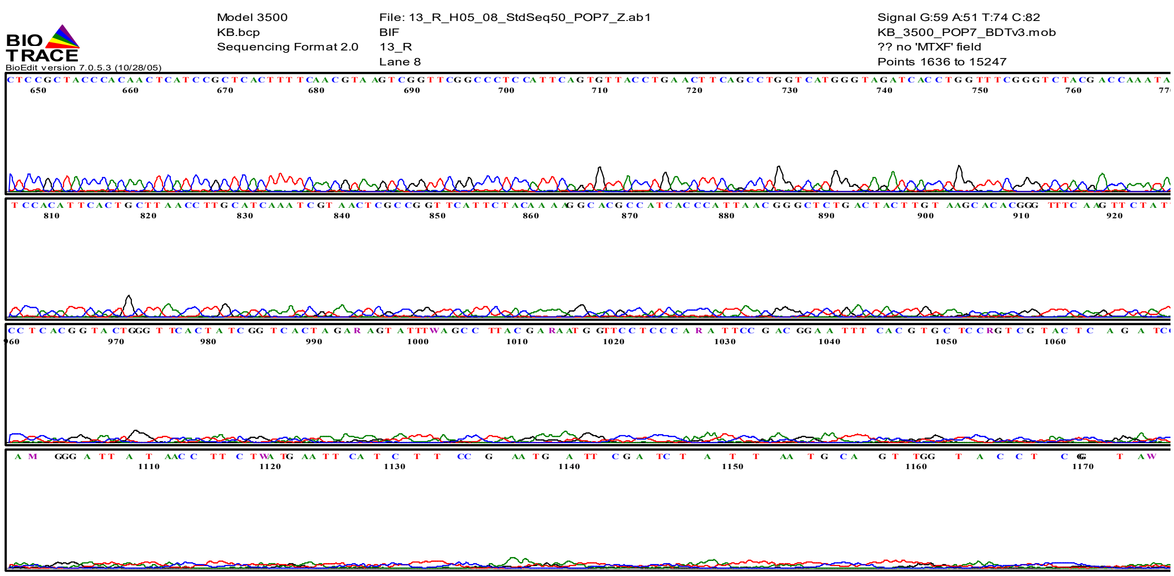
Reverse Primer Sequence

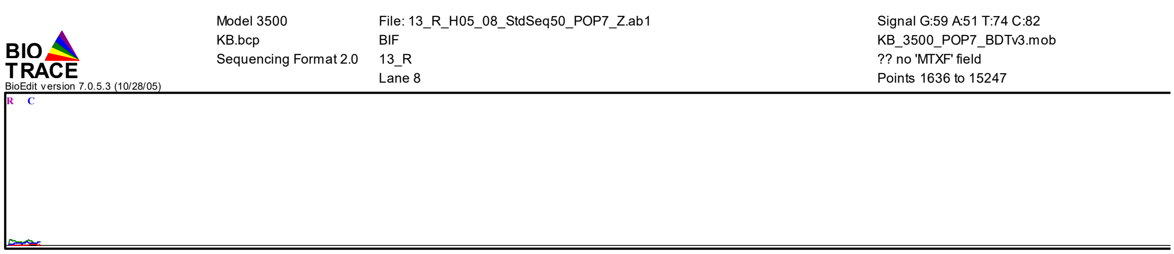
>13_R (Underline genes are omitted during analysis)

