Biomedical Research to Animal Trials

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ABSTRACT

Animal models are a type of model organisms that are mainly used for studying the development and progression of various diseases. Some of the animal models used in preclinical trials and biomedical research include the fruit fly Drosophila melanogaster, nematodes such as Caenorhabditis elegans, Zebrafish, Xenopus and many mammals, such as guinea pigs, mice, rats, dogs, cats, pigs and monkeys, due to their close phylogenetic relationship with humans. Diabetes is a chronic disease that is mainly characterized by abnormal blood glucose levels owing to absolute or relative lack of insulin. In the present study, type 1 diabetes was chemically induced into male SD rats using streptozotocin and type 2 diabetes was studied by knocking out the leptin/leptin receptor gene in C57BL/6 mice. Also, wounds that mimic diabetic ulcers were created on the skin surface of the rats and scaffolds with drugs were used to study wound healing and inflammation. Good research and good animal care are inseparable. A sick or distressed animal does not yield reliable results when compared to a healthy animal. Hence, health surveillance of laboratory animals is an essential part of animal handling. For assessment of the health status of mice, tissues from the skin, liver, kidney, spleen, and intestines were streaked onto plates containing nutrient media and the bacterial and fungal cultures were gram stained for identification. Also, information on the Animal Facility, basics of animal handling such as caging, feeding and bedding of animals, imaging of mice are dealt with in this report.

Keywords: Diabetic models, health status, bacteriology

1. INTRODUCTION

1.1 Overview of Animal Models in Biomedical Research

Despite being different humans and animals share remarkable similarity at the physiological and anatomical level. Due to the presence of same organs (heart, brain, lungs, etc.) and organ systems, animals from mice to monkeys perform similar functions. Humans share 99% of their DNA with mice. Owing to their small size and ease of handling, mice are the most widely used animal models in biomedical research.

Some of the most commonly used animal models and their fields of research include

- Guinea Pigs – nutritional research, crucial for development of vaccines for tuberculosis and diphtheria, replacing heart valves, blood transfusion, studying allergic and respiratory diseases.
- Mice – genetic research and also for studying cancer, diabetes, AIDS, aggressive behaviour, Huntington's disease.
- Primates – for treating infections and diseases associated with human physiological processes such as ageing, reproduction, endocrine function, metabolism, and neurology. Models for studying toxicology, AIDS, Parkinson's disease, malaria

- Rabbits – in the study of cardiovascular diseases, cancer, glaucoma, ear infections, eye infections and diabetes. They are also used as models for cystic fibrosis and cholera
- Rats – in the study of cardiovascular medicine, neural regeneration, wound healing, diabetes and transplantation.

Thus, animals form an important part of biomedical research.

1.2 Drug Discovery

Drug discovery is a process by which a drug candidate is identified and partially validated for the treatment of a specific disease. It deals with mechanism of action, target identification/validation, lead identification/optimization, ADME properties, Pharmacokinetics/Pharmacodynamics and Toxicity. The drug development process consists of pre-clinical trials and clinical trials. Hence for a drug to come into the market it takes about 12 to 15 years.

Pre-clinical trials are performed in order to determine efficacy, toxicity, pharmacokinetics and safety of a particular drug. This information allows researchers to allometrically estimate a safe starting dose of the drug for clinical trials in humans. While wide doses of the drug are tested using in vitro (test tube or cell culture), animal models bridge the gap between invitro studies and clinical trials.

1.3 Diabetic Models

Diabetes mellitus is a disorder wherein the blood glucose levels are abnormally high. Insulin dependent (Type 1) and insulin independent (Type 2) diabetes are the two main forms of Diabetes mellitus. Type 1 diabetes occurs due to destruction of beta cells of the Islets of Langerhans by T-cells whereas Type 2 diabetes is characterised by insulin resistance and is normally found in obese people. Hence, several rodent models have been developed to study the onset and progression of diabetes. Some of them include chemically induced diabetic models (STZ rats), virally induced diabetic models (LCMV under insulin promoter in SD rats), spontaneous autoimmune models (Nonobese Diabetic Mice (NOD) and genetically induced diabetic models such as gene knockout or knockin (db/db mice or ob/ob mice).

