HUMAN PATHOGENIC BACTERIAL STRAINS ISOLATED FROM GOAT MILK

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ABSTRACT

Goat milk is the nutrient rich product. It was consumed by the various part of the world. Usually fresh goat milk is containing fewer microbes. Preserved goat milk has numerous microbes. It was analyzed by using total plate count and draw the statically diagram of the number of colonies present in the plate. The milk was treated as a special milk processing. Managing the safety of milk involves controlling the various sources of contamination, which could be endogenous. Some of the human pathogenic bacteria also present in the processed milk it may cause tuberculosis. 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.

KEYWORDS

16s rRNA, preservation, pathogenic bacteria, Tuberculosis.

INTRODUCTION

Goat milk and their products are nutritionally versatile and of late, have gained wide demand due to their potential nutraceutical properties. Goat milk can get contaminated by various pathogenic or spoilage microorganisms (mainly bacteria) during various stages of processing and storage from farm upto table. Presence of high microbial load in milk can pose major economical loss for local farmers and small hold dairies, as milk price is calculated based on the bacteria count, especially the pathogenic ones (Bonfoh et al., 2003; Metz et al., 2009; Suguna et al., 2011). Previously, some of the pathogenic and spoilage bacteria such has Listeria monocytogenes, Salmonella sp, Campylobacter, Staphylococcus aureus, Bacillus cereus, Escherichia coli, and species of Streptococcus, Staphylococcus and Micrococcus have been isolated from fresh raw goat milk in various parts of the world (Adesiyun et al., 2007; Kagkli et al., 2007). Generally, in fresh goat milk, microbial load is less. However, the count might increase upto 100 fold or more once stored at ambient temperature for an extended period of time (Chye et al., 2004; Suguna et al., 2011). Routinely,
world over, for food safety reasons, microbiological analysis is carried out to monitor and evaluate the level of prevalent pathogenic and spoilage microorganisms.

To our knowledge, no detailed reports are available on the microbiological quality of goat milk. 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 Penang Island, which is envisaged to provide baseline information on the level of contamination and the prevalence of pathogenic bacteria. Results generated in this study is expected to be useful for health conscious consumers as well as the local governing agency to implement appropriate food safety measures to minimize the risk factors associated.

Microbiological analysis

Goat milk samples were analyzed for the prevalence of selected bacterial pathogens. Enumeration of total plate count (TPC), Psychrotrophic count, Coliforms, Presumptive *Escherichia coli*, *Staphylococcus aureus* and yeast and mould counts were carried out by employing standard methods (FDA, 2001; BAM, 2003; Yousef and Carlstrom, 2003). Enumeration of *Coliforms*, *E. coli* and *K. pneumoniae* in goat milk was performed by employing three-tube most probable number (MPN) technique. Positive tubes from MPN were streaked onto eosin methylene blue (EMB) agar (Merck) and incubated at 37ºC for 24h. The typical colony found was confirmed based on their IMViC pattern based on BAM method (FDA, 2001). While, Baird-Parker agar (BPA, Merck) was used to enumerate *Staphylococcus aureus* in the samples. Characteristic black colonies surrounded by a clear zone were selected and subjected to coagulase and thermonuclease tests for confirmation of *S. aureus* (BAM, 2004). For determination of *Salmonella* in samples, International Standard Organization protocol (ISO, 1990) was employed. Presence of *Salmonella* colonies were confirmed using API 20E test kit (Biomerieux, France). For identification of *Listeria* spp., modified method described by the Food and Drug Administration (FDA) was employed (Westoo and Peterz, 1992; FDA, 2001). Selected colonies from each plate of *Listeria* selective agar (Merck) and Palcam *Listeria* selective agar (Merck) were streaked onto Trypticase soy agar (TSA, Merck) and incubated 37ºC for 24 h. Presumptive *Listeria species* isolates were confirmed based on Gram reactions and catalase tests. Isolates, which were Gram-positive and catalase-positives were sub-cultured and identified with API Listeria test kit (BioMerieux, France).