CAWYGACGAWCGMATKAAACGAWTATGGTGGTACAGGAATATCAACCTGTTATCCATCGCCTACGCCT
GTCGGCCYCARCTTAGGACCCGACTAACCCAGAGCGGACGAGCCTTCCTYTGRAAACCTTAGTCAATCGG
TGGACGGGATTCTCACCCGTCTTTCGCTACTCACACCGGCATTCTCACTTCTAAGCGCTCCACATGTCCTT
GCGATCATGCTTCAACGCCCTTAGAACGCTCTCCTACCATTGTCCTACGGACAATCCACAGCTTCGGTAAT
ATGTTTAGCCCCGGTACATTTTCGGCGCAGTGTCACTCGACTAGTGAGCTATTACGCACTCTTTAAATGAT
GGCTGCTTCTAAGCCAACATCCTAGTTGTCTGGGCAACGCCACATCCTTTTCCACTTAACATATATTTTGG
GACCTTAGCTGGTGGTCTGGGCTGTTTCCCTTTCGAACACGGACCTTATCACCCGCGTTCTGACTCCCAA
GTTAAATTGATTGGCATTCGGAGTTTGTCTGAATTCGGTAACCCGAGAAGGGCCCCTCGTCCAAACAGTG
CTCTACCTCCAATAATCATCACTTGAGGCTAGCCCTAAAGCTATTTCGGAGAGAACCAGCTATCTCCAGG
TTCGATTGGAATTTCTCCGCTACCCACAACTCATCCGCTCACTTTTCAACGTAAGTCGGTTCGGCCCTCCA
TTCAGTGTTACCTGAACTTCAGCCTGGTCATGGGTAGATCACCTGGTTTCGGGTCTACGACCAAATACTC
AACGCCCTATTCAGACTCGCTTTCGCTACGGCTCCACATTCACTGCTTAACCTTGCATCAAATCGTAACTC
GCCGGTTCATTCTACAAAAGGCACGCCATCACCCATTAACGGGCTCTGACTACTTGTAAGCACACGGGTT
TCAAGTTCTATTTCACTCCCCYTTCCGGGGTACTTTTCACCTTTCCCTCACGGTACTGGGTTCACTATCGGT
CACTAGARAGTATTTWAGCCTTACGARAATGGTTCCTCCCARATTCCGACGGAATTTCACGTGCTCCRGT
CGTACTCAGATCCMCTCAGAAGAGAAKTASASSSKTKTSSRACTAMGGGATTATAACCTTCTWATGAATT
CATCTTCCGAATGATTCGATCTATTAATGCAGTTGGTACCTCGGTAWGAGAGTGTYCCCTTACACRC

Chromatogram of Reverse (R) primer







Construction of Phylogenetic tree of *Staphylococcus aureus* (23S rRNA gene)

Phylogenetic tree of *Staphylococcus aureus* 23S rRNA is shown in Figure 6. During the time of sequence analysis unnecessary first and last genes were deleted from the sequence. In this research NCBI BLAST homology was 98% of the database. In the tree study isolates represented the evolutionary feature of *S. aureus*. Best match was found in case of *S. aureus* (Copenhagen, Denmark & UK).