1.4 Health Evaluation of Laboratory Mice

Health evaluation of laboratory animals is critical to determine the pathogen status and general health of animals in order to prevent disease in rodents. Serological tests, bacterial cultures, parasitological examinations and histopathology are the commonly used procedures in health monitoring. Our study focusses on the health evaluation of the C57BL/6 mice through bacterial and fungal cultures.

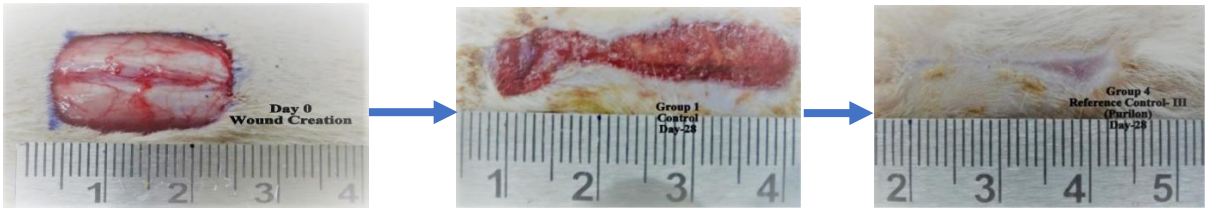
2. METHODS

2.1 STZ Rat Model for Studying Type 1 Diabetes

Streptozotocin, a synthetic antineoplastic [1] agent when administered to male SD (Sprague Dawley) rats causes destruction of the pancreatic β cells (by alkylation of the DNA) and thus eventually induces type 1 diabetes. A single dose of streptozotocin (60mg/kg of body weight of the rat) when administered through the intraperitoneal route induces diabetes in the rats after 5 days. This was confirmed by measuring the blood glucose levels of these rats continuously for 3 days after administration. Measurements were done using a glucometer. The diabetic rats had a significantly high blood glucose levels (greater than 200mg/dl of glucose).

2.2 Full Thickness Excision Model

Artificial full thickness skin wounds (approximately 2cm diameter), that mimic diabetic ulcers were created on the skin surface of SD rats. Measurement of the wounds was done using wound tracing and area of the wound surface was determined starting from day 0 to day 28 on a weekly interval using a tracing sheet. Then the scaffolds containing various drugs were bound around those places that had the wounds. The scaffolds were replaced with new ones occasionally. (first change was done in the first 3 days – day 0, day 3). Finally, in order to determine the contraction of the wound, skin from the wounded portion and also from the surrounding portions was collected for histopathology on day 28. This model was mainly used for understanding wound healing and inflammation.

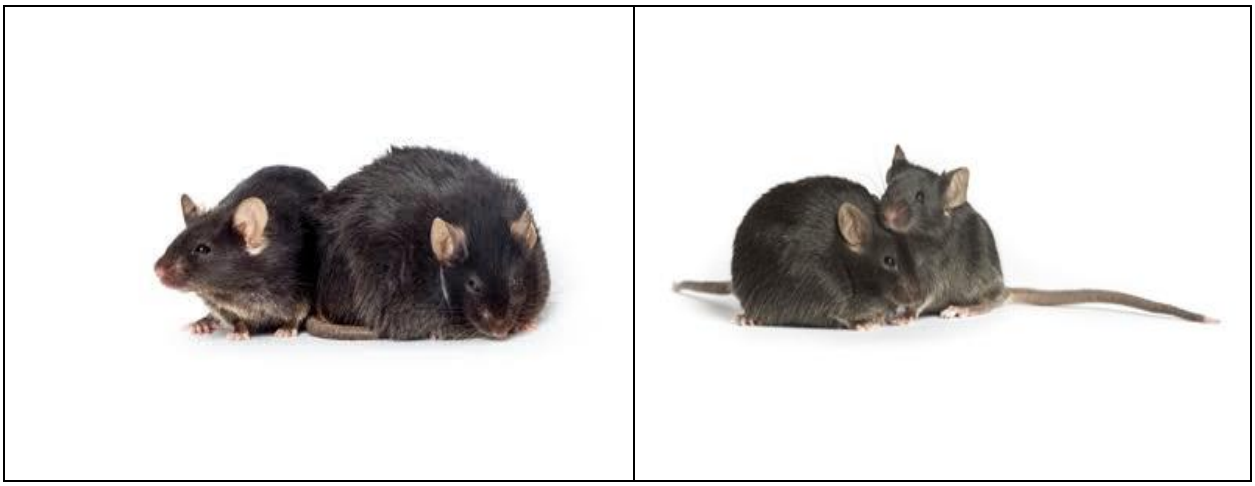


[**Figure 1**: Creation of artificial full thickness skin wounds, Wound Tracing and wrapping them with scaffolds containing drugs and studying the wound contraction]