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. Means were compared by employing analysis of variance (ANOVA, SPSS version15.0) followed by t-test to determine difference among means at 95% confidence level (significance level at P ≤ 0.05).
Goat milk and its products are popular among health conscious consumers and certain ethnic groups. A recent survey showed that there are approximately 42 registered commercial dairy goat farmers in South Africa, 19 of who are in Gauteng province. Donkin identified a need for increased investments in dairy goat schemes to support and expand household milk supply and promote small-scale dairy enterprises at the village level. It is also important to ascertain whether the small-scale dairy enterprises composed of smallholder farmers can compete favorably in the production of safe goat milk.

Managing the safety of milk involves controlling the various sources of contamination, which could be endogenous (organisms entering the milk in vivo) or from some external source (exogenous) after milk had been removed from the udder. Some of the diseases that can be transmitted to humans from milk include *salmonellosis, tuberculosis, brucellosis, listeriosis, Q fever, toxoplasmosis, streptococcal and staphylococcal infections* and *campylobacter* infections. Mastitic agents in goats (endogenous contamination), include coagulase-negative *staphylococcus* species; *Staphylococcus aureus* and *streptococcus* species, e.g. *Streptococcus agalactiae*; *E.coli* and *Pseudomonas species*, and all have been isolated from goat milk. While some may consider coagulase negative *Staphylococcus* intra mammary infections to be co-incidental and an environmental contaminant (non-pathogenic) in goats, others contend that these infections may become chronic and lead to udder sensitivity, elevated somatic cell counts (SCC) and decreased milk production. *Staphylococcus aureus* has been identified as the most pathogenic *staphylococcal* infection both in its subclinical and clinical form in the caprine udder. In this study nearly all *staphylococci* from subclinical cases could be isolated from goat milk removed from the udder. This shows that udder disease remains widespread and consumers of raw milk still run the risk of food poisoning.

Antibiotics are substances used to treat infection caused by microbes. Antibiotics acts by inhibiting or destroying bacterial cells (Demain, 1998) that cause certain disease. In nature, antibiotics are secondary metabolites produced by bacteria (Teasdale *et al.*, 2004) in order to maintain their niche and territory. Only limited groups of microorganisms are responsible for the sources of clinically usable antibiotics. As stated by Cooke and Gibson (1983), only those have an effect on bacterial cells but not the host cells like human categorized as useful antibiotics. Besides, there are only limited numbers of antibacterial actibiotics that can be used to treat bacterial infections (Motta *et al* 2004). Antibiotics can be found easily but only few are useful. Hence, concerted efforts have been carried out by many scientists in order to screen for novel antibiotic producing microbes. Through their efforts, many antibiotics have been successfully discovered in order to combat pathogenic bacteria that cause disease. However, the emergence of new diseases and reemergence of multiple-antibiotic resistant pathogens have rendered the existence used antibiotics ineffectively.

Among the diverse sources of antibiotics, canned sea food is the most important target for scientists in the discovery of novel antibiotics. According to Dulmage and Rivas (1978), canned sea food
microorganisms have continually been screened for their useful biological active metabolites such as antibiotics since long ago.

Based on this concept the present study has been carried out to find the alternate method for the inhibition of pathogenic bacteria.

The types of bacteria present in milk can influence cheese making, shelf-life and can promote health or cause disease in consumers of the milk and milk products. The microbial profile of raw milk can also provide insight into the health status of the lactating dam since it changes during the course of lactation (D'Amico and Donnelly, 2010) and in response to infections such as mastitis (Alawa et al., 2000). However, the complex nature of milk and milk products makes determining what bacteria are present and what influence they exert a challenge. Culture-independent methods of microbial population analysis have grown more sophisticated in recent years. Next-generation sequencing (NGS) is able to generate far more reads than traditional Clone library sequencing (CLS) (Hamady and Knight, 2009). While older studies relied on culturing bacteria for identification (Foschino et al., 2002; Holm et al., 2004; D'Amico and Donnelly, 2010), NGS does not rely on selective media and can provide greater depth and breadth to the study of milk. These new technologies have not been widely applied to the bacteria in goat milk, as yet. Studies such as Callon et al., (2007) which used molecular techniques including single-stranded conformation polymorphism (SSCP) analysis and restriction fragment length polymorphism (RFLP) typing, have found bacteria in raw milk of small ruminants not found in previous culture-based studies which focused on specific groups such as staphylococci (Blagitz et al., 2011) or coliforms (Araya et al., 2008). In light of this, this study used NGS and traditional CLS to determine the microbial diversity in raw goat milk throughout the course of lactation and compare it to that of milk from genetically engineered goats producing the antimicrobial human lysozyme (hLZ) in their milk.