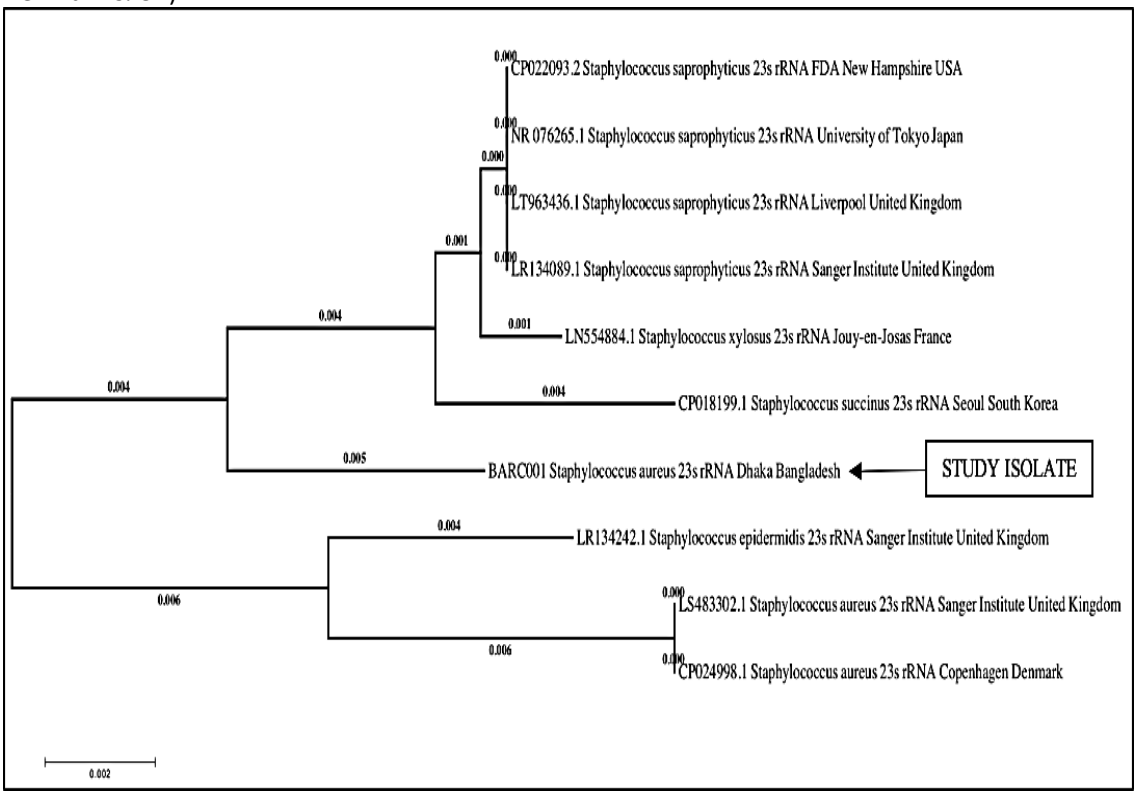


Figure 6: Phylogenetic tree of *Staphylococcus aureus* (23S rRNA)

Phylogenetic tree was constructed using the *Staphylococcus aureus* 23S rRNA gene sequence of the isolates from the present study and related Gene bank sequences. The tree was inferred using the clastalW multiple sequence alignment and Neighbour-Joining method according to Saitou and Nei (1987) using MEGA software (Kumar *et al.,* 2016). Bootstrap value was used as 1000 for tree clustering. The evolutionary history, the optimal tree with the sum of branch length = 0.03591732 is shown. Number at the nodes represents the level of bootstrap support based on the neighbour-joining analysis (Saitou N. and Nei M., 1987). The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura K. *et al.,* 2004) and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1034 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.,* 2016).

Due to limitation of time phylogenetic tree analysis of other organisms cannot be conducted.

Identification and Characterization of *Streptococcus agalactiae* by molecular technique

Identification by DNA amplification using PCR

Out of the 48 samples tested for *Streptococcus* sp. 28 samples (58%), were amplified with the Primer sets of Sag 40 (F) and Sag 445 (R) for 23S rRNA gene region. The positive samples produced bands at 405 bp positions as visualized after electrophoresis on 1.7% agarose gel (Figure 7)

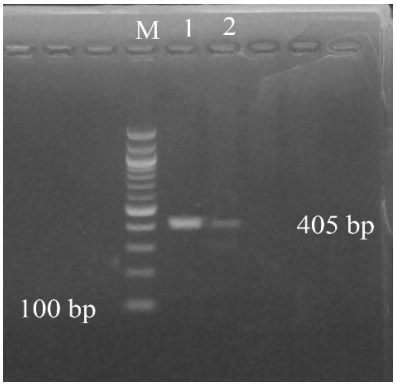


Figure 7: Electrophoresis (1.7% agarose gel) of PCR products containing *Streptococcus agalactiae* with specific primer. *Lane M= 1 kb plus DNA ladder marker. Lanes 1, 2= positive samples (positive band at 405 bp).*

Identification and Characterization of *Streptococcus* sp by molecular technique

Characterization by DNA amplification using PCR

Out of the 48 samples tested for *Streptococcus* sp. 28 samples (58%) were amplified with the Primer sets of Uni 1870 (F); Uni 2308 (R) for 23S rRNA gene region. The positive samples produced bands at 438 bp positions as visualized after electrophoresis on 1.7% agarose gel (Figure 8).

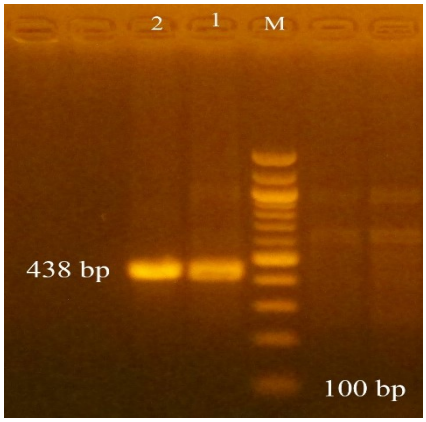


Figure 8: Electrophoresis (1.7% agarose gel) of PCR products containing *Streptococcus* sp. with universal primer. Lane M= 1 kb plus DNA ladder marker. Lanes 1, 2= positive samples (positive band at 438 bp).

