Pathogenic Bacterial Strains Identified and Isolated From Goat Milk

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CHAPTER-I

1. INTRODUCTION

Goat milk and their products are nutritionally versatile,due to their potential nutraceutical properties. Goat milk can get contaminated by spoilage microorganisms (mainly bacteria) during various stages of processing and storage from farm. Previously, some of the pathogenic and spoilage bacteria such has *Listeria* and *Micrococcus* have been isolated from fresh raw goat milk in various parts of the world. However, the count might increase upto 100 fold or more once stored at ambient temperature for an extended period of time

Hence, the main objective to undertake the present study was to screen for the microbiological quality of fresh goat milk collected from two popular, small-scale dairy farms in karakudi, Tamil Nadu, India. Results of in this study are expected to be useful for health conscious consumers, as well as the local economy.

Goat milk samples were analyzed for the prevalence prevalence of selected bacterial pathogens. Enumeration of total plate count (TPC), Enumeration of *Coliforms, E. coli and K. pneumoniae* in goat milk was performed by employing three-tube most probable number (MPN) technique. The typical colony found was confirmed based on their IMViC pattern based on BAM method. For determination of *Salmonella* in samples, International Standard Organization protocol (ISO, 1990) was employed and the modified method described by the Food and Drug Administration (FDA) was employed (Westoo and Peterz, 1992; FDA, 2001). Presumptive *Listeria species* isolates were confirmed based on Gram reactions and catalase tests.

1.2 Statistical analysis

The bacterial counts of milk samples were converted into logarithm of number of colony forming units per ml (log CFU/ml) for statistical analysis, 95% confidence level (significance level at $P \leq 0.05$).

Chapter-II

2. Aim And Objectives

1. To isolate bacteria from preserved goat milk.
2. To identify the isolated bacteria by biochemical and molecular techniques.
3. To standardize DNA isolation.
4. To optimize PCR amplification of 16S rRNA gene.
5. To evaluate antimicrobial activity against human pathogenic bacteria.

Chapter-III

1. Review Of Literature

Conventional histological staining methods, including stain selection and techniques for specific situations, have long been established. DNA isolation is aprocess of purification of DNA from sample using a combination of physical and chemical methods. The first isolation of DNA was done in 1869 by Friedrich Miescher (Dahm, 2008). Currently it is a routine procedure in molecular biology or forensic analyses. For the chemical method, there are many different kits are used for extraction, and selecting the correct one will save time on kit optimization and extraction procedures.

First study about chemical composition of cell was done by Friedrich Miescher. In 1869, he used leukocytes that he collected from the samples on fresh surgical bandages and conducted experiments to purify and classify proteins contained in these cells. During his experiments he identified a novel substance in the nuclei, which he called “nuclein” (Dahm .2005). He then developed two protocols to separate cells’ nuclei from cytoplasm and to isolate this novel compound, nowadays known as DNA, which differed from proteins and other cellular substances. (Holmes, 2001). This scientific finding, along with the isolation protocols used, was published in 1871 in collaboration with his mentor, Felix Hoppe-Seyler (Dahm, 2005). However, it was only in 1958 that Meselson and Stahl (*Meselson et al*, 1958.), developed a routine laboratory procedure for DNA extraction. They performed DNA extraction from bacterial samples of *Escherichia coli* using a salt density gradient centrifugation protocol. Since then, DNA extraction techniques have been adapted to perform extractions on many different types of biological sources (*Meselson et al*, 1958.).

DNA extraction methods follow some common procedures aimed to achieve effective disruption of cells, denaturation of nucleoprotein complexes, inactivation of nucleases and other enzymes, removal of biological and chemical contaminants, and finally DNA precipitation.

DNA precipitation is achieved by adding high concentrations of salt to DNA-containing solutions, as cations from salts such as ammonium acetate counteract repulsion caused by the negative charge of the phosphate backbone. A mixture of DNA and salts in the presence of solvents like ethanol (final concentrations of 70%–80%) or isopropanol (final concentrations of 40%–50%) causes nucleic acids to precipitate. Some protocols include washing steps with 70% ethanol to remove excess salt from DNA. Finally, nucleic acids are resuspended in water or TE buffer (10 mM Tris, 1 mM ethylenediaminetetraacetic acid [EDTA]) (Price *et al* 2009*)* TE buffer is commonly used for long-term DNA storage because it prevents it from being damaged by nucleases, inadequate pH, heavy metals, and oxidation by free radicals. Tris provides a safe pH of 7–8, and EDTA chelates divalent ions used in nuclease activity and counteracts oxidative damage from heavy metals (Herzer 2001).

Agarose gel is a three-dimensional matrix formed of helical agarose molecules in supercoiled bundles that are aggregated into three-dimensional structures with channels and pores through which biomolecules can pass (*Joseph Sambrook et al*).The 3-D structure is held together with hydrogen bonds and can therefore be disrupted by heating back to a liquid state. The melting temperature is different from the gelling temperature, depending on the sources; agarose gel has a gelling temperature of 3542 °C and a melting temperature of 85-95 °C.

Agarose gel has large pore size and good gel strength, making it suitable as an anticonvection medium for the electrophoresis of DNA and large protein molecules. The pore size of a 1% gel has been estimated from 100 nm to 200-500 nm, and its gel strength allows gels as dilute as 0.15% to form a slab for gel electrophoresis (Philip Serwer 1983). Low-concentration gels (0.1 - 0.2%) however are fragile and therefore hard to handle. Agarose gel has lower resolving power than polyacrylamide gel for DNA but has a greater range of separation, and is therefore used for DNA fragments of usually 50-20,000 bp in size. The limit of resolution for standard agarose gel electrophoresis is around 750 kb, but resolution of over 6 Mb is possible with pulsed field gel electrophoresis (*Joseph Sambrook et al*). It can also be used to separate large proteins, and it is the preferred matrix for the gel electrophoresis of particles with effective radii larger than 5-10 nm. A 0.9% agarose gel has pores large enough for the entry of bacteriophage T4 (Philip Serwer 1983).