Lysozyme is a muramidase found in tears, saliva and milk of all mammals that specifically cleaves the 1,4-\(b\)-D-linkage between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan layer of bacterial cell walls, resulting in cell lysis (Masschalck and Michiels, 2003). Lysozyme is present in human milk at much higher levels than the milk of dairy animals (400 mg/ml compared to 0.130 mg/ml in cow milk and 0.250 mg/ml in goat milk (Chandan et al., 1968)) to help protect infants against pathogenic bacteria and promote the formation of a healthy gut microbiota (Lonnerdal, 2003). Goats were genetically engineered to express increased levels of lysozyme in the mammary gland with the intent of improving human health upon consumption of the milk (Magaet et al., 2003). These transgenic goats produce active hLZ in their milk at levels of 270 mg/ml, 68% of the level of human milk (Maga et al., 2006a). Expression of hLZ did not disrupt yield or the gross composition (fat and protein content) of milk (Maga et al., 2006a) and finer analysis demonstrated that the presence of hLZ was the only difference in protein composition between the
milk of transgenic does and their non-transgenic herd mates (Maga et al., 2012). The milk from hLZ goats has been shown to have a longer shelf-life and in vitro slowed the growth of bacterial isolates responsible for causing the spoilage of milk (Pseudomonas fragi) and mastitis (Escherichia coli and Staphylococcus aureus) but not Lactococcus lactis (Maga et al., 2006b) as the milk can still be used to produce cheese (Scharfen et al., 2007). When consumed by animal models, pasteurized hLZ milk beneficially modulates gut microbiota (Maga et al., 2012), improves gut morphology and circulating metabolites in young pigs (Brundige et al., 2010; Cooper et al., 2011) and helps resolve the symptoms of diarrhea (Cooper et al., 2013), all indicating potential human health benefits. One important question to answer is if lysozyme itself is causing these changes or if byproducts of lysozyme presence in milk (different types of bacteria or metabolites) are influencing the antimicrobial action of the milk. In addition, the production of lysozyme in the udder of transgenic goats has the potential to alter the bacterial population of the raw milk, alterations which could have effects on the doe, milk processing and any consumers of the milk. In this study we used CLS and NGS approaches for an in depth characterization of the microbial diversity of raw goat milk and how these populations change in response to the presence of hLZ.

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.

REVIEW OF LITERATURE

Conventional histological staining methods, including stain selection for specific situations, have long been established. Descriptions of the most frequently used staining methods should be sufficient for day-to-day practice. Longer fixation in formaldehyde or in higher concentrations of formaldehyde can lead to sediments of formalin pigment. If the assessment of tissue sections will be affected by such sediments, pretreatment should be considered (Kardasewitsch reaction; Kardasewitsch, 1952). Depending on which tissue is to be investigated, the fixation technique can influence the microscopic image. Thus, for example, the influence of fixation on the development of pulmonary alveoli has been investigated (Hausmann et al. 2004). In some cases, alternative fixing solutions are used: Bouin’s solution, Zamboni solution, “NoTox” (Meyer et al. 1996), pure alcohol, etc. In cases where an electron microscopic investigation is needed, glutaraldehyde is typically chosen as a fixative (3% solution for 24 h at 4°C, followed by phosphate buffer solution; additional fixation in 1% osmium acid, embedded in Epon). It should be noted that fixative
selection and duration can have a direct bearing on potential molecular genetic investigations (Kuhn and Krugmann 1995). Such investigations can be difficult or even impossible and special pretreatment methods are sometimes suggested (Ananian et al. 2010). Immunohistochemical evidence can be found in formalin-fixed tissue, depending on the antigen, as is the case for viral antigens (Lozinski et al 1994), but also in other molecular genetics investigations (Miething et al. 2006). Antigen-conserving methods are also discussed in order to overcome antigen loss or difficult detectability due to autolysis (Pelstring et al. 1991). Microwave pretreatment can accelerate fixation with formaldehyde (Login et al. 1987). In addition to conventional histology, which has long been common practice, immunohistochemical techniques have also found their way into forensic diagnostics (Bratzke and Schröter 1995).

DNA isolation is a process 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. PCR sensitivity detection considered to show the variation between the commercial kits (Yoshikawa, 2011).