2.3 Models for Studying Type 2 diabetes

Leptin is a hormone concerned with the regulation of food intake, appetite and body weight. Absence of leptin or leptin resistance can lead to uncontrolled feeding and weight gain.

Leptin deficient ob/ob mice	Leptin Receptor Deficient db/db mice
• Single autosomal recessive mutation in the obese gene (leptin encoding gene on chromosome 6) causing the change of Arg residue to stop codon that mainly results in premature truncation and functionally inactive protein even with high levels of mRNA [2]. • The animals exhibit obesity, hyperphagia, transient hyper-glycemia, glucose intolerance, and elevated plasma insulin. • They are also hypometabolic, hypothermic, and sub-fertile. • Wound healing is impaired and hormone production from both pituitary and adrenal glands is increased. • This strain is used to model phases I and II of diabetes type II and obesity. • Obesity is characterized by an increase in the number and size of adipocytes. • Although hyperphagia contributes to the obesity, homozygotes gain excess weight and deposit excess fat even when restricted to a diet sufficient for normal weight maintenance in lean mice.	• Single autosomal recessive mutation of Gly to Thr in the leptin receptor gene on the chromosome 4 [2]. • There is abnormal mRNA splicing and it results in non-functional Ob-Rb receptor protein. • Ob-Rb gets replaced by Ob-Ra. • The defective leptin receptor leads to the over-production of extracellular leptin, but lack of intracellular leptin action through Ob-Rb. • They are used to model phase I to phase III of diabetes type II. • Manifest morbid obesity, chronic hyperglycemia, pancreatic beta cell atropy, and they become hypoinsulinic. • The severity of disease on BKS background leads to uncontrolled rise in blood sugar, severe depletion of insulin-producing beta-cells of the pancreatic islets, peripheral neuropathy, myocardial disease and death by 10 months of age. • Exogenous insulin fails to control blood glucose levels and gluconeogenic enzyme activity increases. • Wound healing is delayed, and metabolic efficiency is increased.



2.4 Handling and Care of Animals

2.4.1 Clean Room Facility: The place where the animals are housed. The animal facility corridors are of two types namely: clean corridors (through which we enter into the animal facility) and Service Corridors (where the used cages, food and bedding are placed for their disposed). Both the corridors are unidirectional.

[**Figure 2:** Animal Holding Room]

[**Figure 3**: Clean Corridor]

[**Figure 4**: Service Corridor]

While entering the clean room facility, shoe cover, head cover and face masks are worn to avoid contamination and exposure to animal allergens. This is a respiratory requirement.

Clean room air showers are enclosed chambers placed at the exit and entry of clean rooms. These make use of high velocity jets of air and HEPA (or ULPA) air filter systems to remove loose contaminants from people before they enter the clean room.

2.4.2 Animal Bedding: Animal bedding is mainly provided to absorb, dilute or limit the animal's contact with its excreta, provides insulation allowing the animal to thermoregulate, minimizes the growth of microorganisms and to some extent reduces the intercage ammonia [3]. Bedding is generally manufactured from plant materials such as wood, cotton and corncob (that are subjected to processing) [3].

2.4.3 Animal Feed: Essential nutrients such as carbohydrate, vitamins (vitamin C and vitamin D in the form of cholecalciferol), amino acids, fats, minerals in appropriate ratios are supplied to laboratory animals. Ground corn/ground oats/alfalfa meal/soybean meal/ground wheat are the closed formula diets that are given [3]. Majority of the diets provided to rodents are pelleted because pelletizing results in highest energy content per unit weight and also allows efficient delivery, minimizes waste, decreases need for storage space and also prevents ingredient separation [3]. Acidified water at a pH of 2.5 to 3 is given to laboratory animals to eliminate the spread of bacteria through water.