Polymerase chain reaction (PCR) is a technique used in molecular biology to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

Developed in 1983 by Kary Mullis, (*Bartlett et al* 2003) PCR is now a common and often indispensable technique used in clinical laboratories and research laboratories for a variety of applications (*Saiki et al* 1985). These include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensic sciences and

DNA paternity testing); and the detection of pathogens in nucleic acid tests for the diagnosis of infectious diseases. In 1993, Mullis was awarded the Nobel Prize in Chemistry along with Michael Smith for his work on PCR.

Chapter-IV

3. Material And Methods

4.1 Sample Collection

4.1.1 Milk Samples

Sample collection was carried out on a goat's farm in the karaikudi, Tamil Nadu, India during lactation period after weaning the kids at regular intervals: total of 48 samples of raw goat's milk and 40 samples of pasteurized goat's milk were obtained. On the farm, there were 75 goats of the white short-haired breed in the 1st to 8th lactation. The average daily milk yield is 2–3 liter and the average annual milk yield is 600–800 liters.

4.1.2 Milk Processing

A thorough pre-milking semi-dry udder cleaning is carried out. After milking, the milk is cooled down promptly to 4–6 °C and then stored for 12–24 hours until further processing, i.e. stationary pasteurization in a tank at 72 °C for 20 seconds.

4.2 Sampling

Milk samples were collected after cooling at 4–6 °C and pasteurized milk samples were collected after the heat treatment and subsequent cooling at 4–6 °C. The samples were transported to the laboratory at a maximum temperature of 8 °C and processed.

4.3 Morphological Identification

4.3.1 Staining Technique

Gram's Staining

1. A thin smear of each culture onto separate glass slides was made and air dried and heat fixed.
2. The bacterial smear was covered with a few drops of crystal violet and allowed it to set for 30-60 seconds.

3. The slides were gently rinsed with water.
4. Then the smear was covered with a few drops of Gram's iodine and allowed it to set for 60 seconds and then rinsed with water.
5. Then the slides were rinsed with 95% ethanol (decolorizer), drop by drop, just until the alcohol rinses clear (decolorization). (Be careful not to over-decolorize).
6. The bacteria was stained with a few drops of safranin allowed it to set for 30 seconds then rinsed with water and allowed to air dry
7. The slides were observed under oil immersion.

Capsule Staining

1. Place a small drop of a negative stain (India Ink, Congo Red, Nigrosin, or Eosin) on the slide.

   Congo red is easier to see, but it does not work well with some stains, India Ink generally works, but it has tiny particles that display Brownian motion that must be differentiated from your bacteria. Nigrosin may need to be kept very thin or diluted.

2. Using sterile technique, add a loopful of bacterial culture to slide, smearing it in the dye.
3. Use the other slide to drag the ink-cell mixture into a thin film along the first slide and let stand for 5-7 minutes.
4. Allowed to air dry (do not heat fix).
5. Flood the smear with crystal violet stain (this will stain the cells but not the capsules) for about 1 minutes. Drain the crystal violet by tilting the slide at a 45˚ angle and let stain run off until it air dries.
6. Examine the smear microscopically (100X) for the presence of encapsulated cells as indicated by clear zones surrounding the cells.

Negative Staining

1. Place a very small drop (more than a loop full, less than a free falling drop from the dropper) of nigrosin near one end of a well-cleaned and flamed slide.
2. Remove a small amount of the culture form the slant with an inoculating loop and disperse it in the drop of stain without spreading the drop.
3. Use another clean slide to spread the drop of stain containing the organism.

Simple Staining

1. Using a sterilized inoculating loop, transfer loopful of liquid suspension containing bacteria to a slide (clean grease free microscopic slide) or transfer an isolated colony from a culture plate to a slide with a water drop.
2. Dispersed the bacteria on the loop in the drop of water on the slide and spread the drop over an area the size of the dime. It should be a thin, even smear.
3. Allowed the smear to dry thoroughly.
4. Heat-fix the smear cautiously by passing the underside of the slide through the burner flame two or three times. It fixes the cell in the slide. Do not overheat the slide as it will distort the bacterial cells.

4.4 Motility Test

Hanging Drop Method

1. A drop of the bacterial culture (optimally from a young broth culture) was placed in the middle of a cover slip.
2. A thin line of petroleum jelly was placed around the edge of the cover slide.
3. The depression slide was turned to upside-down (depressed area facing down) and gently touches the cover slide. (The jelly holds the cover slip to the slide and also keeps the suspension from drying out).
4. Then the entire microscope slide/cover slip combination was turned over and observed under microscope.

4.5 Biochemical Tests

Indole Test

1. Indole medium (tryptophan broth) was inoculated with test bacterium and incubated at optimum temperature for 48 hours.
2. After incubation, 4 to 5 drops of Indole Reagent (Kovacs) was added to down the inner wall of the tube.

Methyl Red (MR) Test

MR/VP broth was inoculated with a pure culture of test organism and incubated at optimum temperature for 48 to 72 hours (depends on the growth rate of the bacterium). After incubation, 5 drops of methyl red reagent was added directly to the broth.

Voges-Proskauer Test

1. MR/VP broth was inoculated with a pure culture of test organism and incubated at optimum temperature for 48 to 72 hours (depends on the growth rate of the bacterium).

2. 6 drops (0.6 ml) of 5% α-naphthol, followed by 2 drops (0.2 ml) of 40% KOH was added. (KOH must be added last. If KOH is added first a false positive may be obtained).

3. The tube was vigorously shaken to expose the medium to atmospheric oxygen and allow the tube to remain undisturbed for 5 to 15 min. The tube can be rested at an angle to increase the surface area of the media (greater exposure to atmospheric oxygen).

Citrate Utilization Test

Cimmons citrate agar slant was inoculated with a pure culture of the test organism and incubated at optimum temperature for 48 hours.