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. Most of them follow similar basic steps and include the use of organic and nonorganic reagents and centrifugation methods. Finally, they have developed into a variety of automated procedures and commercially available kits (Carpi et al 2007).
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).

Members of the vibrio genus are gram negative, halophilic bacteria indigenous to coastal marine systems (Thompson et al., 2003). While these common bacteria persist as a natural component of the marine microbial flora, a small percentage of environmental isolates carry the genetic determinants for human pathogenesis (Rivera et al., 2001).

The massive uses of antibiotics in human therapy, bacteria have developed several resistance mechanisms including the efflux of antibiotics (Yala et al., 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 35-42 °C and a melting temperature of 85-95 °C. Low-melting and low-gelling agaroses made through chemical modifications are also available.

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).
The agarose polymer contains charged groups, in particular pyruvate and sulphate. These negatively charged groups create a flow of water in the opposite direction to the movement of DNA in a process called electroendosmosis (EEO), and can therefore retard the movement of DNA and cause blurring of bands. Higher concentration gel would have higher electroosmotic flow. Low EEO agarose is therefore generally preferred for use in agarose gel electrophoresis of nucleic acids, but high EEO agarose may be used for other purposes. The lower sulphate content of low EEO agarose, particularly low-melting point (LMP) agarose, is also beneficial in cases where the DNA extracted from gel is to be used for further manipulation as the presence of contaminating sulphates may affect some subsequent procedures, such as ligation and PCR. Zero EEO agaroses however are undesirable for some applications as they may be made by adding positively charged groups and such groups can affect subsequent enzyme reactions (Joseph Sambrook et al). Electroendosmosis is a reason agarose is used in preference to agar as the agarpectin component in agar contains a significant amount of negatively charged sulphate and carboxyl groups. The removal of agarpectin in agarose substantially reduce the EEO, as well as reducing the non-specific adsorption of biomolecules to the gel matrix. However, for some applications such as the electrophoresis of serum proteins, a high EEO may be desirable, and agarpectin may be added in the gel used (David 2003).

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. It is an easy and cheap tool to amplify a focused segment of DNA, useful for such purposes as the diagnosis and monitoring of genetic diseases, identification of criminals (in the field of forensics), and studying the function of a targeted segment of DNA.

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.

The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase, which the method is named after, are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic
manipulations. PCR is not generally considered to be a recombinant DNA method, as it does not involve cutting and pasting DNA, only amplification of existing sequences.

Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase (an enzyme originally isolated from the bacterium *Thermus aquaticus*). This DNA polymerase enzymatically assembles a new DNA strand from DNA building-blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (also called DNA primers), which are required for initiation of DNA synthesis. The vast majority of PCR methods use thermal cycling, i.e., alternately heating and cooling the PCR sample through a defined series of temperature steps.

In the first step, the two strands of the DNA double helix are physically separated at a high temperature in a process called DNA melting. In the second step, the temperature is lowered and the two DNA strands become templates for DNA polymerase to selectively amplify the target DNA. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions. *Klebsiella pneumonia* over 100 years ago as a cause of community acquired *pneumonia* and is the opportunistic pathogen that can cause *pneumonia*, urinary tract infections and bacteremia (Wu et al., 2012).

**MATERIAL AND METHODS**

**SAMPLE COLLECTION**

**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 l and the average annual milk yield is 600–800 liters.

**MILK PROCESSING**

Goats are machine milked twice daily. A thorough pre-milking semi-dry udder cleaning is carried out. The milking takes place in the designated area of the stable using a pipe milking machine. Milking machine sanitation is performed in a closed circuit way using approved sanitation products. After milking, the milk is cooled down promptly to 4–6 ºC and then stored for 12–24 hours until further processing, i.e. stationary pasteurization in a tank at 72 ºC for 20 seconds. The pasteurized milk is a semi-product for the production of fresh cheese in various flavours.
SAMPLEING

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.

MORPHOLOGICAL IDENTIFICATION

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 minute. 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.

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.
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

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

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’-GGTTACCTTGTACGACTT-3’
**MASTER MIX COMPONENTS**

1. Distilled 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).

**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.
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.

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.

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.