2.5 Caging Systems

2.5.1 Conventional Cages: consists of a shoe box cage with a wire top feeder [4]**.** It is made of plastic with a solid bottom into which contact bedding is placed and directly bedded. Food and water (in bottles) are provided through a stainless-steel wire bar lid [4].

[**Figure 5**: Conventional Caging System found in the Central Animal Facility, IISc]

2.5.2 Individually Ventilated Cages: Air brought in from the animal room is passed through a high-efficiency particulate air (HEPA) filter, delivered to the cage, and then again HEPA filtered out. Since both the supply and the exhaust air are filtered, particulate contamination of the animal housing room is minimized and superior bio-protection is offered to the animals [4]. Also, air changes within the cage helps in removing poisonous gases [4]. More cages can be housed using this method.

[**Figure 6**: Individually Ventilated Caging at the Central Animal Facility, IISc]

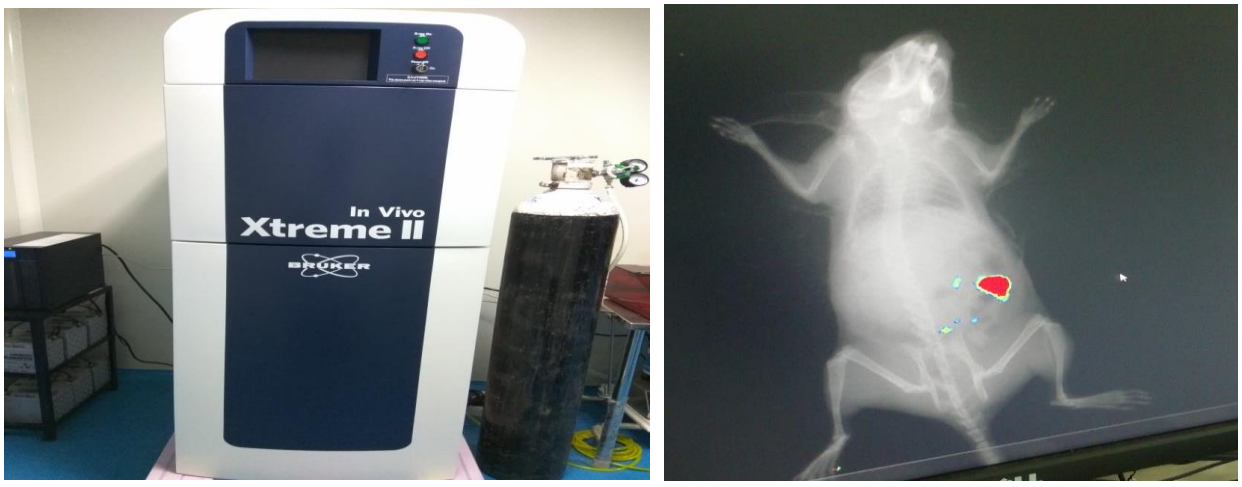
2.5.3 Metabolic Cages: These cages are mainly designed to facilitate the collection of mice urine and fecal samples for metabolic analysis. The most commonly used caging designs house the rat or mouse in a

Plexiglas or glass cylinder with a wire mesh floor [4]**.** A funnel system that is incorporated into the floor of the cage allows the collection of urine and feces separately from each other, facilitating *in vitro* analysis [4].

[**Figure 7**: Metabolic Caging system at Central Animal Facility, IISc]

2.6 Pre-clinical Imaging – Bioluminescence, Fluorescence and X ray imaging

Preclinical imaging refers to the visualisation and study of animal models longitudinally using non-invasive and in vivo techniques.



[**Figure 8**: In Vivo Xtreme imaging equipment (left) and X ray, fluorescent and bioluminescent imaging of a C57BL/6 black mouse (right)]

2.7 Health Surveillance of Animal Models using microbiological techniques.

2.7.1 Preparation of Nutrient Media

Nutrient agar, LB Agar and LB Broth were used for the growth of bacteria whereas Potato Dextrose Agar was used for the growth of fungi. Nutrient agar is a highly enriched media. All kinds of bacteria exhibit growth on nutrient agar. LB Broth/agar is more specific for Enterobacteriaceae and in particular for E. coli.