4.6 Dna Isolation

Procedure

1. The cells were grown overnight in nutrient rich broth (Nutrient broth).
2. 1.5ml of culture was transferred to a tube and centrifuged at 10,000 rpm for 2 minutes.
3. The pellets were collected and repeated the centrifugation with another 1.5ml of culture containing cells.
4. Drained the tubes on a paper towel briefly.
5. 400µl of solution 1 and 100µl of solution 2 and 10µl of 10% SDS were added.
6. 5µl of proteinase K (20mg/ml) was added.
7. Incubate at 55˚c for 2 hours.
8. After incubation it was chilled on ice for 10 minutes.
9. 250µl of 6M Nacl was added.
10. Again it was kept on freezer for 5 minutes.
11. After freezing the sample was spinned at 8000 rpm for 15minutes.
12. 500µl of supernatant was taken and transferred into a new 1.5ml tube.
13. 1ml of 100% ice cold ethanol was added and inverted several times.
14. Again the sample was spinned at 10,000 rpm for 15 minutes.
15. The supernatant was removed and rinse with 500µl of 70% ethanol.
16. The sample was spinned at 10,000rpm for 5 minutes.
17. The supernatant was removed and dry the pellet at room temperature.

18. 100µl of 1X TE buffer was added to the pellet.
19. 5µl of DNA sample was added to the 0.8% agarose gel.
20. Visualized under the UV Transilluminator.

Agarose Gel Electrophoresis

1. 0.24g of agarose in 30ml of TAE buffer was mixed.
2. The agarose solution was boiled till get a clear solution.
3. 1.5µl of EtBr was added the solution gets completely cooled..
4. The clear solution was poured in a gel casting plate with already adjusted gel comb.
5. The casting tray was cooled at room temperature for 30 minutes for solidification.
6. After solidified, 5µl of DNA sample with 2µl of loading buffer were mixed and load in the well.
7. Run the gel 50V for about 20 minutes.
8. Observed the bands in UV light.

Polymerase Chain Reaction

Primer Mix

1. Eubac 5'-AGAGTTTGATCCTGGCTC-3'
2. 1492RA 5'-GGTTACCTTGTTACGACTT-3'

Master Mix Components

1.	Distelled water	-	16µl
2.	10X Assay buffer	-	2.5µl
3.	Primer mix	-	0.5µl
4.	dNTPs mix	-	2µl
5.	Mgcl (30mM)	-	3.0µl
6.	Taq polymerase	-	0.5µl
7.	Template DNA	-	1µl

Pcr Programme For 16s rRNA

Polymerase chain reactions for EUBAC gene can be performed by following the temperature and timing condition programmed in a thermal cycler.

1. Initial denaturation at 95˚c for 5 minutes.
2. Number of cycles 30.

3. Denaturation at 94˚c for 1 minute.
4. Annealing at 45˚c for 45 seconds.
5. Extension at 72˚c for 1 minute.
6. Final extension at 72˚c for 10 minutes.
7. Check the amplified products in 1.5% Agarose gel electrophoresis and the molecular weight was assessed using molecular weight marker (100bp ladder).

4.7 Antimicrobial Activity

1. The broth culture was prepared of test samples and human pathogens.
2. The broth was incubated at over night.
3. The nutrient agar plates were prepared and named properly.The standard antibiotic plates were prepared and named as duplicate plates.
4. The plated were allowed to solidify.
5. After solidification the human pathogens were inoculated by using cotton swab method.
6. Prepare well by using micropipette tips.
7. The test sample were added to the well as concentration about 20µl , 40µl, 60µl.
8. Place the plates in incubator for 24 hours.
9. Observed the plates and note the zone formation.

Sequencing Editing

The obtained sequences were edited based on the electropherogram peak clarities. Sequences with noisy peaks were excluded from the analysis. The sequences were assessed to check the insertion or deletions and codons in MEGA 5.0 software.

Sequencing Characterization

Multiple sequence alignment and pairwise sequence alignment were performed using Clustal W program implemented in MEGA 5.0 in all the sequences. Nucleotide differences were carefully monitored and the differences were observed and edit manually. Sequences were translated into amino acid sequences using vertebrate mitochondrial codon pattern in the MEGA 5.0 for checking the pseudo-gene status. All the sequences were correctly translated into amino acid sequences with their respective starting primers without any internal stop codon.

Blast Search

The amplified sequences of EUBAC were confirmed by similarity index built in the NCBI's BLAST program. Based on the percentage similarity and query coverage against the reference species, the species were confirmed.

Chapter-V

1. Results

5.1 Staining Of Bacteria

TABLE: 1 Illustrated That Staining Of Bacteria

	Staining Techniques	Figure A *Mycobacterium tuberculosis*	Figure B *Pseudomonas aeroginosa*
1.	Gram's staining	Positive	Positive
2.	Capsule staining	Negative	Negative
3.	Negative staining	Negative	Negative
4.	Simple staining	Negative	Negative
5.	Motility test	Negative	Negative
6.	Acid fast staining	Positive	Negative

5.2 Biochemical Tests

Table: 2 Illustrated That Biochemical Tests

	Biochemical Tests	Figure A *Mycobacterium tuberculosis*	Figure B *Pseudomonas aeroginosa*
1.	Indole test	Negative	Negative
2.	Methyl red test	Negative	Negative
3.	Vogesproskauer test	Negative	Negative
4.	Simmon citrate agar test	Negative	Positive

5.3 Antimicrobial Activity

Table: 3 Illustrated That Antimicrobial Activity

	Antimicrobial Activity	*Mycobacterium tuberculosis*	*Pesudomonas aeroginosa*
1.	*Klebsillela pneumoniae*	Positive	Positive

STAINING TECHNIQUES

GRAM'S STAINING

A) B)