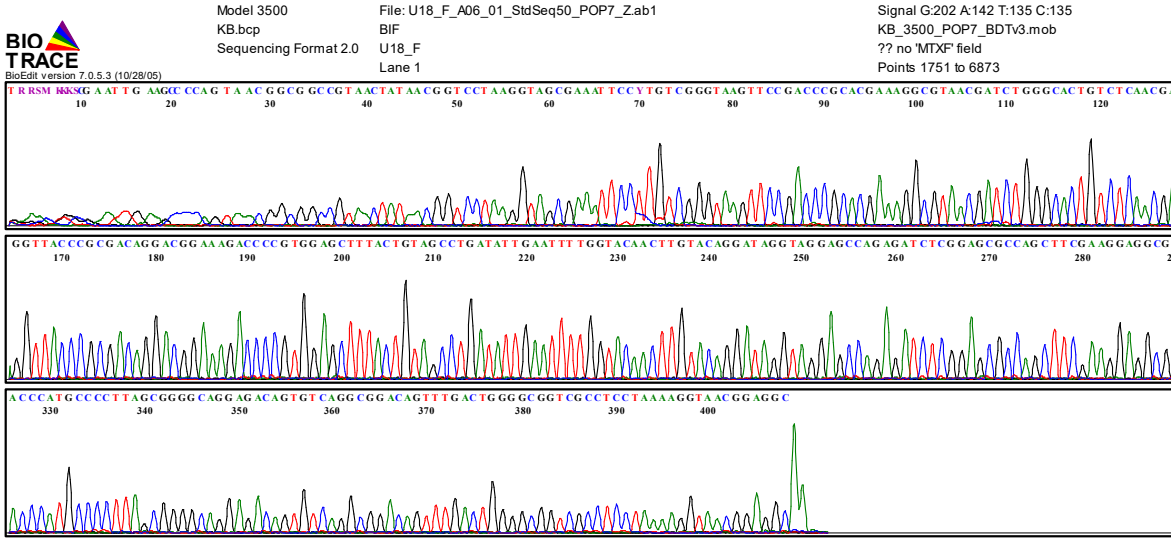
Sequence of *Streptococcus* sp. Forward and Reverse Primer

Forward Primer Sequence

>U18_F (Underline gene are omitted during analysis)

TRRSMKKKSCGAATTGAAGCCCCAGTAACGGCGGCCGTAACTATAACGGTCCTAAGGTAGCGAAATTCC
YTGTCGGGTAAGTTCCGACCCGCACGAAAGGCGTAACGATCTGGGCACTGTCTCAACGAGAGACTCGGT
GAAATTATAGTACCTGTGAAGATGCAGGTTACCCGCGACAGGACGGAAAGACCCCGTGGAGCTTTACTG
TAGCCTGATATTGAATTTTGGTACAACTTGTACAGGATAGGTAGGAGCCAGAGATCTCGGAGCGCCAGC
TTCGAAGGAGGCGTCGGTGGGATACTACCCTGGTTGTATTGAAATTCTAACCCATGCCCCTTAGCGGGG
CAGGAGACAGTGTCAGGCGGACAGTTTGACTGGGGCGGTCGCCTCCTAAAAGGTAACGGAGGC