All the media were autoclaved at 121°C and 15psi for a period of 15 minutes.

2.6.2 Aseptic Laboratory Techniques

- All the basic microbiological techniques such as pouring plating and streaking were carried out in the Laminar Air Flow Hood and the Biosafety Cabinet.
- The working area was sterilized by the UV radiation and alcohol.
- The autoclaved media were allowed to cool to room temperature and were poured into sterile petri plates under aseptic conditions.
- Following solidification, the petri plates were preincubated for 15 hours to check for contamination
- In the absence of contamination, the petri plates were stored at 4°C for later use.

2.6.3 Sampling Animal Tissues and Culturing Microbes

A C57BL/6 mouse (8 to 9 weeks old) was euthanised by cervical dislocation. Tissues from the liver, kidney, spleen, small intestine (jejunum, ileum) and large intestine were streaked onto plates containing nutrient agar, LB Agar and LB Broth media. For detection of fungi, the skin was rubbed gently and streaked onto the Potato Dextrose Agar.

The inoculated bacterial plates were kept for incubation at 37°C for a period of 24 hours whereas the fungal plates were incubated at ambient temperature for 3 to 4 days.

2.6.4 Bacterial Identification

2.6.4.1 Biochemical test – Catalase test

Catalase is an enzyme produced by microbes which live in oxygenated environments (aerobic microbes). These organisms can neutralise the toxic forms of oxygen metabolites such as hydrogen peroxide. Anaerobic organisms lack catalase enzyme. Facultative anaerobes answer positive for catalase test due to their ability to utilize oxygen as the terminal electron acceptor in respiration.

This test can be performed by placing a drop of 3% H_2O_2 on a grease free glass slide and then transferring a loopful of colony onto the slide and mixing it. Presence of bubbles (which signifies the evolution of oxygen) indicates that the organisms are catalase positive whereas absence signifies that they are negative.