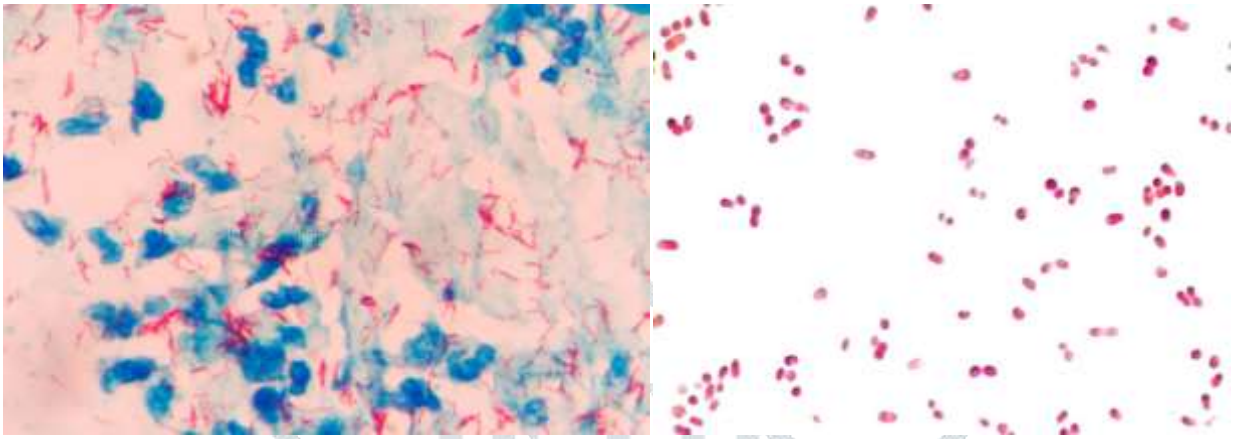


FIGURE 1.1 illustrated that GRAM'S STAINING

The gram staining technique figure A is illustrated that the both gram positive and gram negative for *Mycobacterium tuberculosis.*

The gram staining technique figure B is illustrated that the gram negative for *Pseudomonas aeruginosa*.

CAPSULE STAINING

A) B)

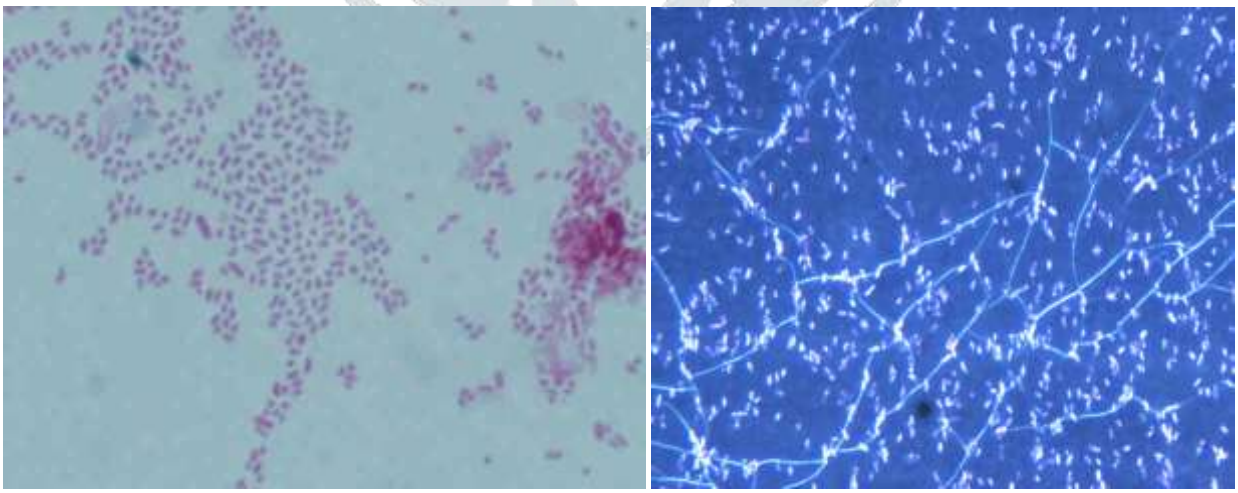


FIGURE 1.2 illustrated that CAPSULE STAINING

The capsule staining technique figure A illustrated that the Negative for *Mycobacterium tuberculosis.*

The capsule staining technique figure B illustrated that the Negative for *Pseudomonas aeruginosa.*

NEGATIVE STAINING

A) B)

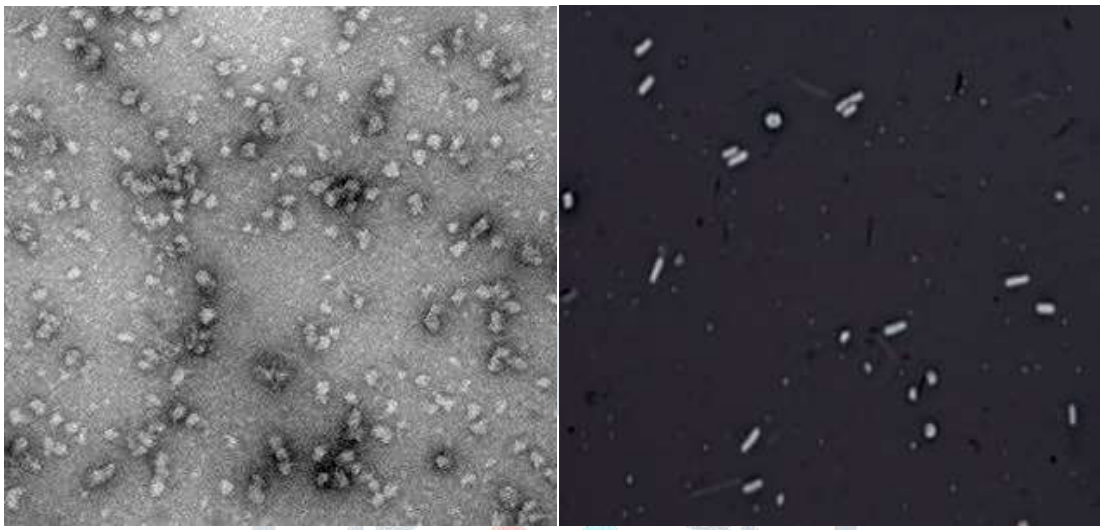


FIGURE 1.3 illustrated that NEGATIVE STAINING

The negative staining technique figure A illustrated that the negative for *Mycobacterium tuberculosis.*

The negative staining technique figure B illustrated that the negative for *Pseudomonas aeruginosa.*

SIMPLE STAINING

A) B)