Chromatogram of Forward (F) primer

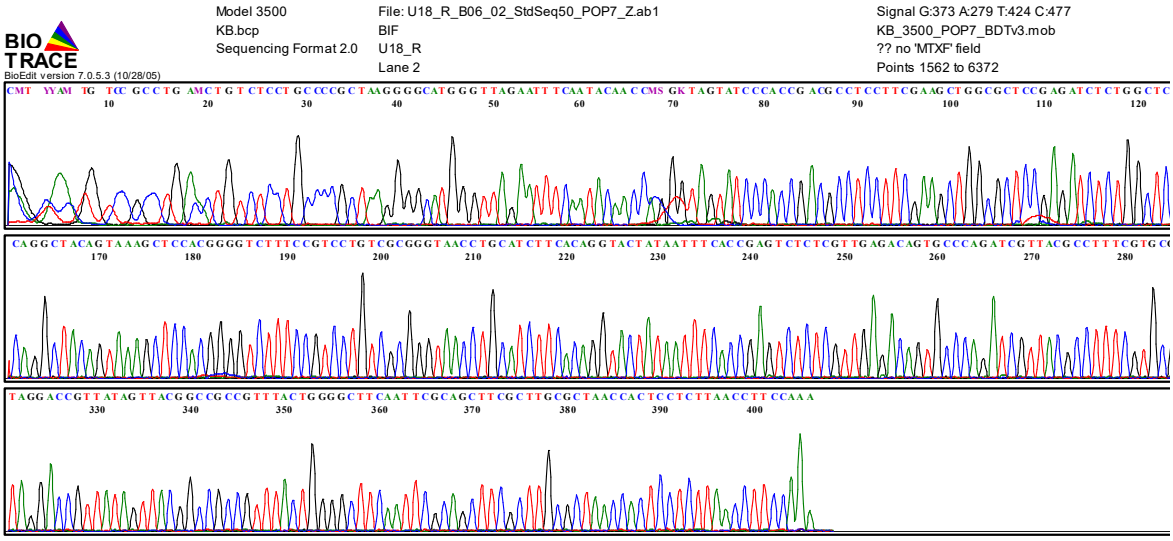


Reverse Primer Sequence

>U18_R (Underline gene are omitted during analysis)

CMTYYAMTGTCCGCCTGAMCTGTCTCCTGCCCCGCTAAGGGGCATGGGTTAGAATTTCAATACAACCM
SGKTAGTATCCCACCGACGCCTCCTTCGAAGCTGGCGCTCCGAGATCTCTGGCTCCTACCTATCCTGTACA
AGTTGTACCAAAATTCAATATCAGGCTACAGTAAAGCTCCACGGGGTCTTTCCGTCCTGTCGCGGGTAAC
CTGCATCTTCACAGGTACTATAATTTCACCGAGTCTCTCGTTGAGACAGTGCCCAGATCGTTACGCCTTTC
GTGCGGGTCGGAACTTACCCGACAAGGAATTTCGCTACCTTAGGACCGTTATAGTTACGGCCGCCGTTTA
CTGGGGCTTCAATTCGCAGCTTCGCTTGCGCTAACCACTCCTCTTAACCTTCCAAA

Chromatogram of Reverse (R) primer



Identification and characterization of *Klebsiella* sp. by molecular technique

Identification of DNA amplification using PCR

Out of the 48 samples tested for *Klebsiella* sp. 30 samples (62.5 %) were amplified with the sets of Universal Primers 27F and 1492R. The positive samples produced bands at 1492 bp positions as visualized after electrophoresis on 1.7% agarose gel (Figure 9).

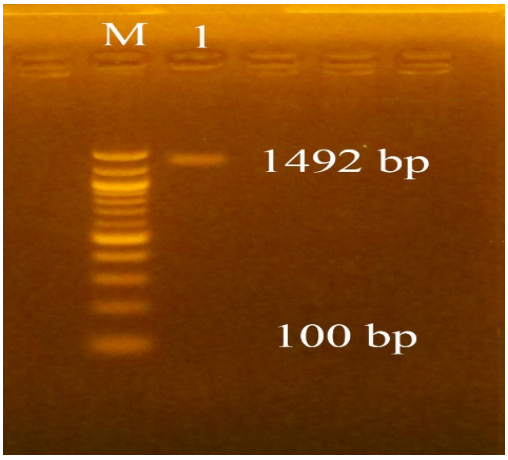


Figure 9: Electrophoresis (1.7% agarose gel) of PCR products containing *Klebsiella* sp with universal primer.
Lane M= 1 kb plus DNA ladder marker. Lanes 1= positive sample (positive band at 1492 bp).

Molecular Detection of *Streptococcus agalactiae* by RT-PCR (Real Time Polymerase Chain Reaction)

Extracted DNA was amplified with Go taq prob and reference primer. PCR max (Eco 48) showed the following graph (Figure 10). The graph indicates that the suspected samples contained *Streptococcus agalactiae.* Samples are arranged as 1, 2, 3, 4 and NTC up to down. The florescence vs cycle (40) given in APPENDIX-IV. Gradually upward graph indicates that samples contained suspected nucleic acid. DNA were detected by florescence.