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<th>STAINING TECHNIQUES</th>
<th>FIGURE A Mycobacterium tuberculosis</th>
<th>FIGURE B Pesudomonas aeroginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Gram’s staining</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>2. Capsule staining</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>3. Negative staining</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>4. Simple staining</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>
5. Motility test | Negative | Negative

6. Acid fast staining | Positive | Negative

ANTIMICROBIAL ACTIVITY TEST

<table>
<thead>
<tr>
<th>ANTIMICROBIAL ACTIVITY</th>
<th>Mycobacterium tuberculosis</th>
<th>Pesudomonas aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Klebsillela pneumoniae</td>
<td>Positive</td>
<td>Positive</td>
</tr>
</tbody>
</table>

DISCUSSION

In recent years, there has been increasing demand for safe and eco-friendly agricultural products (Huh and Kim, 2010). Accordingly, biological control practices are in much greater demand as alternatives to
synthetic pesticides. Biological control practices are particularly important in organic crop production disease control (Cook et al., 2013).

Reports from several studies provide strong evidence that strains of *Bacillus* species, including *B. subtilis* (Kim et al., 2012), *B. amyloliquefaciens* (Kong et al., 2010a), *B. licheniformis* (Govender et al., 2005), *B. pumilus* (Sari et al., 2007), *B. mycoides*, and *B. sphaericus*, significantly reduce disease severity on a variety of hosts in greenhouse or field conditions (Kloepper et al., 2004). *B. subtilis* and *B. amyloliquefaciens* have been used in commercial biological control products due to their excellent antagonistic effects and high stability in harsh environmental conditions (Kwak et al., 2012).

A previous study used tidal flat sediments and jeotgal, a salted-fermented fish product, to find antagonistic microorganisms in Korea. The antagonistic bacteria included *Paenibacillus macerans*, *B. atrophaeus*, and *B. pumilus* from tidal flat sediments, and *B. atrophaeus*, *Paenibacillus sp.* *Virgibacillus pantothenticus*, *B. subtilis*, and other *Bacillus* sp. from jeotgal (Kim et al., 2010).

Consistent with previous reports that *B. atrophaeus* suppresses fungal plant pathogens (Kim et al., 2010), our study demonstrated that three *B. atrophaeus* strains, LB14, HM03, and HM17, were effective for not only inhibiting mycelial growth, but also inhibiting conidial germination of *C. acutatum* and *C. gloeosporioides*. Furthermore, we showed that chinase, protease, siderophore, and phosphate solubilization, associated with LB14, HM03, and HM17, may contribute to suppression of *C. acutatum* and *C. gloeosporioides*.

Fungal cell walls play important physiological roles, in addition to providing structural barriers, in regulating the passage of molecules into cells and their development and survival in response to ambient conditions. The fungal cell wall is a matrix composed of polysaccharides, small amounts of proteins, and other components. Many fungal pathogens in ascomycota and basidiomycota have a mixture of chitin, polymers of glucan, and mannoproteins in their cell walls (Vega and Kalkum, 2012). Many antagonistic bacteria secrete lytic enzymes capable of dissolving fungal cell walls, resulting in exhibition of predatory activity of fungal pathogens (Xu et al., 2014). Strong activity of chitinase and protease in the *B. atrophaeus* strains LB14, HM04, and HM17 are therefore consistent with the observed growth inhibition of *C. acutatum* and *C. gloeosporioides*.

Proteases, in particular, play a key role in the cell lysis process. Proteases bind to the outer mannoprotein layer of the cell wall, open the protein structure, and expose inner glucan layers and chitin microfibrils. Among the three *B. atrophaeus* strains, LB01 (*B. amyloliquefaciens*) exhibited strong protease activity. However, activity of chitinase and protease was almost absent in LB15 (*B. pumilus*). This difference may explain the reduced effectiveness of LB15 compared to the other *B. atrophaeus* and *B. amyloliquefaciens* strains in inhibiting mycelial growth and conidial germination of *C. acutatum* and *C. gloeosporioides*. 
The three *B. atrophaeus* strains, together with *B. amyloliquefaciens* and *B. pumilus* strains isolated from tidal flat sediments, demonstrated a high capacity to solubilize an insoluble phosphate compound. This is an important trait of antagonistic bacteria in supplying phosphate, a major nutrient required for plant growth. Uptake of phosphate by plants is limited due to low solubility of phosphate compounds in soil. 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 by 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.

**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.
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