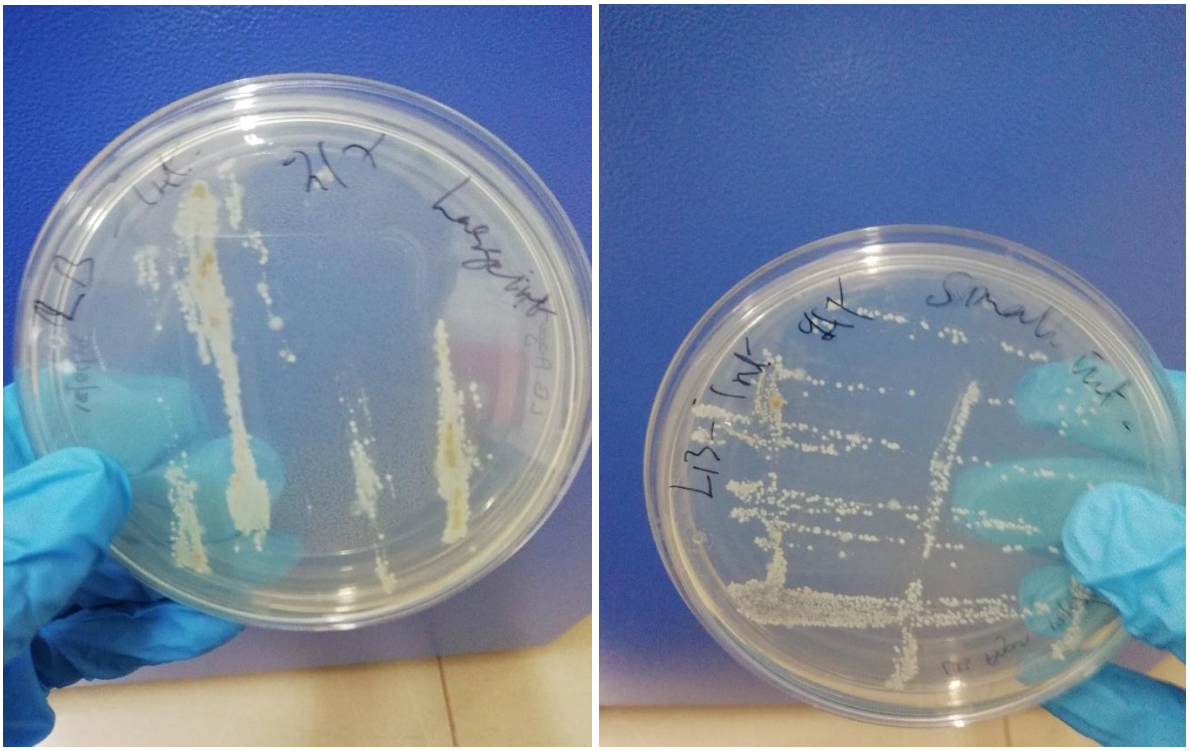
2.6.4.2 Staining of Bacteria

Gram Staining is a differential staining technique developed by Hans Christian Gram that is used to distinguish between gram positive and gram-negative bacteria. The peptidoglycan layer is thicker in gram positive bacteria when compared to their gram-negative counterparts. Crystal Violet and Safranin are the two stains employed in Gram Staining.

Crystal Violet is a primary stain which when added stains all the bacterial cells purple. The mordant used in this staining technique is Gram's Iodine which forms a complex with Crystal Violet (called the CV-I complex) and helps to amplify the intensity of the stain. Ethanol (95%), the decolourising agent removes the primary stain easily from the gram-negative cells due to the presence of a very thin peptidoglycan layer whereas gram positive bacteria successfully retain the Crystal Violet stain. Addition of a secondary or counter stain such as Safranin stains the gram-negative cells pink. Thus, gram positive bacteria appear purple and gram-negative bacteria appear pink when viewed under a compound microscope.

RESULTS

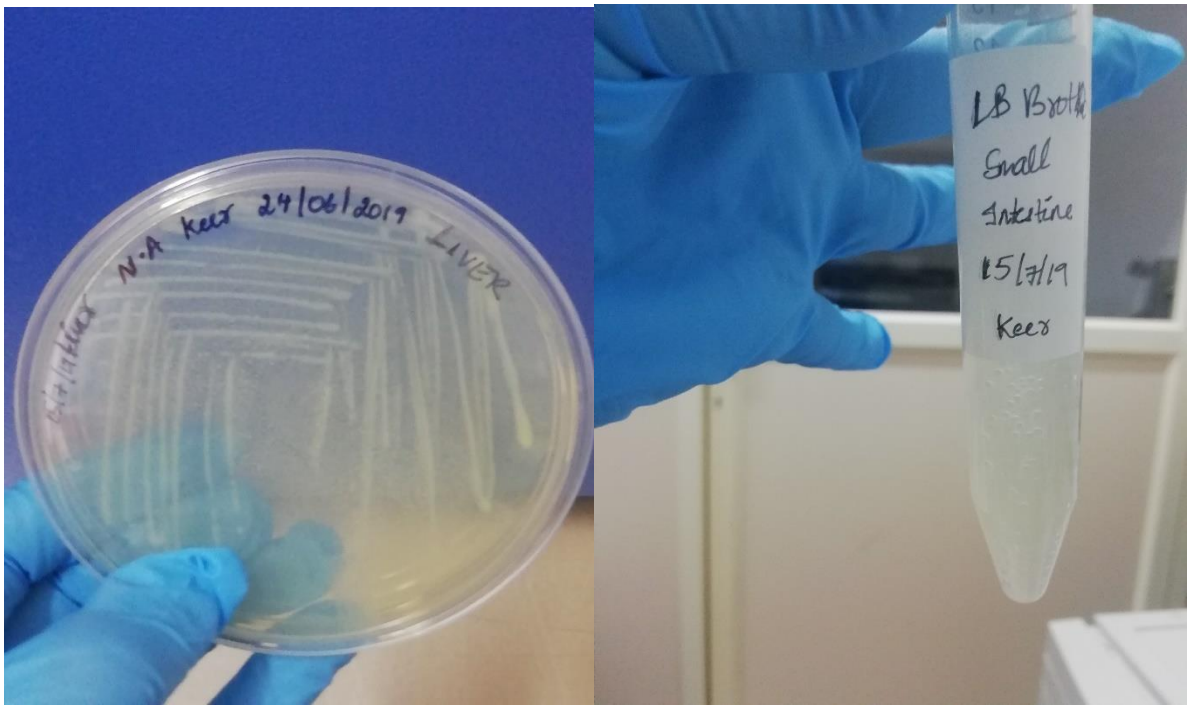
Following incubation, slimy mucoid colonies were observed on the nutrient agar and LB agar plates streaked with small intestine and large intestine samples.