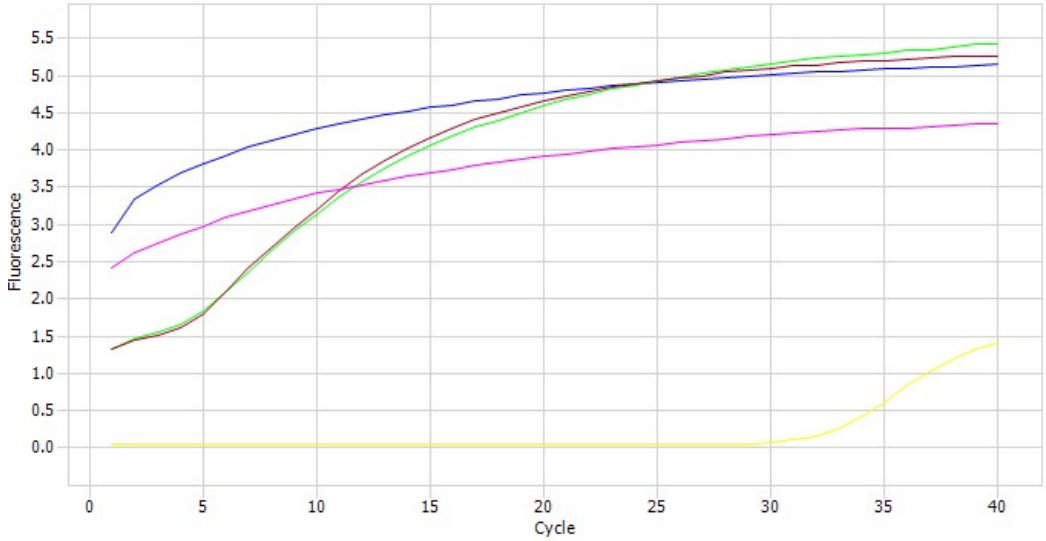


Figure 10: Results of RT- PCR of *Streptococcus agalactiae*

Yellow line is Negative control (NTC), Green, Purple, Pink and Blue lines are sample 1, 2, 3 and 4.

11.3. Development of vaccine

Polyvalent formalin killed vaccine against mastitis was prepared by adding 20 ml of adjuvant (Montanide @ ISA 206) with the mixture of formalin inactivated bacterial isolates of 20 ml volume (4 x 5 ml) as mentioned above in the methodology.

Quality control testing of the experimental vaccine

Sterility test of the vaccine

No growth of any organism on nutrient agar after incubation at 37°C for 24 hours proved that the prepared vaccine was sterile.

Safety test of the vaccine

Not appearance of any lesion, after 14 days, on the odder inoculated intramuscularly with the vaccine proved that the experimental vaccine was safe to use.

Efficacy and potency test of the vaccine

The experiments for efficacy and potency tests of the vaccine could not be performed due to time constraints.

12. Research highlight/findings:

- *E. coli*, *Streptococcus agalactiae*, *Streeptococcus* sp., *Klebsiella* sp. and *Staphylococcus aureus* were isolated and identified from bovine mastitis milk samples.
- Out of the total of 48 samples tested 100% were found positive for *E. coli* and *Staphylococcus* sp. On the other hand, the rate of occurrence of *Streptococcus* sp. and *Klebsiella* sp. was 28% and 30% respectively.
- The organisms were confirmed using PCR and Phylogenetic analysis
- Formalin killed vaccine was prepared from the developed vaccine seeds but the efficacy and potency of the experimental vaccine could not be tested due time constraints.

B. Implementation Position

1. Procurement:

Description of equipment and capital items		PP Target		Achievement		Remarks
		Phy (#)	Fin (Tk)	Phy (#)	Fin (Tk)	
(a) Office equipment		-	-	-	-	-
(b) Lab & field equipment		1	4,90,000	1	4,87,000	-
(c) Other capital items		-	-	-	-	-

2. Establishment/renovation facilities:

Description of facilities	Newly established		Upgraded/refurbished		Remarks
	PP Target	Achievement	PP Target	Achievement	
Molecular Biology Laboratory	-	-	Renovation of existing Molecular Biology Lab	100%	

3. Training/study tour/ seminar/workshop/conference organized: Not applicable

Description	Number of participant			Duration (Days/weeks/ months)	Remarks
	Male	Female	Total		
(a) Training	-	-	-	-	
(b) Workshop	-	-	-	-	

C. Financial and physical progress

Fig in Tk

Items of expenditure/activities	Total approved budget	Fund received	Actual expenditure	Balance/ unspent	Physical progress (%)	Reasons for deviation
A. Contractual staff salary	3,92,500	3,92500	3,92500	0.00	-	-
B. Field research/lab expenses and supplies	11,08,953	11,08,953	11,08,953	0.00	-	-
C. Operating expenses	96,953	96,953	96,953	0.00	-	-
D. Vehicle hire and fuel, oil & maintenance	-	-	-	-	-	-
E. Training/workshop/ seminar etc.	-	-	-	-	-	-
F. Publications and printing	65,000	-	-	0.00	-	-
G. Miscellaneous	49,594	49,594	49,594	0.00	-	-
H. Capital expenses	4,87,000	4,87,000	4,87,000	0.00	100%	-
Total	**22,00,000**	**21,35,000**	**21,35,000**	**0.00**		