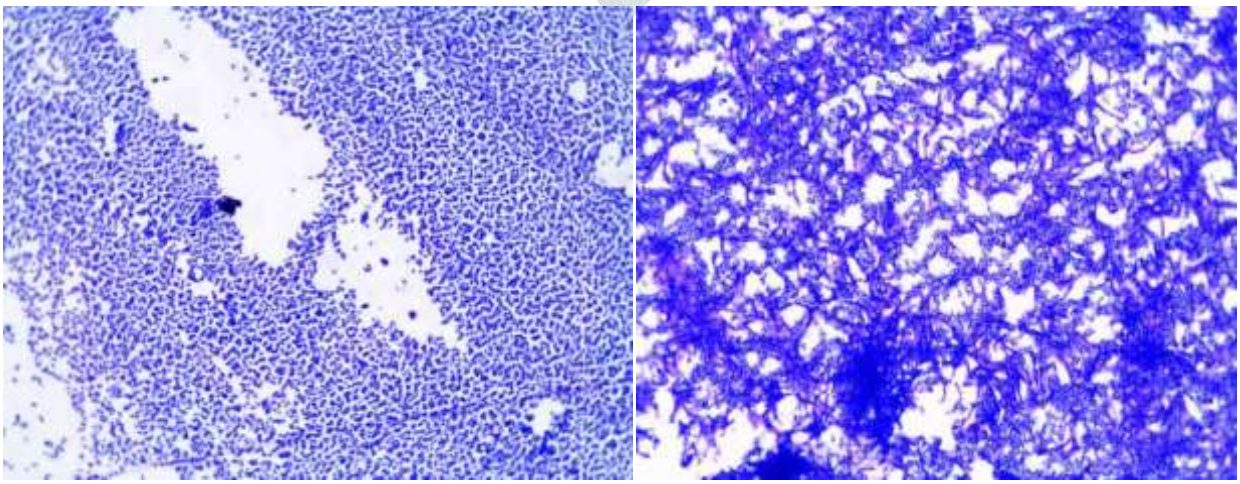


FIGURE 1.4 illustrated that SIMPLE STAINING

The simple staining technique figure A illustrated that the negative for *Mycobacterium tuberculosis.*

The simple staining technique figure B illustrated that the negative for *Pseudomonas aeruginosa.*

MOTILITY TEST

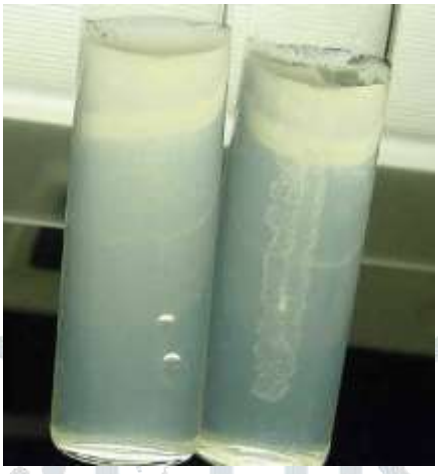


FIGURE 1.5 illustrated that MOTILITY TEST

The methyl test result shown in the figure left is negative for *Mycobacterium tuberculosis.*

The methyl test result shown in the figure right is negative for *Pseudomonas aeruginosa.*

ACID FAST STAINING

A) B)

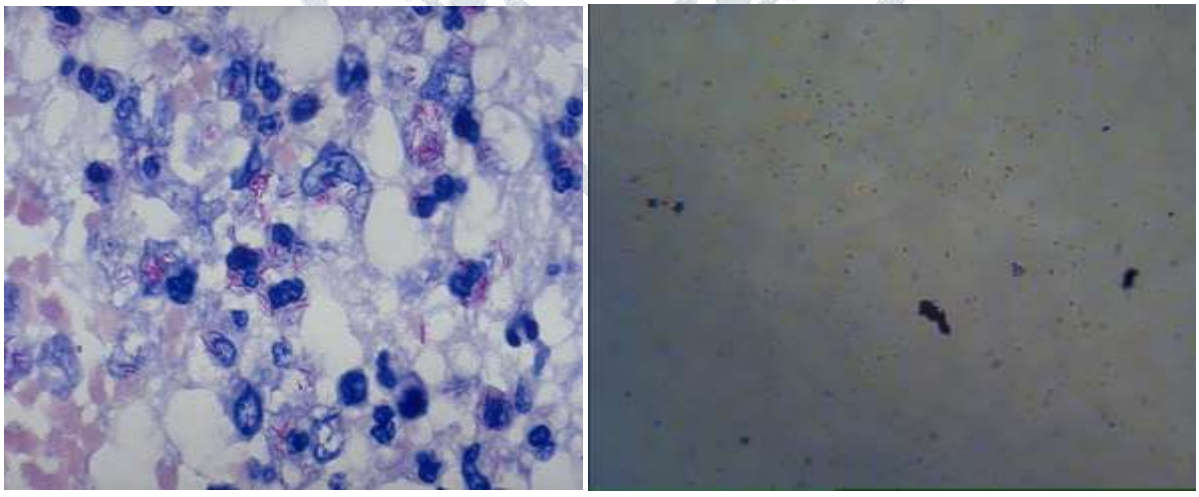


FIGURE 1.6 illustrated that ACID FAST STAINING

The acid fast staining technique figure A illustrated that the positive for *Mycobacterium tuberculosis.* The acid fast staining technique figure B illustrated that the negative for *Pseudomonas aeruginosa*.

BIOCHEMICAL TESTS

INDOLE TEST

A) B)

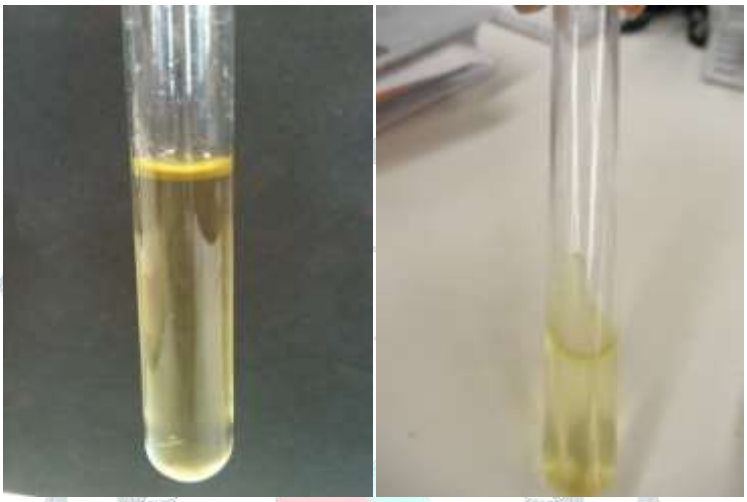


FIGURE 2.1 Illustrated that INDOLE TEST

The indole test result showed in the figure A and B is illustrated that the negative for both *Mycobacterium tuberculosis and Pseudomonas aeruginosa.*