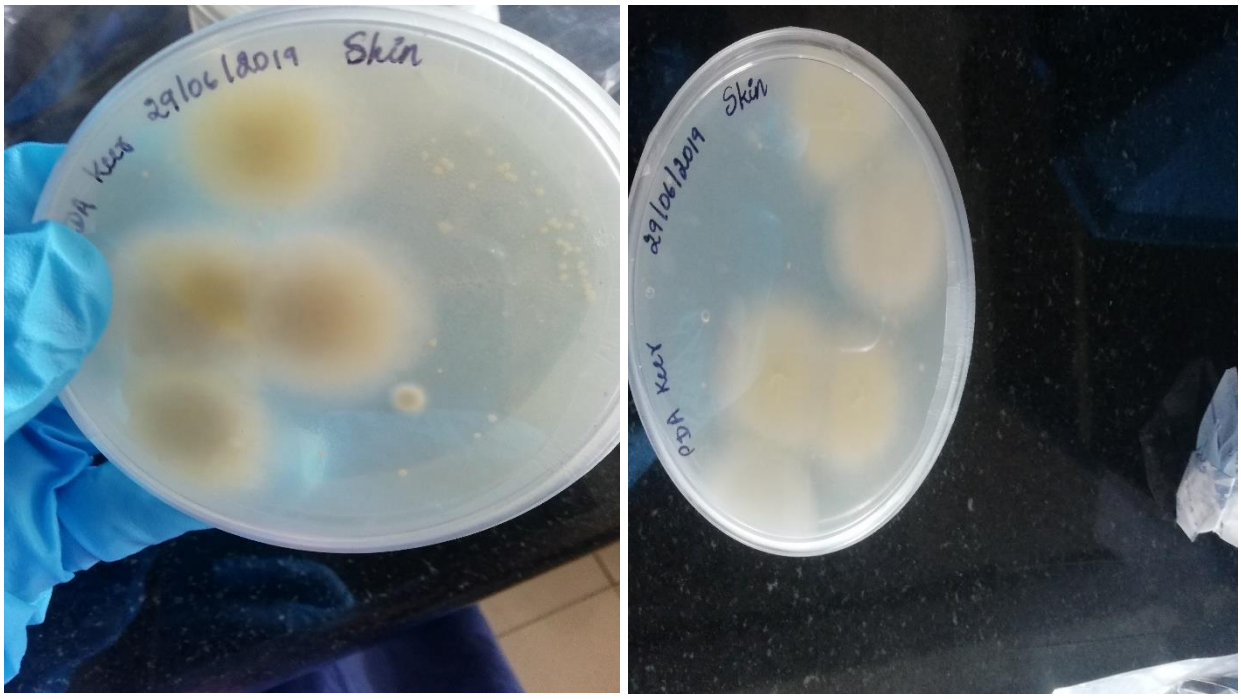
[**Figure 9**: Mucoid colonies found on the LB agar plates streaked with samples from large intestine (left) and small intestine (right)]

[**Figure 10**: Nutrient agar plates showing round mucoid colonies from large intestine (left) and small intestine (right)]

There were no colonies observed on the plate streaked with liver and spleen samples. But when broth containing the liver sample was streaked on the nutrient agar and LB agar plates, colonies were observed.



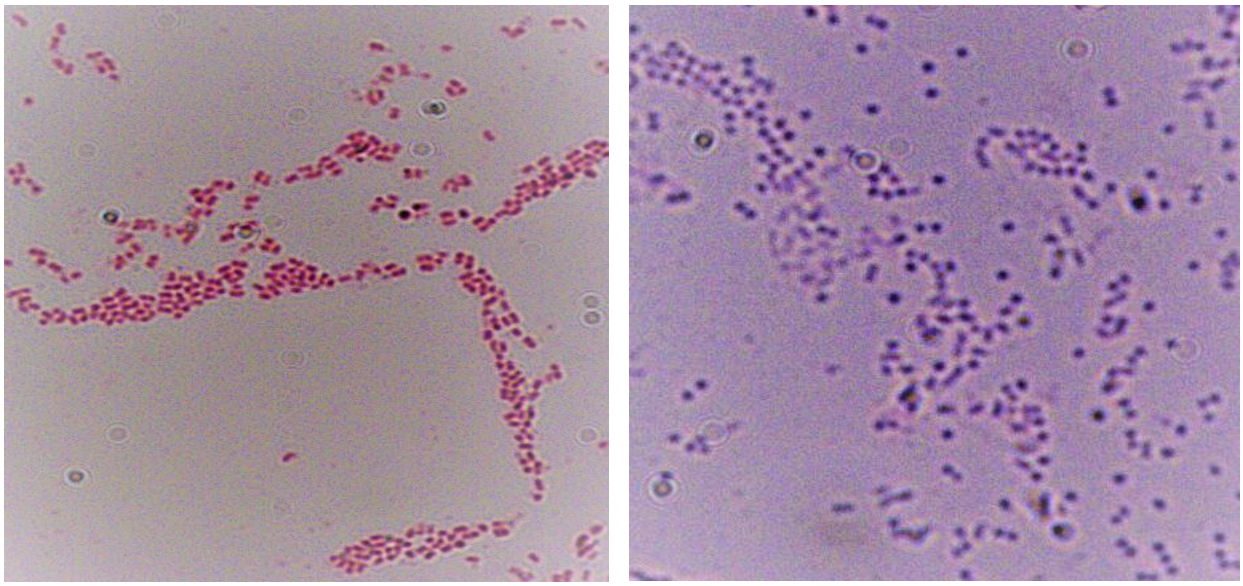
[**Figure 11:** Liver sample streaked onto Nutrient Agar (left) and LB Broth with small intestine sample showing turbidity (right) indicating the presence of bacteria]



[**Figure 12:** Potato Dextrose Agar plates with round light brown fungal colonies]

The bacteria cultured from the liver, small intestine and large intestine samples of mice were catalase positive.

[**Figure 13:** Bacteria from the large intestine, liver and small intestine were found to be catalase positive due to the evolution of bubble resulting from the breakdown of H_2O_2]



[**Figure 14:** Gram negative rod shaped (bacillus) bacteria found in the small intestine (left) and Gram positive bacteria found in the large intestine (right)**]**

Conclusion: As per the results, slimy round mucoid colonies were found to be grown on the nutrient agar and LB agar plates with samples from the intestines of the C57BL/6 black mouse. There were no colonies observed on the plates streaked with spleen and kidney samples. These organs were found to be healthy in the mouse. Therefore, based on the colony characteristics, catalase test and Gram staining, the bacteria found to inhabit the intestines of the mouse under study were E. coli which represented aerobic gram-negative rods and when Gram stained appeared pink. Also, when one of the samples from the large intestine was Gram-stained, it showed the presence of purple coloured circular colonies that represented gram positive cocci.

Future Scope

These bacteria can be more specifically identified using further biochemical tests and other techniques such as real time PCR, microarray, metagenomic assay, Fluorescent In-situ Hybridization (FISH), MALDI-TOF mass spectrometry. A novel method known as 16s ribosome-based identification is used more widely in the recent days to characterise specific intestinal bacteria. This method is based on the principle that 16s rRNA can be employed for the identification of all prokaryotes. Also, the 16srRNA in prokaryotes has hyper variable regions which are highly conserved for a particular species. Following the isolation of genomic DNA from the microbes, if PCR primers specific to the hyper variable regions are designed species specific identification can be made possible. This method could be employed in our study to get more information on gut microbiota along with their characteristics and functions.

ACKNOWLEDGEMENTS

I would like to thank the BEST programme supported in part by Aniket Singh Internship programme, Centre for Biosystems Science and Engineering (BSSE), Dr. Gopalkrishna Hegde and Dr. G.K.Ananthasuresh for giving me such a wonderful opportunity to be a part of the Summer Internship 2019. Sincere thanks to my Supervisor Dr. Sheshagiri Gaonkar for guiding me in this Programme. Further

I would like to thank Ms. Jayshree, Ms. Pallavi, Mr. Vishwas, Mr. Mudasir and Mr. Ameya for the support extended to me during my internship.

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