D. Achievement of Sub-project by objectives: (Tangible form)

Specific objectives of the sub-project	**Major technical activities performed**	**Output (i.e. product obtained, visible, measurable)**	**Outcome**
To isolate and identify the microbial agents causing mastitis in cattle	Sample collection, isolation and identification of bacteria using morphological, biochemical and cultural characteristics.	*E. coli*, *Streptococcus agalactiae*, *Streeptococcus* sp., *Klebsiella* sp. and *Staphylococcus aureus* were isolated and identified from bovine mastitis milk samples. Out of the total of 48 samples tested 100% were found positive for *E. coli* and *Staphylococcus* sp. The rate of occurrence of *Streptococcus* sp. and *Klebsiella* sp. was 28% and 30% respectively.	Not applicable
To characterize the bacterial isolates using molecular identification methods	PCR, Sequencing of the targeted gene and Phylogenetic analysis	The organisms were confirmed using PCR and Phylogenetic analysis	
To develop vaccine against bovine mastitis from the selected bacterial isolates	Development of vaccine seeds, inactivation of the seeds, preparation of vaccine and testing for sterility and safety of the experimental vaccine.	Formalin killed polyvalent vaccine against mastitis was prepared containing the organisms *E. coli*, *Staphylococcus* sp., *Streptococcus* sp. and *Klebsiella* sp. The experimental vaccine was found to be sterile and safe. However, efficacy and potency test of the vaccine could not be carried out due time constraints.	

E. Materials Development/Publication made under the Sub-project:

Publication	Number of publication		Remarks (e.g. paper title, name of journal, conference name, etc.)
	Under preparation	Completed and published	
Technology bulletin/ booklet/leaflet/flyer etc.	-	-	-
Journal publication	-	**1**	Isolation of multi-drug resistant *Klebsiella* sp. from bovine mastitis samples in Rangpur, Bangladesh. *J. Adv. Vet. Anim. Res., 6(3): 362–365, September 2019*
Information development	-	-	-
Other publications, if any	-	-	-

F. Technology/Knowledge generation/Policy Support (as applied):

i. **Generation of technology (Commodity & Non-commodity)**
Formalin killed polyvalent vaccine developed however; more research with extra fund is needed before using the vaccine to control mastitis in Bangladesh.

ii. **Generation of new knowledge that help in developing more technology in future**

Formalin killed polyvalent vaccine against mastitis was prepared containing the organisms *E. coli*, *Staphylococcus* sp., *Streptococcus* sp. and *Klebsiella* sp. However, the vaccine needs to go through efficacy and potency tests in animals before declaring the vaccine to be effective against mastitis in the dairy cows in Bangladesh.

iii. **Technology transferred that help increased agricultural productivity and farmers' income**

Not applicable

iv. **Policy Support:**
Not applicable

G. Information regarding Desk and Field Monitoring

i) **Desk Monitoring:**
Done

ii) **Field Monitoring (time & No. of visit, Team visit and output):**

- First monitoring in 17.03.18 at 9.00 AM
- Second monitoring in August 2017
- Comments/Output: Progress of the project was rated as poor. Suggested to expedite the activities.

H. Lesson Learned (if any)

This type of project, related to polyvalent vaccine production using local isolates needs more time and adequate funding.

I. Challenges

To keep pace with the time was a big challenge.

Signature of the Principal Investigator
Date …….

Seal

Counter signature of the Head of the organization/authorized representative
Date …….
Seal

Referemces

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APPENDIX-I

RT-PCR thermal condition and cycle

Application	Quantification/DNA Binding Dye/DNA/Standard Curve				
Instrument	Eco™ Real-Time PCR system				
Software	Eco™ Software v5.2.13.0				
Last Date Saved	10/13/2018 7:44 PM				
Stage	**Step**	**Temperat**	**Duration**	**Cycles**	
UDG Incubation	Step 1	95	00:02:00	1	
PCR Cycling	Step 1	95	00:00:15	40	
PCR Cycling	Step 2	60	00:01:00	40	
Total Program Length	1 Hour 11 Minutes 55 Seconds				
Total Cycle Count	40				