METHYL RED TEST

A) B)

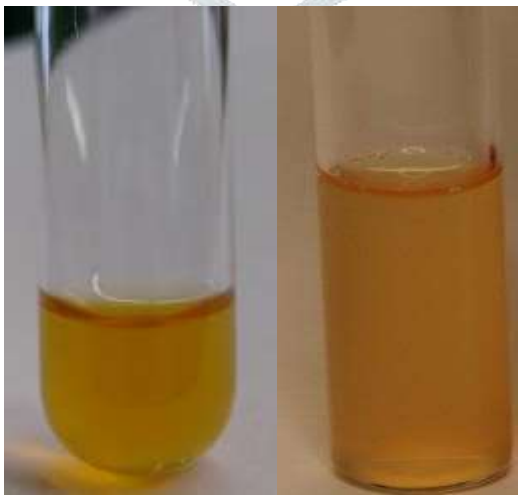


FIGURE 2.2 Illustrated that METHYL RED TEST

The methyl test result showed in the figure A and B is illustrated that the negative for both *Mycobacterium tuberculosis and Pseudomonas aeruginosa.*

VOGESPORSKAUER TEST

A) B)

FIGURE 2.3 illustrated that VOGESPORSKAUER TEST

The voges-proskauer test result showed in the figure A and B is illustrated that the negative for both *Mycobacterium tuberculosis and Pseudomonas aeruginosa.*

CIMMON CITRATE TEST

FIGURE 2.4 Illustrated that CIMMON CITRATE TEST

The cimmon citrate agar test result showed in the figure left is negative for *Mycobacterium tuberculosis.*

The cimmon citrate agar test result showed in the figure right is positive for *Pseudomonas aeruginosa.*

DNA ISOLATION

The DNA was isolated from two species of Bacteria by standardized procedure showed clear bands in the 0.8% agarose gel electrophoresis.

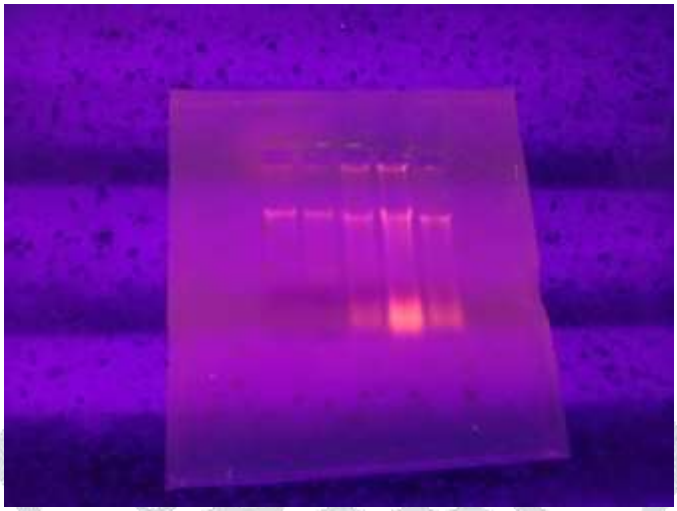


FIGURE 3.1 DNA ISOLATION

Genomic DNA isolated from two species (lane 3 is *Mycobacterium tuberculosis and lane 4 is Pseudomonas aeruginosa).*

PCR AMPLIFICATION

DNA region of 16S rRNA gene was successfully amplified using universal primers.

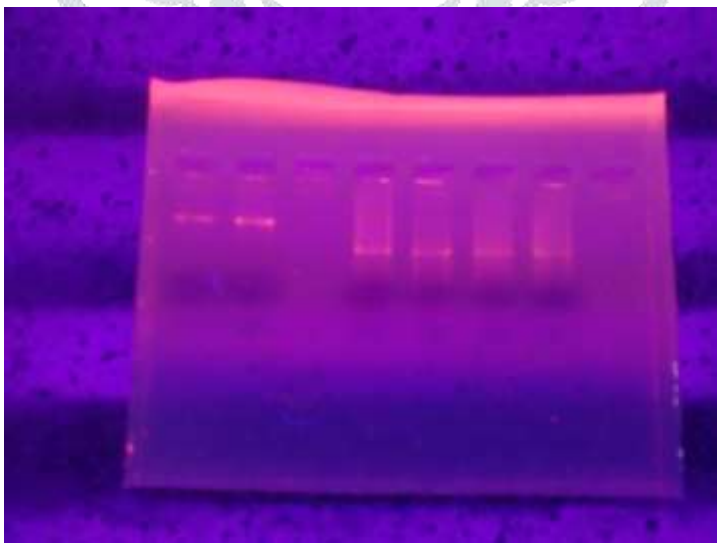
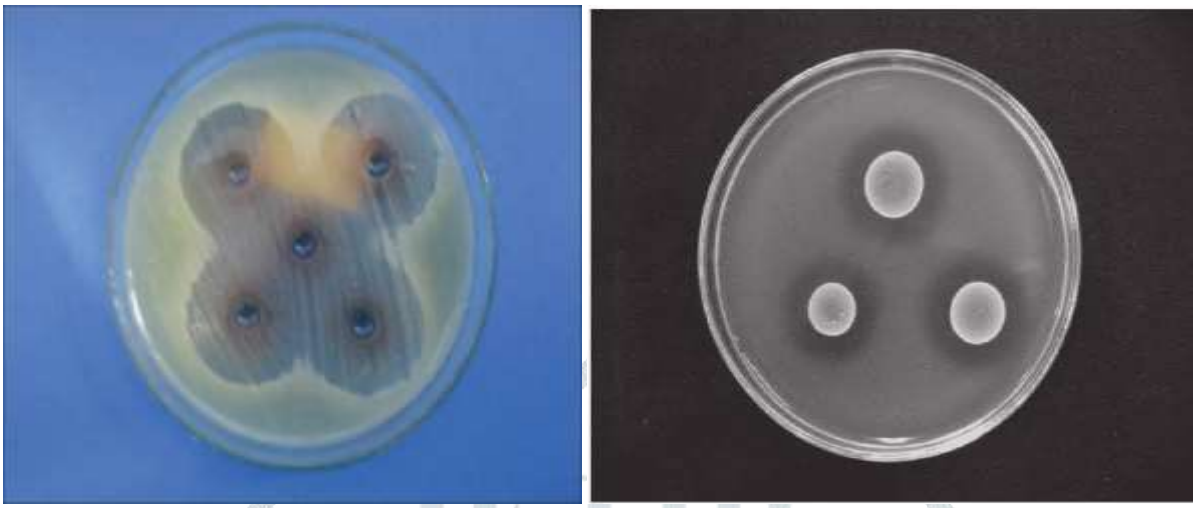


FIGURE 4.1 PCR AMPLIFICATION

Agarose gel electrophoresis of amplified 16S rRNA gene (lane 4&5 amplified EUBAC gene).

ANTI MICROBIAL ACTIVITY

A) B)



Sequence Obtained After Sequencing for 16s rRNA Gene

>*Mycobacterium tuberculosis* (Present study)

GTGCCCGAGCAACACCCACCCATTACAGAAACCACCACCGGAGCCGCTAGCAACGGCTGTCCCGTCGTGG
GTCATATGAAATACCCCGTCGAGGGCGGCGGAAACCAGGACTGGTGGCCCAACCGGCTCAATCTGAAGGT
ACTGCACCAAAACCCGGCCGTCGCTGACCCGATGGGTGCGGCGTTCGACTATGCCGCGGAGGTCGCGACC
ATCGACGTTGACGCCCTGACGCGGGACATCGAGGAAGTGATGACCACCTCGCAGCCGTGGTGGCCCGCCG
ACTACGGCCACTACGGGCCGCTGTTTATCCGGATGGCGTGGCACGCTGCCGGCACCTACCGCATCCACGA
CGGCCGCGGCGGCGCCGGGGGCGGCATGCAGCGGTTCGCGCCGCTTAACAGCTGGCCCGACAACGCCAGC
TTGGACAAGGCGCGCCGGCTGCTGTGGCCGGTCAAGAAGAAGTACGGCAAGAAGCTCTCATGGGCGGACC
TGATTGTTTTCGCCGGCAACTGCGCGCTGGAATCGATGGGCTTCAAGACGTTCGGGTTCGGCTTCGGCCG
GGTCGACCAGTGGGAGCCCGATGAGGTCTATTGGGGCAAGGAAGCCACCTGGCTCGGCGATGAGCGTTAC
AGCGGTAAGCGGGATCTGGAGAACCCGCTGGCCGCGGTGCAGATGGGGCTGATCTACGTGAACCCGGAGG
GGCCGAACGGCAACCCGGACCCCATGGCCGCGGCGGTCGACATTCGCGAGACGTTTCGGCGCATGGCCAT
GAACGACGTCGAAACAGCGGCGCTGATCGTCGGCGGTCACACTTTCGGTAAGACCCATGGCGCCGGCCCG
GCCGATCTGGTCGGCCCCGAACCCGAGGCTGCTCCGCTGGAGCAGATGGGCTTGGGCTGGAAGAGCTCGT
ATGGCACCGGAACCGGTAAGGACGCGATCACCAGCGGCATCGAGGTCGTATGGACGAACACCCCGACGAA
ATGGGACAACAGTTTCCTCGAGATCCTGTACGGCTACGAGTGGGAGCTGACGAAGAGCCCTGCTGGCGCT
TGGCAATACACCGCCAAGGACGGCGCCGGTGCCGGCACCATCCCGGACCCGTTCGGCGGGCCAGGGCGCT
CCCCGACGATGCTGGCCACTGACCTCTCGCTGCGGGTGGATCCGATCTATGAGCGGATCACGCGTCGCTG
GCTGGAACACCCCGAGGAATTGGCCGACGAGTTCGCCAAGGCCTGGTACAAGCTGATCCACCGAGACATG
GGTCCCGTTGCGAGATACCTTGGGCCGCTGGTCCCCAAGCAGACCCTGCTGTGGCAGGATCCGGTCCCTG
CGGTCAGCCACGACCTCGTCGGCGAAGCCGAGATTGCCAGCCTTAAGAGCCAGATCCGGGCATCGGGATT
GACTGTCTCACAGCTAGTTTCGACCGCATGGGCGGCGGCGTCGTCGTTCCGTGGTAGCGACAAGCGCGGC
GGCGCCAACGGTGGTCGCATCCGCCTGCAGCCACAAGTCGGGTGGGAGGTCAACGACCCCGACGGGGATC
TGCGCAAGGTCATTCGCACCCTGGAAGAGATCCAGGAGTCATTCAACTCCGCGGCGCCGGGGAACATCAA

AGTGTCCTTCGCCGACCTCGTCGTGCTCGGTGGCTGTGCCGCCATAGAGAAAGCAGCAAAGGCGGCTGGC
CACAACATCACGGTGCCCTTCACCCCGGGCCGCACGGATGCGTCGCAGGAACAAACCGACGTGGAATCCT
TTGCCGTGCTGGAGCCCAAGGCAGATGGCTTCCGAAACTACCTCGGAAAGGGCAACCCGTTGCCGGCCGA
GTACATGCTGCTCGACAAGGCGAACCTGCTTACGCTCAGTGCCCCTGAGATGACGGTGCTGGTAGGTGGC
CTGCGCGTCCTCGGCGCAAACTACAAGCGCTTACCGCTGGGCGTGTTCACCGAGGCCTCCGAGTCACTGA
CCAACGACTTCTTCGTGAACCTGCTCGACATGGGTATCACCTGGGAGCCCTCGCCAGCAGATGACGGGAC
CTACCAGGGCAAGGATGGCAGTGGCAAGGTGAAGTGGACCGGCAGCCGCGTGGACCTGGTCTTCGGGTCC
AACTCGGAGTTGCGGGCGCTTGTCGAGGTCTATGGCGCCGATGACGCGCAGCCGAAGTTCGTGCAGGACT
TCGTCGCTGCCTGGGACAAGGTGATGAACCTCGACAGGTTCGACGTGCGCTGA

>*Pseudomonas aeruginosa* (Present study)

ATGGCAGGAGCTGACCCCGTCCCTGAGCCTCAACTGCGCGGTGTTTCCCAACTCAGGACCGAAGACGCAA
GCCGTCGACGGCGATTACGCCTGGGCGCTCTGGCGTCCCTACTCCTGCTGCCAGCGCAAGGGGCAGATCT
TCCTCGGCAGTACCGACTTCCAATAAGGACACGGAGACGAATCATGCGAATGAACATCACCTCGGTCGCA
CTGACGTGGCTGCTCGCAGCGCAACTTGCCCAGGCCGACGACCCGATCAACGTGTCCAAGACCGGCACGG
TGCTCAGCGACGAGGTCCTCTACAGCATTGGCGGCGGCAGCGCGGTGAGCATGGGCAGCGCCGGCCAGAT
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CTGGAGAACCAGCTCAACGGTGCCACACAGGGTTTCCAGAACATCATGGGCTCAGTCATCCAGAACGCGA
CCGGCGCGGTCATGTCGCTGCCGGCGTTGATCATCCAGCGCGCGAACCCTCAGCTCTACAACCTGATCAC
CAATGGCATCCTGCAGGCGCGGATCGACTACGACCGCTCGAAAGGGACTTGCAGAGCGATCGCCGAGAAG
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AACCTGGGTCGGTGGAGACAAGGCCGGCGGCTCCGGCCAGAAGCCCATTCGCATCGTCAACGACGTGACC
CGGGCGGGCTACAACCTGTTGACCAGCCGCTCAGTGAATGACTCGTCGAGCGTGCCTTCCGCCACATGCA
ACAATGGCCTGGTCTGCAACACCTGGTCCTCCCCCCAGGAAGCCGCCGCGTTCGCCACCCGGGTACTGGG
CGAACAACAGCAACAGACCTGCGAAGGCTGCCAGAAGACGGTGACGGCTGCAGGCGTCGGCCTCACCCCG
CTGATCCAGGAGACCTACGACAAGAAGCTCCAGTCGCTGCAGGAGCTGCTGTCGAAGAGCAAACCACTGA
CTGCAGAGAACCTGGCTGCGGCCGGCACCGATGCTCTGCCAATTACCCGCGGCGTCATCGAGGCGCTGCG
CGACGAGCGTGACCAGGACGTCCTGGCGCGCCGCCTGGCGTCCGATGTCTCCCTGATGGACGTGCTCAGC
AAGGCACTGCTACTGCAGCGCCTGATGTTCGCCGGCGCCAAAGAGCCCAACGTCGCCGCCAACGGCCTGG
CCACCCAAGCCGTCGATCAGCAGACCAGCCTCCTGCAGCAGGAGATCTCGAACCTCAAGACCGAACTGGA
ACTCCGTCGCGAGTTGGCCAGCAATTCGCCCATGCGGGTCATCGAGCGCGGGCAACAGCGCGCCTCAGGG
TCCAGTGGCGTGTTCGAGTCGGCGCCCGATGCTGATCGCCTCGATCGCCTGCAGGCCCCCTCTGCCGCCG
GCGGCAAGTCGGGAGGGAGACCGTGA

Chapter-VI

6. Discussion

In recent years, there has been increasing demand for safe and eco-friendly agricultural products. This systematic rewiew aims to assess the evidence of bacterial effects of goat milk on human pathogenic bacteria. In the goat milk processing, there is some problem accumulating due to the unstability of milk nature.

Many bacteria are known to increase solubilization of insoluble phosphate forms by releasing organic acids and phosphatase enzymes (*Halder et al.,* 1990). Therefore, phosphate solubilizing bacteria, especially those with antifungal activities, may promote crop productivity, by not only providing plant-absorbable forms of phosphate, but also by effectively protecting plants from fungal soil-borne diseases (*Dey et al.*, 2004).

In addition, production of siderophore by the three *B. atrophaeus* strains may be a key factor in promoting plant growth and protecting plants from human pathogens. Iron, an essential cofactor for cellular processes, is abundant in nature, but iron bioavailability is very limited in soils due to low solubility under aerobic conditions and in the presence of a neutral pH. A number of bacteria, including plant pathogens, produce iron-chelating siderophores. Siderophores produced by antagonistic microorganisms may inhibit the growth of plant pathogens, but may enhance plant growth by increasing iron in the root zone. Studies have demonstrated that beneficial bacteria-producing siderophores stimulate plant growth and inhibit germination of a soil-borne fungal pathogen (*Alexander et al., 1991*). Similarly, the variable efficiency in inhibiting conidial germination of *C. acutatum* and *C. gloeosporioides* may be correlated with siderophore production b y the *B. atrophaeus* and *B. pumilus* strains, although the effect of strong antifungal substances produced by *B. atrophaeus* strains should also be considered. Further evaluation of LB14 should be performed under different environmental conditions, cultural practices to obtain more knowledge of efficacy in the field. Knowledge of the mechanisms and performance of antagonistic microorganisms will be helpful in developing reliable biological systems for disease control.

Chapter-VII

1. Summary And Conclusion

Two bacterial species, *Mycobacterium tuberculosis, Pseudomonas aeruginosa were* collected by karaikudi goat farm. The morphological characters of these two species were compared and found they are distinct. DNA was isolated from fresh broth culture by standardized method and the purity was checked. The PCR conditions for the amplification of EUBAC and 16S rRNA gene were standardized. All the sequences were checked for species confirmation by BLAST in NCBI. Based on the similarity search the two species were identified as *Mycobacterium tuberculosis, Pseudomonas aeruginosa*. This study serves as a basis for future studies possibly involving the conservation and management of the species.

Chapter-VIII

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