Dye	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6	Cycle 7	Cycle 8	Cycle 9	Cycle 10	Cycle 11	Cycle 12	Cycle 13	Cycle 14	Cycle 15	Cycle 16	Cycle 17	Cycle 18	Cycle 19
Green	2.880007	3.34231	3.519588	3.689879	3.819573	3.929488	4.038529	4.118423	4.207088	4.281491	4.351535	4.411277	4.473271	4.517032	4.573188	4.608299	4.660885	4.687731	4.73625
Green	1.328606	1.479678	1.542883	1.651734	1.831785	2.079789	2.359604	2.633048	2.901466	3.140895	3.369546	3.568356	3.745846	3.913674	4.056305	4.191749	4.304792	4.40215	4.504251
Green	1.321765	1.456359	1.514239	1.618237	1.794311	2.096821	2.413888	2.688327	2.946685	3.207025	3.454114	3.665895	3.856071	4.02076	4.165494	4.28481	4.404061	4.505564	4.584437
Green	2.42033	2.614328	2.737939	2.872685	2.979505	3.089204	3.182007	3.261251	3.336213	3.418867	3.475622	3.538046	3.596641	3.647828	3.698021	3.743554	3.7999	3.841995	3.873472
Green	0.045431	0.044777	0.045986	0.044537	0.044953	0.044443	0.044267	0.044297	0.043697	0.04389	0.043967	0.043573	0.04371	0.043354	0.043645	0.043127	0.043418	0.043195	0.043304

Cycle 20	Cycle 21	Cycle 22	Cycle 23	Cycle 24	Cycle 25	Cycle 26	Cycle 27	Cycle 28	Cycle 29	Cycle 30	Cycle 31	Cycle 32	Cycle 33	Cycle 34	Cycle 35	Cycle 36	Cycle 37	Cycle 38	Cycle 39	Cycle 40
4.765111	4.796526	4.828856	4.85931	4.876697	4.907131	4.9337	4.945876	4.975598	4.998267	5.008364	5.027895	5.045528	5.06064	5.078469	5.089412	5.097538	5.109424	5.122261	5.138839	5.146072
4.598144	4.680151	4.747854	4.818747	4.864054	4.935836	4.969417	5.025741	5.06603	5.107551	5.162041	5.194561	5.227653	5.256272	5.28572	5.3076	5.338617	5.348086	5.386701	5.413895	5.42886
4.665592	4.726608	4.78091	4.852996	4.886083	4.920947	4.964331	4.995809	5.045824	5.076105	5.096025	5.125663	5.140997	5.166789	5.185843	5.197444	5.224754	5.245921	5.250039	5.249306	5.263665
3.917473	3.948448	3.977475	4.016627	4.043467	4.072989	4.10063	4.130407	4.15079	4.177388	4.204919	4.237251	4.248535	4.26207	4.28739	4.290951	4.295487	4.30793	4.326595	4.358084	4.354987
0.043449	0.043263	0.043101	0.043422	0.0429	0.043873	0.045032	0.046838	0.051519	0.058977	0.074891	0.10488	0.161376	0.26166	0.412606	0.60839	0.822851	1.025135	1.189092	1.317157	1.414816

APPENDIX II

Questionnaire for the surveillance/investigation of Bovine Mastitis

1. Particulars of the owner: Date: ……

- Name: ……..…… Vill: ……Post: ……District: ……..
- Mobile: ……

2. Particulars of Farm & House:

Small	Medium	Large

- Type of floor: Concrete ☐ Other …….
- Hygienic condition: Poor ☐ Good ☐ Very Good ☐
- Feeding: Ready feed Other……
- Time of Feeding: …… Time /day
- Watering: …… Time /day
- Floor Cleaning: …… Time /day
- Washing house by Antiseptic powder ☐ Disinfectant ☐ Water ☐
- Washing of Animal daily ☐ weakly ☐ monthly ☐

3. Disease History:

- Deworming: ☐
- History:
- Clinical sign:

- **Milk Color:**
- **Test:**
- Types of Disease: Bacterial ☐ Viral ☐ Fungal ☐ Parasitic ☐ hers
- Name o ☐ ase: ……

1. Vaccination:

Name of vaccine	Age (day)	Remarks

5. Treatment given: Yes ☐ No ☐

Name of medicine used	For	Remarks

Opinion of farmer about disease: