MOLECULAR DETECTION OF RESPIRATORY PATHOGENS IN RED EARED SLIDER TURTLES.

T H E S I S

Submitted

In partial fulfillment of the requirements for the Degree of

MASTER OF VETERINARY SCIENCE

IN

ANIMAL BIOTECHNOLOGY

BY

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2021

DECLARATION OF THE STUDENT

I hereby declare that the experimental research work and interpretation of the thesis entitled **"MOLECULAR DETECTION OF RESPIRATORY PATHOGENS IN RED EARED SLIDER TURTLES."** or part thereof has not been submitted for any of the other degree or diploma of any university, nor the data has been derived from any thesis or publications of any university or scientific organization. The sources of material used and all assistance received during the course of investigation have been duly acknowledged.

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This is to certify that the thesis entitled **"MOLECULAR DETECTION OF RESPIRATORY PATHOGENS IN RED EARED SLIDER TURTLES"** submitted by **MISS MISSAL TEJASHREE SURENDRA** to the Maharashtra Animal Sciences University, Nagpur, in partial fulfillment of the requirement for the degree of Master of Veterinary Science (M.V.Sc.) has been approved by the Student's Advisory Committee after examination in collaboration with the External Examiner.

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LIST OF ABBREVIATIONS

AGE : Agarose gel electrophoresis

BLAST : Basic Local alignment search tool

Bp : Base pair

cDNA : Complementary DNA

Concn : Concentration

DNA : Deoxyribonucleic acid

dNTPs : Deoxynucleotide triphosphates

DW : Distilled water

EDTA : Ethylene diamine tetra acetate

EU/ml : Equivalent Unit per milli litre

***et al.* : Et Alia (and others)**

NCBI : National Center for Biotechnology Information

NSS : Normal saline solution

PBS : Phosphate buffer saline

P:C:I : Phenol:Chloroform:Isoamyl Alcohol

PCR : Polymerase chain reaction

PPE : Personal Protective Equipments

RES: Red Eared Slider

RT-PCR : Reverse Transcriptase PCR

TAE : Tris acetate EDTA buffer

Taq : *Thermus aquaticus*

TE : Tris-EDTA Buffer

viz : Namely

UNITS OF MEASUREMENT

% : Percentage

°C : Degree Celsius

µg : Microgram

µL : Microlitre

hr : Hours

I.U. : International Unit

kb : Kilobase

lbs:Pounds

min : Minute

ml : Mililitre

mM : Milimolar

mm : Milimeter

MW : Molecular weight

Nm : Nanometer

N : Normality

pg : Picogram

pH : Potential of Hydrogen

pmole : Picomole

rpm : Rotation per minute

sec : Seconds

U : Units

V : Volts

v/v : Volume by volume

vol. : Volume

w/v : Weight by volume

1. INTRODUCTION

Trachemys scripta elegans or commonly known as Red Eared Sliders are freshwater turtles belonging to the order Testudine, suborder Cryptodira and family Emydidae (Boyer and Innis, 2019). The name comes from the characteristically red post orbital stripes and their ability to easily slide off rocks and logs into water (Burger, 2009). This species is native to southern United States and northern Mexico and they have been included in the list of "World's 100 Most Invasive Species" published by the IUCN. The red-eared slider is now found on every continent except Antarctica (Salzberg, 2000). These turtles have been gaining popularity as house pets in urban and suburban areas over the past few years because of their ample availability at local aquarium stores and their compact size. These turtles are usually kept in pairs in most households. Since these are freshwater turtles, they require ample area to swim and prefer being in water for longer hours. They tend to come on dry shore or logs to bask which helps in regulating their body temperature and to rid of any external parasites on their bodies when in the wild (Morreale and Gibbons, 1986).

Although Chelonians have a huge lifespan, both aquatic and terrestrial species are susceptible to numerous health problems in captivity. Being ectotherms, these organisms are unable to regulate their body temperature and thus are prone to conditions caused due to low environmental temperatures. Environmental temperatures have a significant influence on the physiological condition, metabolism, growth, behavior and immunity in ectotherms (Rojas *et al.*, 2005). Proper management and care of these reptiles facilitate healthier and relatively disease free lives as compared to the ones poorly kept. Adequate amount of basking under a UV lamp, a protein rich diet and thermoregulation helps keeping these turtles healthy. Commonly occurring disorders in captive chelonians include hypocalcemia, stunted growth, metabolic disorders, respiratory infections and gastric infections. Majority of these conditions can be avoided by following adequate managemental practices and

regular health check-up. Chelonian diet should comprise of a variety of high protein diet and multivitamin and calcium supplementation.

Respiratory tract infections have been commonly observed in captive chelonians. Common symptoms expressed during any respiratory infection by the turtles include mucoid discharge from nostrils, swollen eyelids, lethargy, lopsided swimming and anorexia.

The causes of infections could be viruses, bacteria, fungi and parasites. Herpes virus associated upper respiratory tract diseases and necrotizing stomatitis has been reported (Sim *et al.,* 2015). An upper respiratory tract disease (URTD) caused bacteria belonging to the *Mycoplasma* spp. is observed commonly in turtles (Palmer *et al.,* 2016). Captive chelonians which are kept at subnormal temperatures have also known to be susceptible to mycotic pulmonary diseases (Origgi and Jacobson, 2000).

Upper Respiratory Tract Disease like syndrome (URTD-LS) has been observed to be affecting Eastern Box Turtles; the signs included ocular and nasal discharge along with swollen eyelids. Infection of a *Mycoplasma* spp and differentiation of mycoplasma associated URTD from other suspected causes of URTD-LS and aural abscessation in box turtles was confirmed by PCR (Feldman *et al.*, 2006).

Terrapene Herpesvirus 1 (Ter HV1), box turtle adenovirus (BTADV), and an un-named *Mycoplasma* spp. (BTMyco) have also been associated with mortality in free ranging Eastern Box Turtles in Urbana, Illinois, United States of America (Adamovicz *et al.*, 2018)

Lung eye trachea disease associated virus (LETV) and gray patch disease (GPD) associated virus is known to cause illness and death in juvenile maricultured green turtles (*Chelonia mydas*) and are a major health concern, especially in turtles reared for release into the wild. This virus has been classified as Chelonian Herpesvirus 1 by the International Committee on the Taxonomy of Viruses [ICTV] (Rebell *et al.,* 1975). Clinical signs of Chelonian herpes virus and *Mycoplasma agassizii* infections may overlap, and the infections can be difficult to distinguish. The most common signs seen in both herpes virus and *Mycoplasma* infections are

rhinitis, conjunctivitis, and stomatitis. However, stomatitis might not be associated with mycoplasmosis (Jacobson, 2007).

Upon aerobically culturing oral swabs of severely infected and moribund Eastern Box Turtles a heavy bacterial growth of multiple organisms including *Escherichia coli, Vibrio alginolyticus, Morganella morganii* and *Aeromonas hydrophila* was observed (Sim *et al.,* 2016). Even in healthy chelonians supposedly pathogenic gram negative bacteria are said to be a part of their normal microflora. (Heynol *et al.*, 2015)

Many organisms associated with these respiratory infections are zoonotic in nature e.g. *Citrobacter* spp, *Escherichia coli*, *Aeromonas* spp, *Salmonella* spp. The transmission of these zoonotic infections to owners could occur if adequate personal hygiene measures are not adopted. Prevention will require a comprehensive One Health approach involving human, animal, and environmental health (Bosch *et al.*, 2016).

Very scarce literature about the respiratory pathogens of red eared slider turtles is available from Mumbai and India. The purpose of this study was to shed some light over the diagnosis of the disease and detection of specific pathogens which would further help facilitate an adequate therapeutic approach. The turtles suffering from such mixed respiratory tract infections require intensive supportive care along with good management practices of turtle keeping.

This study was therefore designed with the following objectives,

1. Isolation and identification of pathogens associated with respiratory tract of Red Eared Slider turtles.
2. Molecular characterization of representative isolates using molecular techniques.

2. REVIEW OF LITERATURE

Red eared slider turtles, a subspecies of pond sliders, have compact size which makes it easier to keep them in aquariums and small ponds in households. The popularity of this species among the pet owners in India is ever increasing. But the lack of knowledge about proper management practices and behavior of the reptile might lead to adverse health issues. It is very likely that the owners of diseased pet abandon the animal to survive in the wild. This species, being highly invasive, is said to displace the native turtle species and also transmit diseases to the native population (Pendlebury *et al.*, 2006). Under such scenario, it becomes important to ensure that there is perfect diagnosis of the disease condition and appropriate treatment of the pet.

Red eared sliders are supposed to easily get sick, especially in captivity. Most common conditions these animals suffer include: swollen eyelids, ocular infections, hypovitaminosis A, abscesses, shell rot and respiratory diseases. Most often one condition might lead to onset of the other due to decreased immune response and further aggravate the situation. Following appropriate guidelines while handling these reptiles, can help to easily avoid most illnesses associated with sliders. (turtleholic.com, redearedslider.net).

Respiratory diseases are frequently observed in captive chelonians, which range from acute to chronic in nature, the exact cause of these infections need to be known to tackle them adequately.

Pathogens of Zoonotic Importance:

Reptiles have known to be carriers of certain zoonotic pathogens. These zoonotic diseases are easily transmitted to the owners who handle their pets without following proper hygienic practices.

Bosch *et al.*, (2016) reported a total of 15 multistate outbreaks during 2006–2014, of turtle-associated salmonellosis in humans in the United States of America. They recognized that exposure to small pet turtles is a source of human salmonellosis.

The zoonotic transmission of *Salmonella* bacteria to children were reported in these outbreaks since turtles are a popular reptilian pet among children.

Heo *et al.,* (2016) reported that *Citrobacter freundii* was isolated and characterized by conventional pathogenicity tests, such as proteolysis, biofilm formation and hemolysis, PCR assays of virulence genes and antimicrobial disk diffusion tests. This zoonotic *Citrobacter freundii* infection can occur if pet turtle owners fail to hygienically handle the turtle or the turtle's environment.

Rzeżutka *et al.,* (2020) detected the presence of *Cryptosporidium* DNA in one sample of intestinal scraping collected from a red-eared slider. A phylogenetic analysis of a 18SSU rRNA gene fragment showed 100% sequence identity between the *C. parvum* strain isolated from the turtle and other *C. parvum* strains previously detected in cattle from the Lublin province. There was no clinical evidence that the red-eared slider turtle was truly infected rather than being merely a mechanical parasite carrier.

Thomas *et al.,* (2020) studied Spotted Turtles which were lethargic and showed reduced/stopped food intake for a week. Eye inflammation was observed, and a slimy secretion surrounded the eyes, nostrils and feet of the spotted turtles. These turtles were found tobe infected with *Aeromonas hydrophila* and *Citrobacter portucalensis* which are potential zoonotic pathogens with multidrug resistance.

Bonacina *et al.,* (2021) collected serum samples from 49 *Trachemys scripta elegans* living in a natural park of northern Italy and tested by a microagglutination test to measure detectable antibodies against different *Leptospira* serovars. It was not clear if *Leptospira* can cause disease in chelonians or if these animals can serve as reservoirs of leptospirae. Considering that chelonians often share the same environment with other animals and humans, and considering the One Health perspective, investigations to better understand the role of chelonians as a source of *Leptospira* infection are necessary.

Respiratory tract infections in chelonians:

Respiratory tract infections in chelonians are mainly characterized by symptoms like nasal discharge, respiratory distress, anorexia and blepharedema. These infections in chelonians are most likely caused due to pathogens like Herpes virus, Ranavirus, Picornaviruses, Ferlaviruses *Mycoplasma*, *Klebsiella, Pseudomonas, Aeromonas, Escherichia coli, Salmonella,* and *Proteus* spp. (Feldman., 2006). Chelonian Herpes virus and *Mycoplasma agassizzi* are found to be the most common respiratory pathogens in turtles (Soares *et al.*, 2004).

Viral diseases of respiratory tract in Chelonians:

Cox *et al.,* (1980) documented that a painted turtle (*Chrysemys picta*) which died in captivity had marked necrosis in the liver and lungs with numerous intranuclear inclusion bodies in hepatocytes and respiratory epithelial cells. Electron microscopy revealed presence of herpesvirus-like particles in cells in affected tissues.

Braune *et al.,* (1989) reported a highly fatal diphtheroid-necrotizing stomatitis in tortoises of unknown etiology. These tortoises suffered from dyspnea and anorexia, due to massive diphtheroid membranes in oral and pharyngeal cavities. Histologically, eosinophilic intranuclear inclusion bodies were found in epithelial layers of oral and tracheal mucous membranes. Furthermore, electron microscopy revealed herpesvirus like particles in affected cells.

Pettan-Brewer *et al.,* (1996) discovered using electron microscopy, that herpesvirus particles were observed in intranuclear inclusions and cytoplasm. Viral stomatitis, tracheitis, and bronchopneumonia complicated by bacterial infection were diagnosed by them. Although respiratory disease is common in desert tortoises, this was believed to be the first report of association with a viral infection.

Westhouse *et al.,* (1996) documented that a free-living adult male gopher tortoise (*Gopherus polyphemus*) was found on Sanibel Island, Florida (USA), with signs of upper respiratory disease. Necropsy after euthanasia and transmission electron microscopy of formalin fixed trachea and lung was performed. Intracytoplasmic viral particles were observed within necrotic cells in the tracheal

lumen and epithelial cells of the lung. Mature extracellular virions were surrounded by an envelope and were 150 to 220 nm in diameter. Virions and cytoplasmic inclusions were morphologically similar to those of the Family Iridoviridae.

Muro *et al.,* (1998) documented an epidemic of chronic rhinitis in a population of 50 captive spur-thighed tortoises (*Testudo graeca graeca*) from Palafrugell (Girona, Spain), in which eight animals died and 12 were euthanatized to perform necropsies and post-mortem studies. The main clinical sign was a bilateral, seromucous rhinitis often accompanied by stomatitis and glossitis. Lesions were restricted to the respiratory system and oral cavity. Marked epithelial hyperplasia and presence of a severe mixed inflammatory infiltrate in the epithelium of the oral, nasal, and tracheal mucosae were observed. Electron microscopy demonstrated the presence of intracytoplasmic and intranuclear viral particles of the size, shape, and distribution pattern typical of a herpesvirus.

Curry *et al.,* (2000) conducted an experiment in which, Lung eye trachea disease (LETD)-associated herpesvirus (LETV) was used as a model chelonian herpesvirus to test viral infectivity after exposure to seawater. The LETV virus preparations grown in terrapene heart (TH-1) cells were dialyzed for 24 to 120 hrs. against aerated artificial or natural seawater or Hank's balanced salt solution (HBBS). Fresh TH-1 cells were inoculated with dialyzed LETV and on day 10 post-infection, cells were scored for cytopathic effect. Virus samples dialyzed up to 120 hrs. were positive for the herpesvirus DNA polymerase gene by polymerase chain reaction.

Origgi and Jacobson (2000) stated that Herpes viral disease is more common in chelonians as compared to other reptiles. The disease resulted in significant mortality and was said to be epizootic in turtles. The turtles suffering from Herpes virus infection exhibited harsh respiratory sounds, caseated material covering the globe, around the glottis and within the trachea.

Marschang *et al.,* (2001) conducted a study in which a total of 16 chelonid herpesviruses that were isolated between 1992 and 1998 were compared with one another on the basis of serology and restriction enzyme digestion patterns of viral DNA. The viruses were found to stem from tortoises of three different species in four

different European countries and the United States of America. The majority of the isolates were similar to one another. One isolate, however, differed strongly from all others both serologically and in the restriction cleavage pattern of its DNA, showing that there are at least two different sero- and genotypes of herpesviruses that infect tortoises.

Johnson *et al.*, (2005) documented that Herpesvirus infection in a captive California desert tortoise (*Gopherus agassizii*) was detected by light microscopic observation of intranuclear inclusion bodies in various tissues followed by transmission electron microscopic observation of herpesvirus-like particles, and amplification of herpesvirus nucleic acid sequences using polymerase chain reaction. Using an indirect enzyme linked immunosorbent assay, anti-tortoise herpesvirus antibodies were detected one month after initial onset of clinical signs. This novel herpesvirus is distinct from the previously described tortoise herpesvirus (tortoise herpesvirus-1, THV-1) sharing 83% sequence identity of 60 amino acids of a portion of the DNA polymerase gene and 79% sequence identity across 120 amino acids of a portion of the ribonucleotide reductase gene. Similar to THV-1, this novel herpesvirus, tortoise herpesvirus-2 (THV-2), also clusters with the alphaherpesviruses.

Johnson *et al.*, (2007) designed an experimental transmission study to determine whether a causal relationship exists between a Ranavirus (BSTRV) isolated from a Burmese star tortoise that died and the lesions observed in that tortoise. A pilot study was performed with 3 box turtles (*Terrapene ornata ornata*) and 3 red-eared sliders (RESs; *Trachemys scripta elegans*) to assess their suitability in a larger study. Three of four IM-inoculated RESs developed clinical signs (nasal and ocular discharge, oral plaques, conjunctivitis and hyphema and extreme lethargy). A Ranavirus was isolated from kidney homogenates of 3 euthanatized turtles; DNA sequences of a portion of the major capsid protein gene were amplified by polymerase chain reaction. Virions compatible with Ranavirus were observed

within necrotic cells of the spleen of 1 IM-inoculated turtle using transmission electron microscopy.

Johnson *et al.,* (2008) studied the instances of Ranaviral diseases in chelonians which is said to be causing mass mortality events in reptiles. A portion of the major capsid protein gene from 2003-2005 was sequenced and was found to be identical to each other and to Frog Like Virus 3 across 420 base pairs. These results suggested that amphibians and chelonians were infected by a similar virus.

Stacy *et al.,* (2008) studied that Lung Eye Trachea Disease and Grey Patch Disease in *Chelonia mydas* turtles (Green sea turtles) are Herpes viral in origin. A nested PCR targeting a conserved region of the DNA dependent DNA polymerase was conducted. Phylogenetic analysis of these products revealed that all the chelonian herpes viruses belong to the subfamily α-herpesvirinae.

Ossiboff *et al.,* (2015b) found a high incidence of herpesvirus infection in bog turtles and smaller numbers of positive wood (5) and spotted (1) turtles. Sequence and phylogenetic analysis revealed three previously uncharacterized alphaherpesviruses. Glyptemys herpesvirus1 was the predominant herpesvirus detected and was found exclusively in bog turtles in all states sampled. Glyptemys herpesvirus2 was found only in woodturtles. Emydidherpesvirus2 was found in a small number of bog turtles and a single spotted turtle from one state. Based on these findings, Glyptemys herpesvirus1 appeared to be a common infection in the study population, whereas Glyptemys herpesvirus2and Emydid herpesvirus2 were not as frequently detected. Emydid herpesvirus2was the only virus detected in more than one species.

Ossiboff *et al.,* (2015a) documented that a captive, juvenile, female northern map turtle (*Graptemys geographica*) was found dead following a brief period of weakness and nasal discharge. Postmortem examination identified pneumonia with necrosis and numerous epithelial, intranuclear viral inclusion bodies, consistent with herpesviral pneumonia. Similar intranuclear inclusions were also associated with foci of hepatocellular and splenic necrosis. Polymerase chain reaction (PCR) screening of fresh, frozen liver for the herpesviral DNA-dependent DNA polymerase

gene yielded an amplicon with 99.2% similarity to recently described emydid herpesvirus 1 (EmyHV-1).

Sim *et al.,* (2015) identified a novel herpesvirus, Terrapene herpesvirus 1 (TerHV1) and serves as the first herpesvirus sequenced in the genus Terrapene. Similar to the other herpesviruses of the Order Testudines, TerHV1 clusters with the genus Scutavirus of the subfamily Alphaherpesvirinae.

Winzeler *et al.,* (2015) conducted quantitative PCR analyses of DNA from liver tissue, ocular, oral, nasal, and cloacal swabs were all positive for ranavirus, and sequencing of the template confirmed infection with a FV3-like ranavirus. Histopathologic examination of postmortem tissue samples revealed ulceration of the oral and tracheal mucosa, intracytoplasmic epithelial inclusions in the oral mucosa and tongue sections, individualized and clusters of melanomacrophages in the liver, and bacterial rods located in the liver, kidney, heart, stomach, and small intestine. This was the first report of morbidity and mortality of a mud turtle with a systemic ranaviral infection.

Adamovicz *et al.,* (2018) studied the pathogenic factors behind the declining numbers of free ranging Eastern Box Turtles. Single and co pathogens detected included Ranavirus, Terrapene herpesvirus 1, adenovirus, and *Mycoplasma* sp.

Bacterial diseases of respiratory tract in Chelonians:

Respiratory bacterial infections are the most commonly occurring diseases in Chelonians. These infections range from acute to chronic type and are primary infections or could be secondary to preexisting conditions.

Snipes *et al.,* (1980) recorded that *Pasteurella* sp was consistently found to be associated with respiratory lesions in captive tortoises with signs of respiratory disease but was also found to be part of the gastrointestinal and nasal flora of healthy tortoises. It was hypothesized that respiratory disease in captive desert tortoises involves a commensal bacterium with the potential to be an opportunistic pathogen when the tortoise is stressed by a captive environment.

Schumacher *et al.,* (1993) used an enzyme-linked immunosorbent assay (ELISA) for the detection of *M. agassizii*-specific antibodies in desert tortoises was developed with a monoclonal antibody with specificity for desert tortoise immunoglobulin light chain. Plasma samples from one group of tortoises were tested immediately before and 1 month after challenge either with nasal exudate containing *M. agassizii* or with a purified preparation of *M. agassizii*. Plasma samples from a second group of known healthy and sick tortoises were also tested. In the first group, the ELISA detected seroconversion in individual tortoises following challenge with *M. agassizii*. In the second group, ELISA results were positively correlated with the health status of the tortoises, as determined by clinical and pathologic findings. In addition, the ELISA revealed that tortoise antimycoplasma antibodies were specific for *M. agassizii* when samples were assayed against *M. agassizii, M. pulmonis, M. testudinis*, and *M. gallisepticum* antigens. The observed direct correlation between the presence of nasal mucosal lesions and *M. agassizii*-specific antibodies proved that the ELISA reliably diagnosed *M. agassizii* infection in desert tortoises.

Lederle *et al.,* (1997) conducted a study in which a population of desert tortoises (*Gopherus agassizii*) at Yucca Mountain (Nevada, USA) was monitored during four sampling periods using enzyme-linked immunosorbent assays (ELISA) to determine the percentage of individuals that had been exposed to *Mycoplasma agassizii*, a causative agent of upper respiratory tract disease. From 15 to 23% of samples per period tested positive for exposure to the mycoplasma. However, few clinical signs of upper respiratory tract disease were noted.

Jensen *et al.,* (2003) reported the anatomopathological and microbiological findings in a case of fatal pulmonary and cutaneous *Mycobacterium kansasii* infection in a captive Chinese soft shell turtle (*Pelodiscus sinensis*). To the authors' knowledge, this is the first report of *M kansasii* infection in a reptile.

Jacobson, (2007) isolated *Mycobacterium marinum* from hepatic and splenic granulomas in two australian snakenecked turtles (*Chelodina longicollis*), from the lung of a loggerhead sea turtle (*Caretta caretta*) with a pulmonary nodule

and from tissues of one of five hawksbill sea turtles (*Eretmochelys imbicata*) with visceral granulomas containing acid-fast organisms. *Mycobacterium kansasii*, was identified in a captive Chinese soft-shelled turtle (*Pelodiscus sinensis*), the turtle developed several small white foci on its carapace, showed signs of respiratory distress, and subsequently died. At necropsy several white papular lesions were found on the neck and carapace, and white nodules were found within the pulmonary parenchyma. The lung nodules consisted of granulomas, with acid-fast bacteria in their centers.

Lecis *et al.*, (2011) screened free-living European tortoises (spur-thighed tortoises *Testudo graeca*, Hermann's tortoises *Testudo hermanni*, marginated tortoises *Testudo marginata* temporarily housed in a wildlife center in Italy. They molecularly characterized 13 *Mycoplasma* isolates detected in all *Testudo* spp. studied, and three PCR-positive animals showed typical URTD clinical signs at the time of sampling.

Guthrie *et al.,* (2013) collected blood samples and nasal swabs from 40 free-ranging Texas tortoises on public and private lands in Texas, USA, from May to October 2009. ELISA, PCR and culture of the samples collected were carried out. Current infection with *M. agassizii* was confirmed in one tortoise that had mild clinical signs of URTD and was positive by ELISA (antibody titer >512), PCR, and culture. The clinical isolate was confirmed as *M. agassizii* by restriction fragment length polymorphism and immunobinding.

Silbernagel *et al.*, (2013) studied the occurrence of *Mycoplasma* infections in Red Eared Slider turtles introduced in California and observed that this infection was transmitted to the native Western pond turtles. Mycoplasmas were also found to be associated with lower body weight of the turtles.

McGuire *et al.*, (2014) studied a gopher tortoise (*Gopherus polyphemus*) population with a historically high prevalence of antibodies to *Mycoplasma agassizii* to assess long-term effects of upper-respiratory-tract disease (URTD) on tortoise behavior. 15-yr recapture data suggest that, despite high prevalence of *M. agassizii*, population density has not decreased over time. However, emigration,

especially of tortoises with severe clinical disease, may play an important role in dispersal and persistence of pathogens.

Ossiboff *et al.*, (2015) studied the presence of Mycoplasmal infection as a part of health assessment of endangered bog turtles using PCR. Sequence analysis of portions of the 16S–23S intergenic spacer region and the 16S ribosomal RNA gene revealed a single, unclassified species of Mycoplasma that has been previously reported in eastern box turtles, ornate box turtles (*Terrapene ornata ornata*), western pond turtles (*Emys marmorata*), and red-eared sliders (*Trachemys scripta elegans*).

Aplasca *et al.*, (2019) carried out a health evaluation of native and non-native invasive turtle species and isolated a *Mycoplasma* spp. from red eared slider turtles.

Sandmeier *et al.*, (2019) evaluated cause of injury and quantified levels of three potential mycoplasmal pathogens (*Mycoplasma agassizii*, *Mycoplasma testudineum*, and an emydid mycoplasma) in three-toed box turtles (*Terrapene carolina triunguis*) using a quantitative PCR (qPCR) assay for the emydid mycoplasma, with a similar specificity and sensitivity as the existing qPCR assays for *M. agassizii* and *M. testudineum.*

Pathogenic bacterial profiling from upper respiratory tract:

Evans (1983) conducted a study in which chronic bacterial pneumonia was diagnosed in two free-ranging Eastern box turtles. Mucoid exudation into the upper respiratory tract and bilateral, caseating pneumonia were seen grossly. Microscopically, chronic, active inflammation with pseudomembrane formation occurred in the nasal sinuses and lungs while caseating granuloma-like structures were also observed in alveoli and associated infundibulae. A mixture of gram-negative bacteria including *Morganella morganii*, *Acinetobacter calcoaceticus*, *Serratia marcescens* and *Pseudo monas* sp. were isolated from both turtles.

Santoro *et al.*, (2006) conducted bacteriological examination of 70 nesting green turtles (*Chelonia mydas*) from Tortuguero National Park, Costa Rica was performed to investigate nasal and cloacal aerobic bacteria. A total of 325 bacterial isolates were obtained, including 10 Gram-negative and three Gram-positive genera.

Hacioglu *et al.,* (2012) performed a study on pathogenic and non-pathogenic oral and cloacal microflora of free ranging freshwater turtles belonging to two different species which revealed the presence of organisms like *Aeromonas* sp., *Proteus* sp. *Vibrio* sp., *Escherichia coli, Klebsiella pneumoniae, Citrobacter freundii,* and *Enterobacter* spp.

Liu *et al.,* (2013) captured free-ranging Red-eared Sliders (*Trachemys scripta elegans*) from farm ponds located in the Flint Hills of Kansas and a zoo pond in Emporia, Kansas, USA, to evaluate their enteric bacterial flora and associated antibiotic resistance. Bacteria obtained from cloacal swabs were composed of six different Gram-negative genera.

Heynol *et al.,* (2015) studied the bacterial flora from oral swabs of two different species namely common musk turtles (*Sternotherus odoratus*) and West African mud turtles (*Pelusios castaneus*). Most commonly occurring oral bacteria were *Citrobacter* spp., *Aeromonas* spp, *Chryseobacterium* spp. and *Salmonella* spp.

Sim *et al.,* (2016) could isolate *Escherichia coli, Vibrio alginolyticus, Morganella morganii,* and *Aeromonas hydrophila* upon aerobically culturing the oral swabs from the moribund Eastern Box turtles.

Umbrasko et al., (2020) studied the *Emys Orbicularis* species to gather knowledge about their external and internal microbes. Bacterial communities largely consisted of two phyla, the Proteobacteria and Firmicutes. Most common found bacteria were *Pseudomonas* spp. (40%) and *Proteus* spp. (27%).

Molecular identification of respiratory pathogens in Chelonians:

Brown *et al.,* (1995) conducted a sequencing analysis of the 16S rRNA genes of two mycoplasmas, *Mycoplasma agassizii* and *Mycoplasma testudinis* and used it for taxonomic comparisons between different *Mycoplasma* sp.

VanDevanter *et al.,* (1996) developed a consensus PCR which targeted a herpesviral DNA-directed DNA polymerase and successfully amplified a short (215- to 315-bp) region of 21 of 22 different herpesviral species, it allowed for the determination of partial DNA polymerase-coding sequences from 14 herpes viruses

(12 animal and 2 human) for which no DNA polymerase sequence data have previously been reported.

Une *et al.*, (2000) used a PCR based method using a herpesvirus consensus primer for identification of herpesviral infection in tortoises. A single band of about 230 bp was detected in PCR products from two out of twenty swabs taken from the oral cavity, three out of three paraffin-embedded tissue sections from the liver (two cases) and oral mucosa (one case), and one out of two fresh tissue samples from the oral mucosa. Nucleotide sequencing of these PCR products indicated that the herpesvirus present in these tortoises might belong to the alphaherpesvirinae. PCR using swabs and biopsy tissues was a sensitive and highly specific method for the diagnosis of herpesviral infections in tortoises.

Soares *et al.*, (2004) confirmed the presence of *M. agassizii* by restriction digestion of the PCR product. A 307-bp fragment of the Chelonian Herpesvirus (ChHV) UL5 homologue gene was sequenced and found to show most similarity to equine herpesvirus type 1. A prevalence of 15.8 and 8.2% was found for *M. Agassizii* and ChHV, respectively. Comparison of the carriage of both *M. agassizii* and ChHV in different species of tortoises correlated the presence of *M. agassizii* with *Testudo horsfieldii* and ChHV with *Testudo marginata* and *Testudo graecaiberia*. An association of ChHV with stomatitis was also found. Mixed infections with both agents were detected.

Feldman *et al.*, (2006) carried out the detection of a *Mycoplasma* sp by PCR, studied the range of clinical signs of disease and differentiated the mycoplasma associated with URTD-LS from other causes of URTD-LS and aural abscessation in box turtles.

Salinas *et al.*, (2011) conducted a study using polymerase chain reaction for 63 tortoises (59 spur-thighed tortoise, *Testudo graeca*; three Greek tortoise, *Testudo ibera*; and one Russian tortoise, *Agryonemys horsfieldii*) with clinical signs of rhinitis-stomatitis complex to identify the causative agent. Both ChHV and *M. agassizii* are considered pathogenic in captive tortoises and both are a threat to wild

populations. However, neither agent was detected from most of the symptomatic tortoises evaluated, indicating that other agents could be involved in the rhinitis-stomatitis complex.

Jacobson *et al.,* (2012) measured the prevalence of antibodies to Testudinid herpesvirus (TeHV) 3 in wild populations of desert tortoises in California after following field observations of oral lesions similar to those seen in captive tortoises with herpesvirus infection**.** DNA was extracted from the tongue and from the nasal mucosa of two wild adult male desert tortoises, with gross lesions consistent with trauma and puncture wounds which were necropsied. Sequencing of polymerase chain reaction products of the herpesviral DNA-dependent DNA polymerase gene and the UL39 gene respectively showed 100% nucleotide identity with TeHV2, which was previously detected in an ill captive desert tortoise in California. Although several cases of herpesvirus infection have been described in captive desert tortoises, our findings represent the first conclusive molecular evidence of TeHV2 infection in wild desert tortoises. The serologic findings supported cross-reactivity between TeHV2 and TeHV3.

Origgi *et al*., (2015) reported the first *de novo* sequence assembly and analysis of the genome of *Testudinid herpesvirus 3* (TeHV3). Viral isolation, TeHV identification, phylogenetic analysis and pathological characterization of the associated lesions, were performed. The results revealed the existence of at least two distinct TeHV3 geno-groups apparently associated with different pathologies in tortoises and the first evidence for a putative homologous recombination event having occurred in a chelonian herpesvirus.

Palmer *et al.,* (2016) documented URTD associated with *Mycoplasma* sp. in two free-living, three-toed box turtles (*Terrapene carolina triunguis*) in Missouri, US. Both turtles were *Mycoplasma* sp. positive by PCR and had URTD-like clinical signs, including nasal and ocular discharge, palpebral edema, lethargy, and weight loss, during a 6–8-wk period.

Kolesnik *et al.,* (2017) conducted a screening of 1015 captive chelonians across Europe for the presence of *Mycoplasma* spp., herpesviruses, ranaviruses,

picornaviruses, and ferlaviruses with the help of PCR. Out of which they found out that there was a significant correlation between mycoplasma and herpes virus infection.

Marschang and Kolesnik (2017) used standard PCR methods for the detection of herpesviruses and mycoplasma, samples included oral swabs and nasal washes as well as various tissues and were obtained from both clinically ill and apparently healthy animals.

Adamovicz *et al.*, (2018) detected the presence of Herpes viral DNA using TaqMan qPCR assays and Mycoplasma targeting the 16S rRNA gene using conventional PCR.

DiGeronimo *et al.*, (2019) collected choanal-cloacal swabs of Indochinese box turtles among which two were positive for *Mycoplasma* sp. by polymerase chain reaction. Sequencing of the 16S rRNA gene and 16S-23S rRNA intergenic spacer was 99% homologous to an unclassified *Mycoplasma* sp. previously documented in free-ranging and captive North American species of the family Emydidae.

Ballouard *et al.*, (2021) used a PCR assay to detect the presence of herpesvirus and *Mycoplasma spp* in a population of western Hermann's tortoise (*Testudo hermanni hermanni*). This study revealed the presence of herpesvirus in 7 free-ranging individuals. Additionally, *Mycoplasma agassizii* was detected in 15 of the 18 populations sampled with a frequency ranging from 2.5 to 25%. Exotic spur-thighed tortoises showed high frequency of *Mycoplasma* infection in captivity (18%) and in individuals (50%) found in native Hermann's tortoise sub-populations, suggesting that this species could be a significant vector.

3. MATERIAL AND METHODS

The present study was conducted to identify the bacterial pathogens causing respiratory infections in captive Red Eared Slider turtles from Mumbai. The recovered isolates were identified using conventional methods comprising of biochemical characterization and then were further subjected to confirmation by Polymerase Chain Reaction (PCR) targeting 16S rDNA. The details of material used and methods followed are described below.

3.1 Material

3.1.1 Glassware

The glassware made up of neutral glass required for the present investigation was obtained from M/s Borosil, India. Before using, all of the glassware was treated with 1% HCl for overnight, thoroughly rinsed with distilled water, allowed to dry and then sterilized in hot air oven at 160°C for one hour as per the standard sterilization protocols.

3.1.2 Plastic ware

The plastic ware required for the present research viz. centrifuge tubes, microcentrifuge tubes, PCR tubes, pipette tips etc. was procured from M/s Tarsons, India, Kolkata. All the plastic ware was autoclaved at 120°C at 15 lbs pressure for 15 min before use.

3.1.3 Chemicals and reagents

The chemicals and reagents used during present investigation were obtained from following manufacturing companies.

1) M/s Takara Co. Ltd. (Japan)

2) M/s Fermentas Life Science (Canada)
3) M/s Hi Media Laboratories Private Limited, Mumbai (India)
4) M/s Merck, Mumbai (India)
5) M/s Eurofins Genomics India Pvt. Ltd.
6) M/s Sisco Research Laboratories, Private Limited, Mumbai (India)
7) M/s Thermo Fisher Scientific, Pvt. Ltd. (USA)

The chemicals and reagents required for PCR and other molecular biology work were of molecular grade.

3.1.4 Media

The following media were used for the isolation and identification of bacteria.

1) Brain Heart Infusion (BHI) Agar (HiMedia)
2) Mac-Conkey Agar (HiMedia)
3) Blood Agar (HiMedia)
4) Eosin Methylene Blue (EMB) Agar (HiMedia)
5) Nutrient Agar (HiMedia)
6) Simmons Citrate Agar (HiMedia)
7) Christensen's Urea Agar (HiMedia)
8) MR-VP Broth (HiMedia)
9) Indole-Nitrate Broth (HiMedia)
10) Brain Heart Infusion (BHI) Broth (HiMedia)

3.1.5 Equipment

A variety of equipment like thermal cycler, automatic gel documentation system, gel electrophoresis apparatus, refrigerated centrifuge, etc. was used during the present work. The details of equipment used have been mentioned in Appendix I.

3.1.6 Specimens

This study focused on identification of respiratory pathogens from red eared slider turtles. Therefore, oral swabs from turtles showing signs of respiratory distress were collected in duplicates (Plate 1). A total of 31 turtles, with respiratory symptoms, presented to several veterinary clinics were included in the study. Oral swabs were collected in duplicate; one set was subjected to isolation of bacterial pathogens, while the other was used for DNA extraction.

3.2 Methods

3.2.1 Collection, transport and preservation of samples

Majority of the samples were collected from private veterinary practitioners for diagnosis and treatment of the respiratory infections in red eared slider turtles. One set of the oral swabs was collected in MEM with 2% FBS for DNA extraction and the other was collected in Nutrient broth for bacterial isolation. Both swabs were transported to the laboratory immediately by maintaining a cold chain. The swabs for DNA extraction were stored at -20ºC and the swabs for bacterial isolations were directly incubated at 37°C for 18 - 24 hrs. for isolation of bacterial pathogens.

3.2.2 Identification of pathogens using conventional bacteriological techniques

The swabs incubated in nutrient broth were further used for isolation of bacterial pathogens by streaking on blood agar. Isolated colonies thus obtained on blood agar were observed for their morphological characters and subjected to Gram's staining and further biochemical tests such as Methyl Red (MR), Voges-Proskauer (VP) , Indole, Nitrate, Catalase, Oxidase, Citrate and Urease.

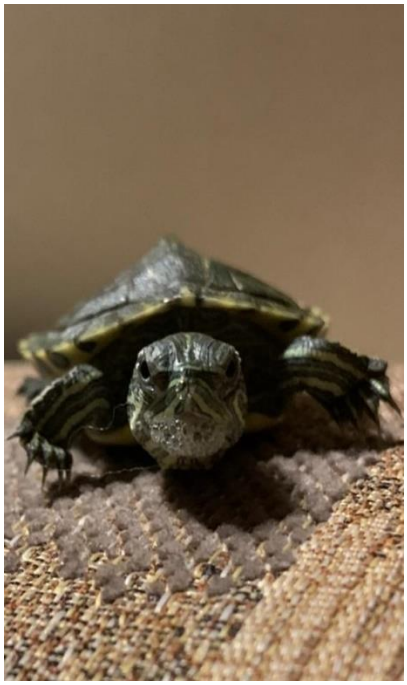

Plate 1. Collection of Oral Swabs and Turtle exhibiting signs of respiratory infection

a. Isolation on Blood Agar

The samples which showed turbidity in nutrient broth after incubation were inoculated on 5% sheep Blood Agar by following standard procedures. A loopful of the broth culture was streaked on the agar plates for isolation of bacteria and was incubated at 37°C for 18 – 24 hrs. Smears were prepared from the isolated colonies and were stained using Gram's Staining technique.

b. Gram's Staining

Air-dried and heat-fixed smears of bacterial isolates w were flooded with crystal violet stain for one minute. The slide was washed in a gentle and indirect stream of distilled water for two seconds. The slide was flooded with the mordant: Gram's iodine for one minute. The side was washed again in a similar manner before flooding it with decolorizing agent for 15 seconds. The slide was decanted and flooded with counterstain, safranin for 30 seconds to one minute and decanted and then air dried and observed under the microscope at 100x magnification under oil immersion lens. The gram-negative bacteria stained pink/red and gram-positive bacteria stained blue/violet.

c. Catalase Test

A loopful of colony was placed on the surface of a clean, dry glass slide. Then a drop of 3% H_2O_2 was added and immediate production of gas bubbles was considered as positive test, whereas in negative test no gas bubbles were produced.

d. Oxidase Test

Standard oxidase discs (Hi Media Laboratories Pvt. Ltd., Mumbai) were used to perform the test. The loopful of culture from single colony was just placed on the disc. Immediate development of blue colour was considered as positive test whereas negative test showed no colour change.

e. Methyl Red (MR) Test

The test was performed using Methyl red (MR) broth (HiMedia Pvt. Ltd.) in which pure culture was inoculated. The tubes were incubated at 37°C for 48 hours. After incubation five drops of Methyl red indicator solution was added to the test tube. Positive results were indicated by red colour however, yellow colour indicated negative results.

f. Voges-Proskauer (VP) Test

The test was performed using Voges-Proskauer (VP) broth in which pure culture was inoculated. The tubes were incubated for 48 hours at 37°C. After incubation 3 ml of Barritt's B reagent (alpha naphthol) and one ml of Barritt's A (40% KOH) reagent was added. The tube was exposed to atmospheric oxygen for 10-15 minutes. Positive results showed pink colour in two to five minutes and negative result was indicated by yellow or copper colour at the surface.

g. Indole Test

The test was performed by using five ml Indole broth in which pure culture was inoculated and incubated at 37°C for 48 hours. After incubation 0.5 ml of Kovac's reagent was added by slightly tilting the test tube and adding it slowly from the sides of the tube. Pink layer of xylene was considered as positive reaction and no colour change with yellow coloured ring at the surface indicated the test as negative.

h. Nitrate reduction Test

The test was performed by using five ml of Nitrate broth in which the pure culture was inoculated and incubated for 48 hours at 37°C. After incubation one ml of Sulphanilic acid and one ml of α-naphthylamine reagent was added to the broth

culture. Development of a distinct red colour (which may turn to brown rapidly) was considered as positive test. No change in colour indicated that the test was negative.

i. Citrate Test

Simmons Citrate agar slants were inoculated from 18 – 24 hrs. old colony cultures and incubated at 37° C and observed up to seven days. A positive reaction was observed by change in colour of the slant from green to blue with growth while negative showed no colour change of the slant and absence of growth.

j. Urease Test

Christensen's Urea agar slants were inoculated from 18 – 24 hrs. old colony cultures and incubated at 37°C and observed up to seven days. A positive reaction was observed by development of pink colour in the slant and no colour change indicated a negative reaction.

3.2.3 Molecular detection of Respiratory pathogens by Polymerase Chain Reaction (PCR)

The samples collected and the swabs isolated from turtles showing respiratory symptoms were subjected to molecular techniques, namely Polymerase Chain Reaction (PCR), for detection of the pathogens.

a. Extraction of DNA from swabs

DNA was extracted from swabs collected in MEM with 2% FBS using the procedure described by Van Devanter *et al.*, (1996). The procedure employed was as follows:

MEM containing swab was incubated with DNA extraction buffer at 65°C for overnight with an equal volume of Phenol-Chloroform (1:1)

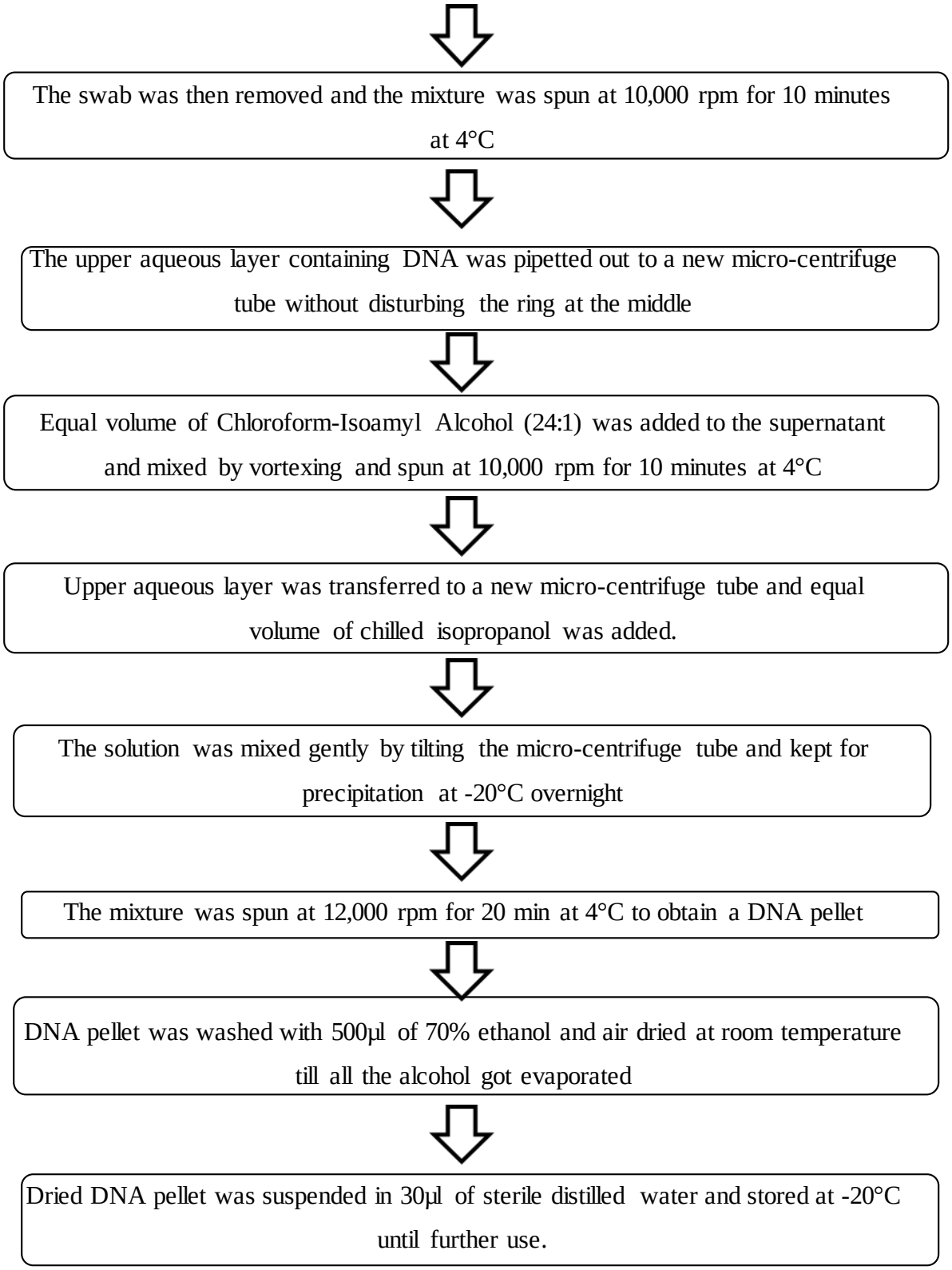

Figure 3.1 DNA Extraction From Swabs

b. Extraction of DNA from bacterial isolates

DNA was extracted from pure bacterial cultures as per the protocol described by Sambrook and Russell (2006). The procedure employed was as follows,

The isolates were subcultured in 1 ml of BHI broth by incubating at 37°C under for 16-20 hrs.

⇩

The cells were pelleted out by centrifugation @ 10,000 rpm/min for 10 min and re-suspended in 0.5ml of TE buffer by vortexing.

⇩

For disrupting the cell membrane, 100µl of 10 % SDS and five µl of Proteinase K (10mg/ml) was added, vortexed briefly and incubated at 37°C for 1 hour in water bath.

⇩

Equal amount of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added, vortexed for 10 sec. and centrifuged at 10, 000 rpm for 10 mins at 4°C

⇩

The upper aqueous phase containing DNA was carefully transferred to a fresh micro centrifuge tube and DNA was precipitated by addition of 0.6 volume of Isopropanol.

⇩

The tubes were then kept at -20° C for three hours or overnight followed by centrifugation at 12,000 rpm for 15 mins.

⇩

The supernatant was discarded, leaving about 20 µl above the pellet which was washed with one ml of 70% Ethanol and centrifuged at 11,000 rpm for 10 min.

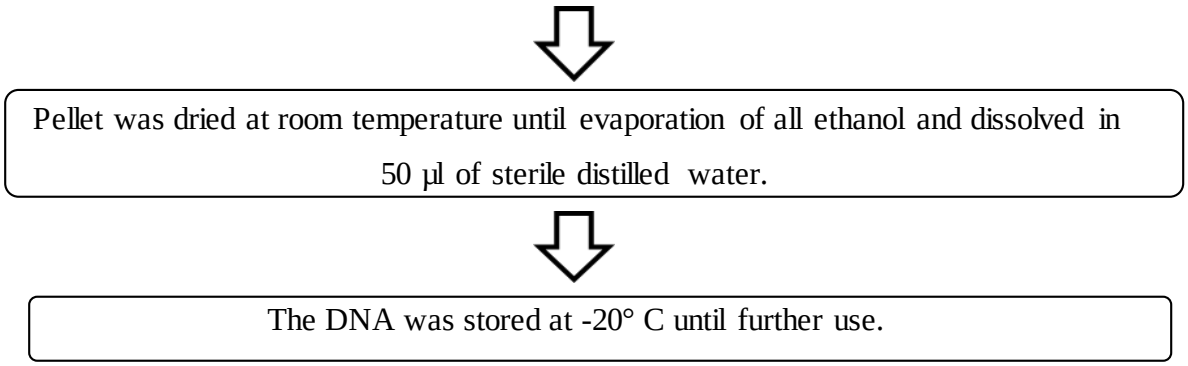

Figure 3.2 DNA Extraction From Bacterial Isolates

c. Quantification of DNA

The DNA extracted from the swabs and pure cultures were quantified spectrophotometrically at 260 nm and 280 nm using Epoch Biotek using two µl of the DNA sample and the $A_{260:}A_{280}$ ratio was calculated.

3.2.4. Universal 16S r DNA PCR

The genomic DNA extracted from pure bacterial cultures were subjected to polymerase chain reaction targeting the 16S rDNA region of the chromosome.

a. Universal 16S r DNA Primers for Prokaryotic DNA

The primers used for detection of bacterial pathogens by PCR targeted the conserved region of the bacterial genome which gives rise to the 16S subunit of the ribosomal DNA (rDNA) of all the bacterial species. Sequences of the primer set used are mentioned in table 3.1. These primers amplified a product of ~1400 bp.

Table 3.1 Universal Prokaryotic Primer 16S r DNA

Primer	**Oligonucleotide sequence**
Pro F	5'- GAGTTTGATCCTGGCTCA – 3'
Pro R	5'- ACGGCTACCTTGTTACGACTT -3'

b. Reaction Mixture

A total of 25 µl reaction mixture was prepared for 16S rDNA PCR using above primer set as mentioned in table 3.2.

Table 3.2 Reaction mixture for 16s r DNA PCR

Sterile water	10 µl
2 X PCR Master Mix	12.5 µl
Primer Pro- F (0.1 mM / µl)	0.75 µl
Primer Pro- R (0.1 mM / µl)	0.75 µl
Template DNA	1 µ l
Total	**25.00 µl**

c. Cycling Conditions

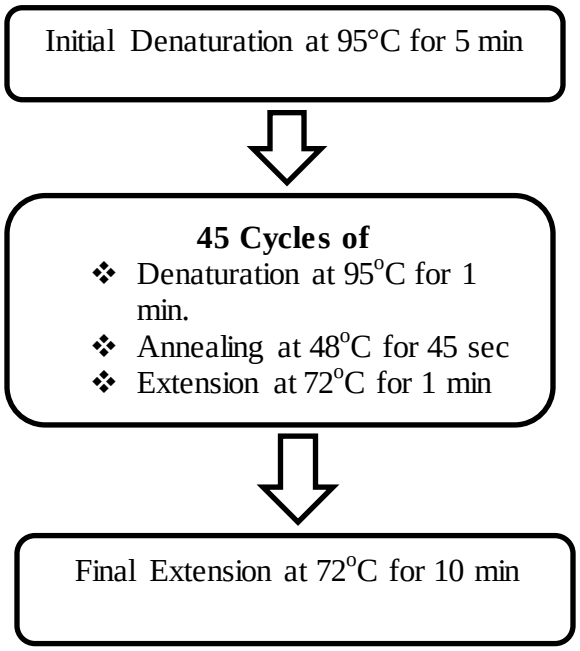

Figure 3.3 Cycling conditions for Universal 16S rDNA PCR

3.2.5 Characterization of *Pseudomonas spp.*

The isolates identified as *Pseudomonas* by conventional methods and 16S rDNA PCR were further characterised by OprF gene specific PCR.

a. Oligonucleotide primers

Table 3.3 Oligonucleotide primers for *Pseudomonas* OprF gene PCR

Primer	Oligonucleotide sequence
OprF F	CAGATGCGACCGAAACATAG
OprF R	CTGTCGCTGTTGATGTTGGT

b. Reaction mixture

The PCR was set in a final volume of 25 µl consisting of reactants shown in table 3.6

Table 3.4 Reaction mixture for *Pseudomonas* for OprF gene

Component	**Quantity (µl)**
Sterile water	19 µl
10X PCR buffer+ $MgCl_2$ (25 mM)	2.5 µl
dNTPs mix (10 mM)	1 µl
Primer OprF - F (0.1 mM / µl)	0.6 µl
Primer OprF - R (0.1 mM / µl)	0.6 µl
Template DNA	1 µl
Taq DNA polymerase (5 U /µl)	0.3 µl
Total	**25.00 µl**

c. Cycling conditions

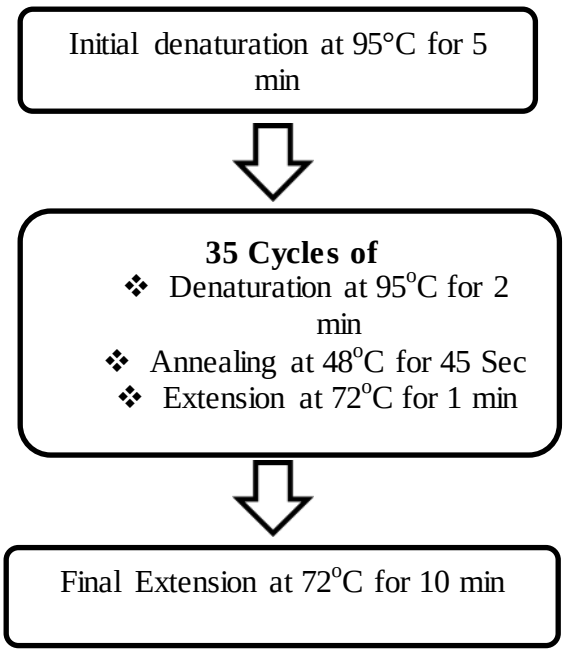

Fig. 3.4 Cycling conditions for OprF gene PCR

3.2.6 Detection of *Mycoplasma spp*.by PCR

The presence of Mycoplasmal infection in the respiratory tract of the turtles was detected by applying a genus specific PCR for *Mycoplasma* spp.

a. Oligonucleotide primers

The amplification of 710 bp genus specific of 16S rDNA region of Mycoplasmal DNA using an oligonucleotide primer sequence as per Van Kuppeveled *et al.*, (1992).

Table 3.5 Oligonucleotide primers for *Mycoplasma* 16S rDNA gene

Primer	Oligonucleotide sequence
Myco F	5'-ACT-CCT-ACG-GGA-GGC-AGC-AGT-A -3'
Myco R	5'-TGC-ACC-ATC-TGT-CAC-TCT-GTT-AAC-CTC -3'

b. Reaction Mixture

The PCR was set in a final volume of 25 µl consisting of reactants shown in table 3.6

Table 3.6 Reaction Mixture for *Mycoplasma* 16S rDNA PCR

Reagents	Volume
Sterile water	9.9 µl
2X PCR Master Mix	12.5 µl
Primer Myco- F (0.1 mM / µl)	0.5 µl
Primer Myco- R (0.1 mM / µl)	0.5 µl
Template DNA	0.6 µ l
Total	25.00 µl

c. Cycling conditions for *Mycoplasma* PCR

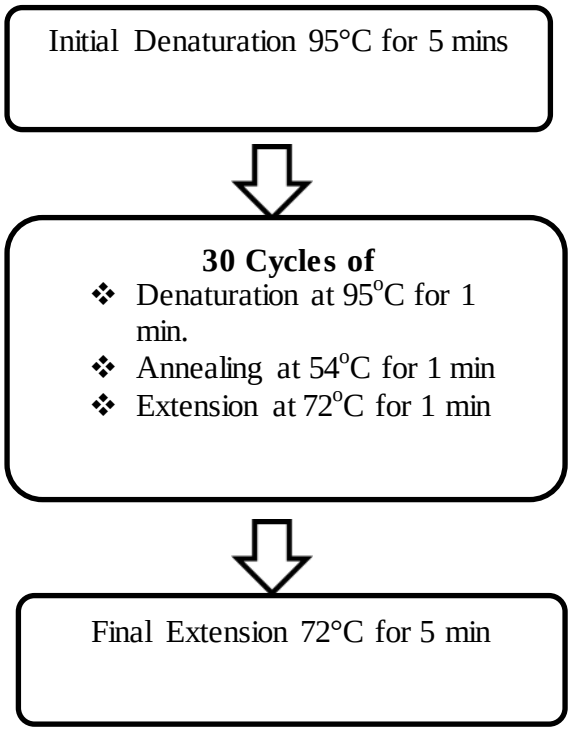

Fig 3.5 Cycling conditions for *Mycoplasma* 16S rDNA PCR

3.2.7 Visualization of PCR products

The gel casting platform was placed on a levelled surface and open sides were sealed with adhesive tape. The gel comb was then placed across the gel casting platform, so that the teeth of the comb remained 1mm above the base of the platform. Agarose gel (1%) was prepared by boiling molecular biology grade agarose (SRL, India) in 1 X Tris Acetate EDTA (TAE) buffer to dissolve it completely. After cooling to about 50°C, ethidium bromide (Bangalore Genie) was added to the agarose solution to a final concentration of 0.5µg/ml. This molten agarose was then poured onto the gel-casting platform and was kept undisturbed for about 45 minutes to allow the gel to solidify. After the gel solidification, the comb was taken out carefully to avoid any damage to the wells and adhesive tape was removed. The gel with the gel-

casting platform was then submerged in the electrophoresis tank with sufficient quantity of electrophoresis buffer above the surface of the gel (about one mm level) and with the wells at the cathode end of the tank. As the PCR master mix already possessed the loading dye, about five µl of PCR product was directly loaded into the well. Simultaneously, 100 bp and 1 kb DNA ladder Dye plus (DSS Takara, India) was also loaded in one of the wells (depending upon the size of amplicon). Electrophoresis was performed at the rate of 5 V/cm^2 of the gel and the progress of mobility was monitored by the migration of indicator dye. At the end of electrophoresis the gel was visualized under UV transilluminator, to check the amplicon of desired molecular weight and results were recorded using gel documentation system (Gel Doc EZ Imager, Bio-Rad).

3.2.8 Sequencing of the PCR Products

The amplified PCR products were subjected for sequencing and further BLAST analysis tools were used. PCR products were sent to Genomebio for commercial sequencing by Sanger's method.

4. RESULTS AND DISCUSSION

The present investigation dealt with detection of organisms causing respiratory infections in captive Red Eared Slider turtles using different conventional and molecular techniques. Conventional methods included bacterial isolation and identification by biochemical characterisation, while molecular techniques comprised of identification of pathogens by polymerase chain reaction targeting the conserved 16S rDNA region of the genome using universal and genus specific primers and sequencing of representative PCR products for confirmation of the pathogens.

4.1 Sample Collection

Red Eared Slider turtles suffering with respiratory infections showed clinical signs of nasal discharge, respiratory distress, anorexia and blepharedema. Turtles presented with such symptoms to the veterinary clinics were included in the study. Choanal swabs were collected from turtles showing signs of respiratory distress. The external nares of these turtles are extremely narrow, thus causing difficulty in collection of nasal swabs. The choana, which is the opening between the nasal cavity and the nasopharynx, is placed inside the oral cavity. This leads to the seeping of nasal secretions into the oral cavity from where they can be easily soaked onto small thin collection swabs (McArthur *et al.,* 2004). All the studies performed on identification of respiratory pathogens in turtles till date have approved the method of collection of oral swabs instead of nasal swabs (Johnson *et al.*, 2007; Heynol *et al.*, 2015; Sim *et al.*, 2016).

The oral swabs were collected in duplicates from 31 turtles showing signs of acute to chronic type of respiratory distress and symptoms, for bacteriological examination (Plate 1).

4.2 Identification of Bacterial Pathogens by Conventional Methods

4.2.1 Isolation of Bacteria

A total of 31 oral swabs were collected from turtles showing signs of acute to chronic type of respiratory distress and symptoms. A total of 30 isolates were

recovered from 31 swabs when subjected to isolation on blood agar plates. Differences in the cultural characteristics of the isolates and staining morphology were recorded and are presented in table 4.1.

Table 4.1 Characterization of Bacterial Isolates based on Gram Staining and Colony Morphology

Sample No.	Colony Characteristics on BA	Gram Staining Reaction	Bacterial Morphology
T7	Small and rough	Gram Negative	Bacilli
T8	Swarming	Gram Negative	Bacilli
T9	Small and rough	Gram Negative	Bacilli
T10	Swarming	Gram Negative	Bacilli
T11	Small and mucoid	Gram Negative	Bacilli
T12	Small and rough	Gram Negative	Bacilli
T13	Small and mucoid	Gram Negative	Bacilli
T14	Circular flat	Gram Negative	Bacilli
T15	Greyish circular	Gram Negative	Cocobacilli
T16	Swarming	Gram Negative	Bacilli
T17	Swarming	Gram Negative	Bacilli
T18	Greyish circular	Gram Negative	Cocobacilli
T19	Greyish circular	Gram Negative	Cocobacilli
T20	Circular and mucoid	Gram Negative	Bacilli
T21	Circular flat	Gram Negative	Bacilli

T22	Small and mucoid	Gram Negative	Bacilli
T23	Swarming	Gram Negative	Bacilli
T24	Circular and mucoid	Gram Negative	Bacilli
T25	Greyish circular and big	Gram Negative	Bacilli
T26	Swarming	Gram Negative	Bacilli
T27	Swarming	Gram Negative	Bacilli
T28	Greyish circular	Gram Negative	Cocobacilli
T29	Greyish circular and big	Gram Negative	Bacilli
T30	Swarming	Gram Negative	Bacilli
T31	Swarming	Gram Negative	Bacilli
T32	Circular and mucoid	Gram Negative	Bacilli
T33	Greyish circular and big	Gram Negative	Bacilli
T34	Swarming	Gram Negative	Bacilli
T36	Colorless and small	Gram Negative	Cocci
T37	Swarming	Gram Negative	Bacilli

Various types of colonies were observed on blood agar. Majority of them were small, circular and mucoid (n=11). Swarming type of growth was also presented by 12 isolates which is characteristically exhibited by *Proteus* spp. Haemolytic colonies were also observed in a few isolates (n= 8). Small, circular, convex, white to gray colonies were showed by one isolate on blood agar,

Preliminary identification of the isolated colonies was done by Gram's staining. Out of 31 isolates observed only one (T35), which appeared as small, circular, convex, white to gray colonies on blood agar, was found to be a Gram positive bacillus (Plate 2). All the remaining smears showed Gram negative bacilli, cocci or cocobacillary rods (Plates 3 and 4).

Morphologically similar organisms were reported by several authors (Evans, 1983; Liu *et al.*, 2013). Delli Paoli Carini *et al.*, (2017) found that the predominant bacterial pathogens isolated from nasopharyngeal swabs of captive green turtles were gram negative. Hidalgo-Vila *et al.*, (2020) isolated 19 species of Gram negative organisms out of 35 samples collected from red eared slider turtles in Spain. Ciccarelli *et al.*, (2020) identified 13 common gram negative pathogens from 14 swab samples of loggerhead sea turtles. Umbrasko *et al.*, (2020) profiled the bacteria post Gram staining and were able to identify six gram negative bacteria and one gram positive isolate from oral cavities of European pond turtle (*Emys orbicularis*)

Isolated organisms (n=30) were preserved on BHI slants and were simultaneously streaked on MacConkey's Agar (MA) and Eosin Methylene Blue (EMB) Agar and were incubated for 18 - 24 hrs. at 37°C. Growth on MA and EMB was documented for further differentiation and identification. Out of 31 isolates, eight organisms developed pink colonies on MA indicating lactose fermentation (Plate 5). Growth on EMB was only observed with three isolates. The colonies were found to be black, circular and shiny (Plate 6). Three of the isolates showed green pigmentation on BHI agar indicating the growth of *Pseudomonas* spp. (Plate 7).

4.2.2 Biochemical characterization of the isolates

All the pure isolates were subjected to biochemical tests for species identification. The tests performed were Methyl Red, Voges-Proskauer, Indole, Nitrate, Catalase, Oxidase, Citrate and Urease (Plates 8 - 15). A diverse range of results were obtained from all samples which are tabulated in Table 4.2.

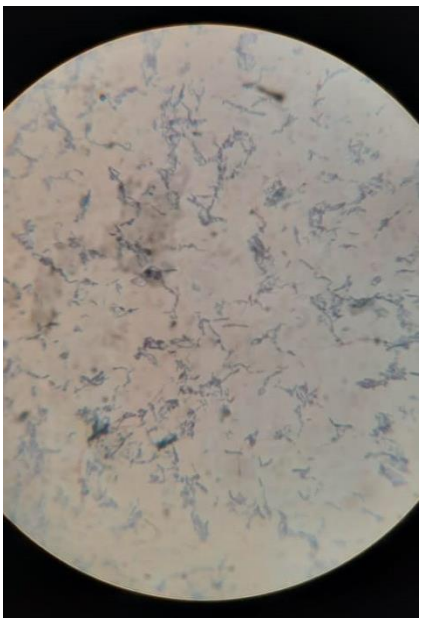

Plate 2. Gram Positive Bacteria

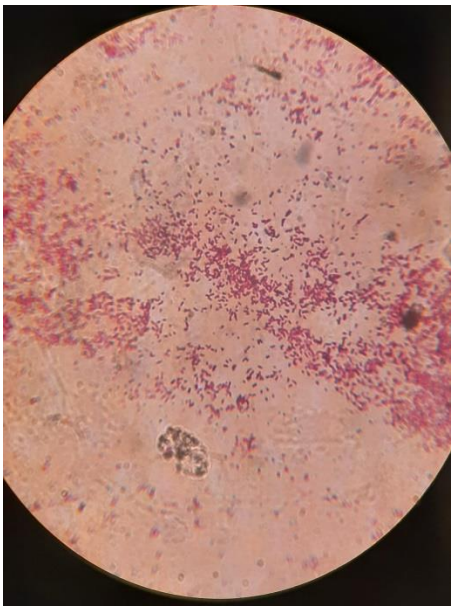

Plate 3. Gram Negative Bacilli

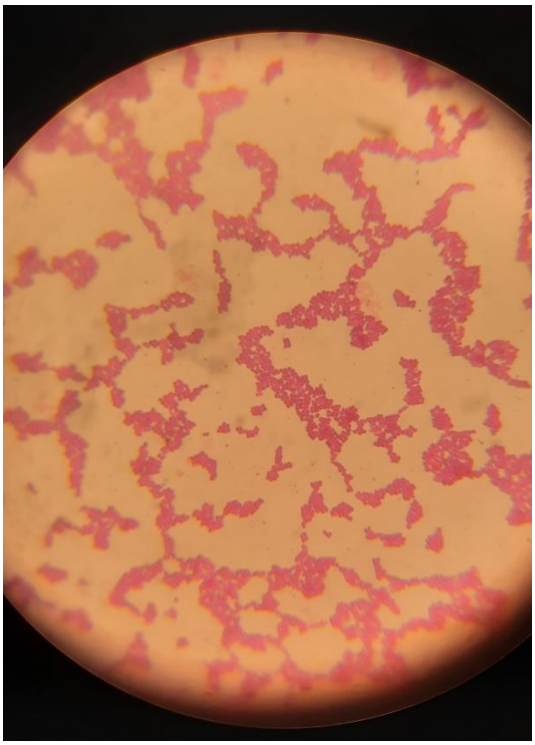

Plate 4. Gram Negative Cocci

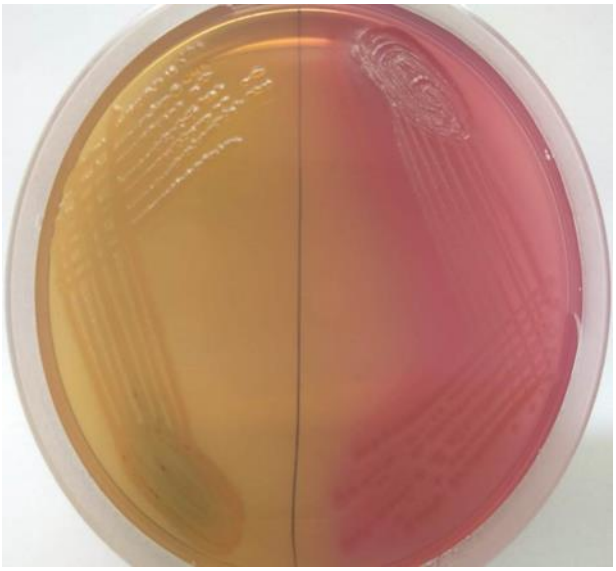

Plate 5 Colonies on MacConkey Agar.

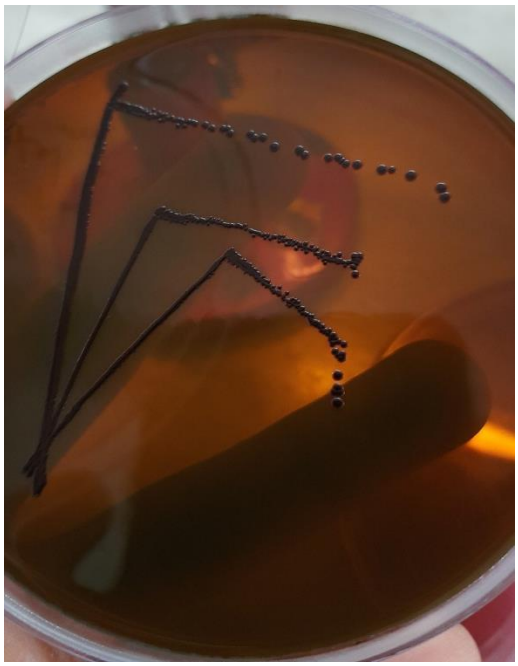

Plate 6. Colonies on EMB Agar

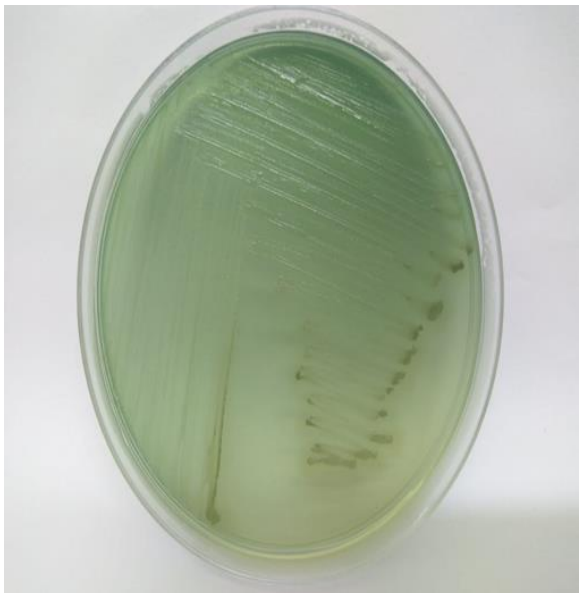

Plate 7. *Pseudomonas* Colonies on BHI Agar

The staining reactions, colony morphologies and results of biochemical tests of the 31 isolates were collectively analyzed for the identification of eight different species of bacteria. It was found that, 11 of the isolates belonged to *Proteus* spp., four were found to be *Aeromonas* spp., three each were identified as *Pseudomonas* spp., *Klebsiella* spp, *Serratia* spp and *Escherichia* spp, while one each were identified as *Moraxella* spp and *Citrobacter* spp. Isolate T35 showed a gram positive staining reaction. Gram positive staining reaction and biochemical tests suggested that it could belong to *Corynebacterium* spp., further screening by using selective medium and molecular techniques is required for confirmation. Various workers (Keymer, 1978; Evans, 1983; Soccini and Ferri, 2004; Ferronato *et al.*, 2009; Hacioglu *et al.*,.2012; Heynol *et al.*, 2015) have reported the presence of similar pathogens in turtles based on the cultural and biochemical characteristics of the isolated organisms.

Plate 8. Methyl Red Test

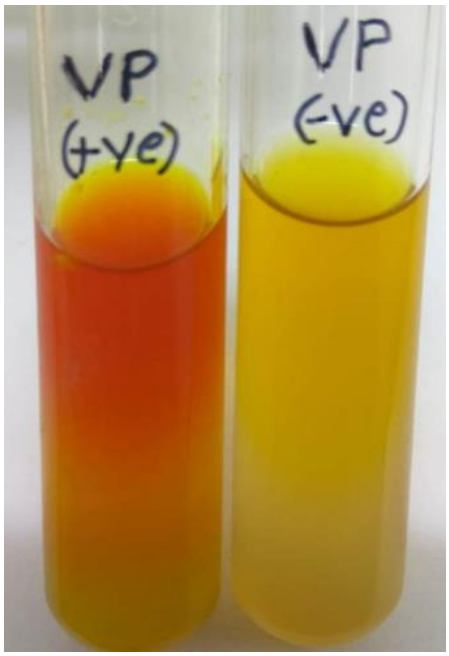

Plate 9. Voges-Proskauer Test

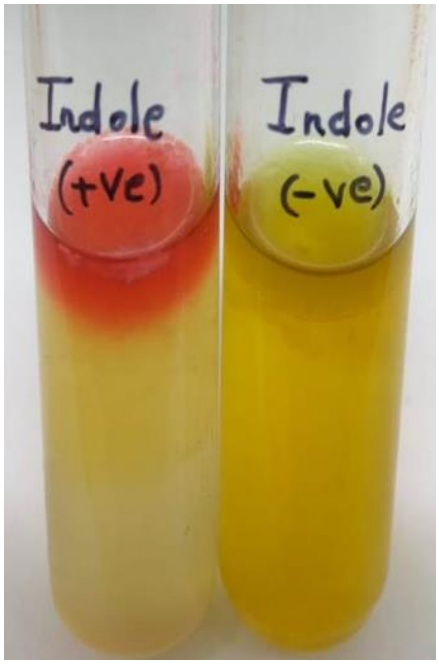

Plate 10. Indole Test

Plate 11. Nitrate Test

Plate 12.. Urease Test

Plate 13.Citrate Test

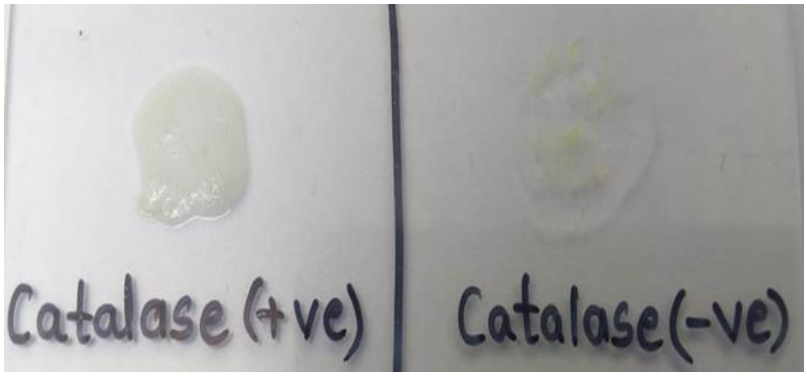

Plate 14. Catalase Test

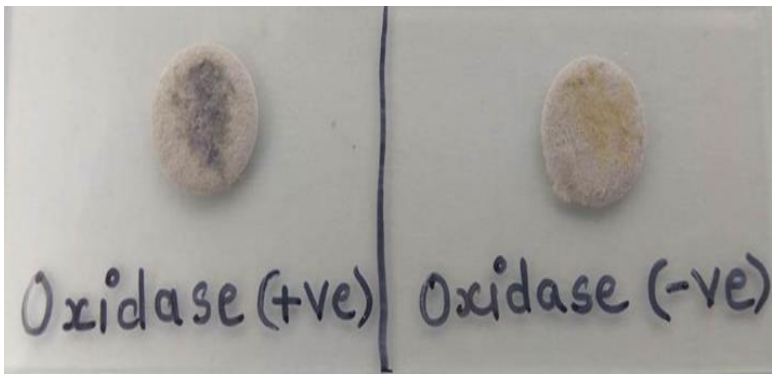

Plate 15. Oxidase Test

Table 4.2 Biochemical Characterization of Bacterial Isolates and Growth on Differential Media

Sample No	MR	VP	Indole	Nitrate	Urease	Citrate	Oxidase	Catalase	BA	MA	EMB	Genus
T7	-	-	-	+	-	+	+	+	H	NL	NG	*Pseudomonas* spp
T8	+	+	-	+	+	-	-	+	NH	NL	NG	*Proteus* spp
T9	-	-	-	+	-	+	+	+	H	NL	NG	*Pseudomonas* spp
T10	+	+	-	+	+	-	-	+	NH	NL	NG	*Proteus* spp
T11	+	+	-	+	-	+	-	+	NH	NL	NG	*Serratia* spp
T12	-	-	-	+	-	+	+	+	H	NL	NG	*Pseudomonas* spp
T13	+	+	-	+	-	+	-	+	NH	NL	NG	*Serratia* spp
T14	+	-	-	+	-	+	-	+	NH	LF	NG	*Citrobacter* spp
T15	+	-	+	+	-	-	-	+	NH	LF	Circular black	*Escherichia* spp
T16	+	+	-	+	+	-	-	+	NH	NL	NG	*Proteus* spp
T17	+	+	-	+	+	-	-	+	NH	NL	NG	*Proteus* spp
T18	+	-	-	+	+	+	+	+	H	NL	NG	*Aeromonas* spp
T19	+	-	-	+	+	+	+	+	H	NL	NG	*Aeromonas* spp
T20	-	+	-	+	+	+	-	+	NH	LF	NG	*Klebsiella* spp
T21	+	-	-	+	-	+	-	+	NH	LF	NG	*Citrobacter* spp
T22	+	+	-	+	-	+	-	+	NH	NL	NG	*Serratia* spp
T23	+	+	-	+	+	-	-	+	NH	NL	NG	*Proteus* spp
T24	-	+	-	+	+	+	-	+	NH	LF	NG	*Klebsiella* spp
T25	+	-	+	+	-	-	-	+	NH	LF	Circular black	*Escherichia* spp
T26	+	+	-	+	+	-	-	+	NH	NL	NG	*Proteus* spp
T27	+	+	-	+	+	-	-	+	NH	NL	NG	*Proteus* spp

T28	+	-	-	+	+	+	+	+	H	NL	NG	*Aeromonas* spp
T29	+	-	-	+	+	+	+	+	H	NL	NG	*Aeromonas* spp
T30	+	+	-	+	+	-	-	+	NH	NL	NG	*Proteus* spp
T31	+	+	-	+	+	-	-	+	NH	NL	NG	*Proteus* spp
T32	-	+	-	+	+	+	-	+	NH	LF	NG	*Klebsiella* spp
T33	+	-	+	+	-	-	-	+	NH	LF	Circular black	*Escherichia* spp
T34	+	+	-	+	+	-	-	+	NH	NL	NG	*Proteus* spp
T36	ND	ND	-	+	-	ND	+	+	-	-	-	*Moraxella spp*
T37	+	+	-	+	+	-	-	+	NH	NL	NG	*Proteus* spp

(ND-No Data; NG- No Growth; H- Hemolytic; NH- Non-hemolytic; LF-Lactose Fermenting; NLF-Non-Lactose Fermenting)

The biochemical characterization showed results corresponding with *Aeromonas* spp and *Citrobacter spp* were reported also by Thomas *et al.,* (2020) from an endangered spotted turtle during a study of multidrug resistance and plastic colonization of these bacteria. Identification of a vast range of bacterial organisms using biochemical tests and API (Analytischer-Profil-Index, BioMereux, Nürtingen, Germany) system was done by Heynol *et al.,* (2015). They were able to identify *Citrobacter* spp., *Aeromonas* spp., *Chryseobacterium* spp., *Salmonella* spp., *Proteus* spp., *Serratia* spp., *Pseudomonas* spp., *Acinetobacter* spp., *Klebsiella* spp., *Providencia* spp. and *Moraxella* spp. Out of the 19 bacterial species isolated by Hidalgo-Vila *et al.,* (2020) one was identified as *Proteus vulgaris* and *Klebsiella pneumoniae* each, two were found to be *Citrobacter koseri,* while *Aeromonas hydrophila* was found to be the most common pathogen (n=7). Biochemical identification of 13 bacterial isolates from respiratory swabs of sea turtles, done by Ciccarelli *et al.,* (2020), using API-system (Biomérieux, France) lead to the identification of *Pseudomonas aeruginosa, Pseudomonas putrefaciens, Citrobacter freundii, Klebsiella oxytoca* and *Aeromonas hydrophila* which are similar to those recorded in our study.

Out of the 30 isolates identified, seven organisms including *Aeromonas* spp (4) and *Pseudomonas* spp (3) exhibited hemolysis on blood agar.

The biochemical reactions exhibited by the bacteria could help in identifying the pathogens up to genus level. For accurate treatment of the condition, it is necessary that the pathogens be detected precisely. Therefore, molecular tools were adopted for further confirmation of the pathogens up to species level.

4.3 Molecular Identification of Respiratory Pathogens

4.3.1 Molecular Characterization of Bacterial Isolates

The DNA extracted from all the bacterial isolates (n=30) were utilized in a PCR assay for identification of the species of the bacteria. This assay targeted a region of DNA which codes for 16S rDNA. This segment of the bacterial genome is conserved within all the bacterial species and so can be used to identify any prokaryotic DNA. Further sequencing of the PCR amplicons and BLAST analysis (NCBI) of these sequences leads to identification of the species of individual bacterial isolates.

The primers targeting the 16S rDNA region (~1400 bp fragment) were used as mentioned in table 3.1. All the 30 isolates showed amplification of ~1400 bp products (Plate 16). The PCR products were further subjected to Sanger's sequencing. Approximately 500 bp sequences of four PCR products could be obtained. The chromatograms for these four isolates are mentioned in Figures 4.1, 4.2, 4.3, 4.4. These sequences were subjected to BLAST analysis to discover their identity. These four sequences from different isolates showed homology with four different species of organisms. The percent identity and the accession numbers of the sequences present in the NCBI database, with which our sequences show homology are mentioned in table 4.3. The sequences obtained were submitted to NCBI and the accession numbers are awaited.

The isolates were thus identified as *Pseudomonas aeruginosa* (sample T12), *Citrobacter amalonaticus* (sample T14), *Escherichia fergusonii* (sample T15) and *Proteus mirabilis* (sample T17). The results of molecular identification corresponded with those of biochemical assays, although it was difficult to identify the species of organisms using biochemical characterization. These organisms have been reported as few of the common pathogens causing respiratory illnesses in turtles (Al Bahry *et al.*, 2012; Yan *et al.*, 2013; Wendt *et al.*, 2017; Chung *et al.*, 2017).

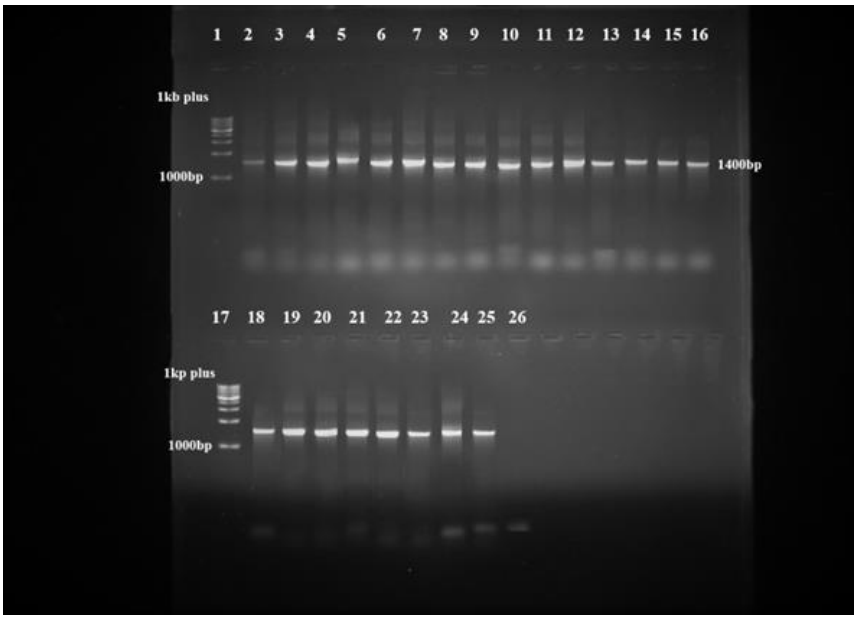

Plate 16. Universal Prokaryotic 16s rRNA PCR Assay
Lane 1 and 17-1 kb plus ladder, Lane 2 to 16- Isolates, Lane 18 to 25- Isolates, Lane 26- Negative control

Plate 17. OprF PCR Assay of *Pseudomonas*
Lane 1-100bp Ladder, Lanes 2-4 Isolates, Lane 5 Negative Control

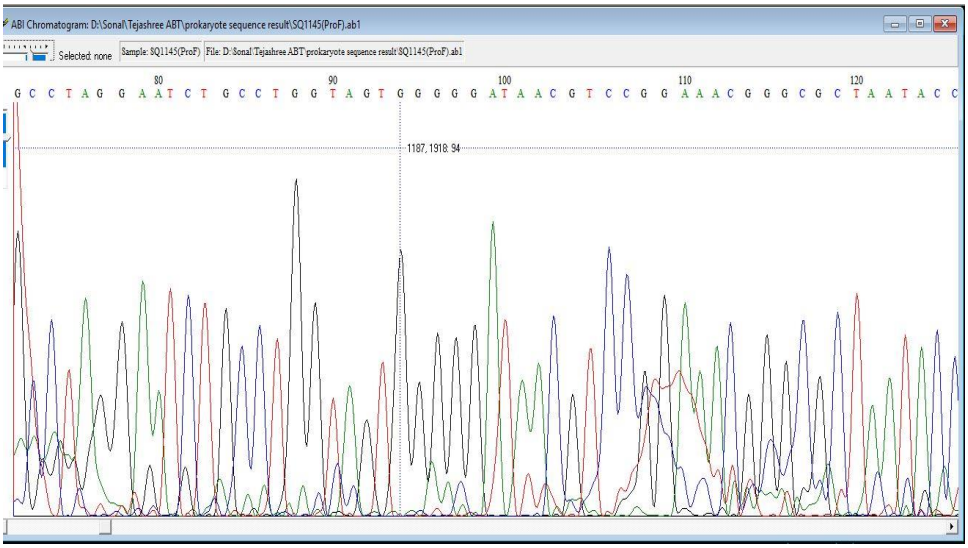

Figure 4.1 Chromatogram of Isolate T12

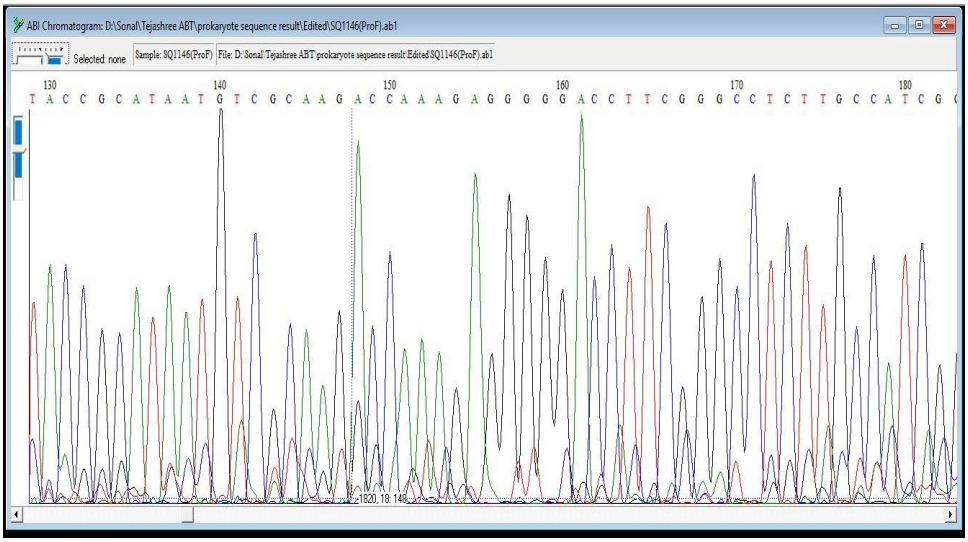

Figure 4.2 Chromatogram of Isolate T14

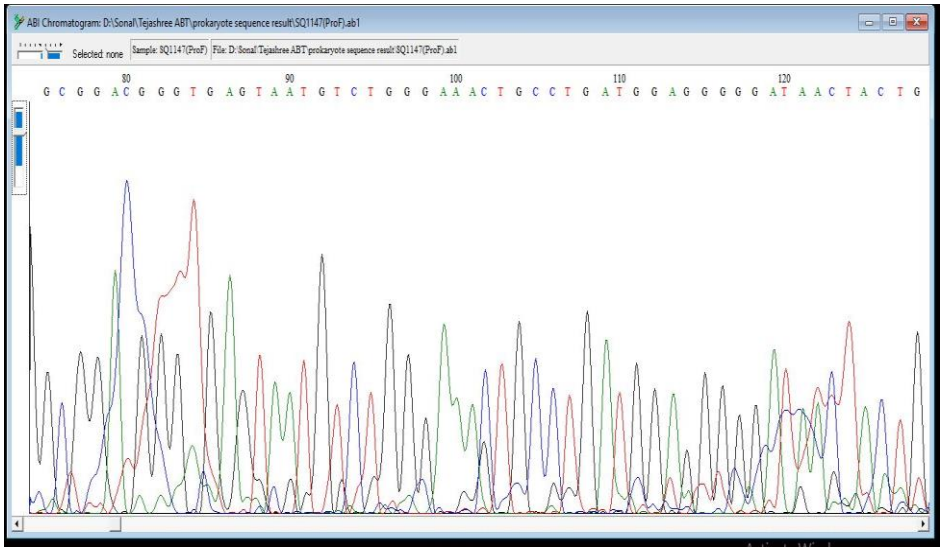

Figure 4.3 Chromatogram of Isolate T15

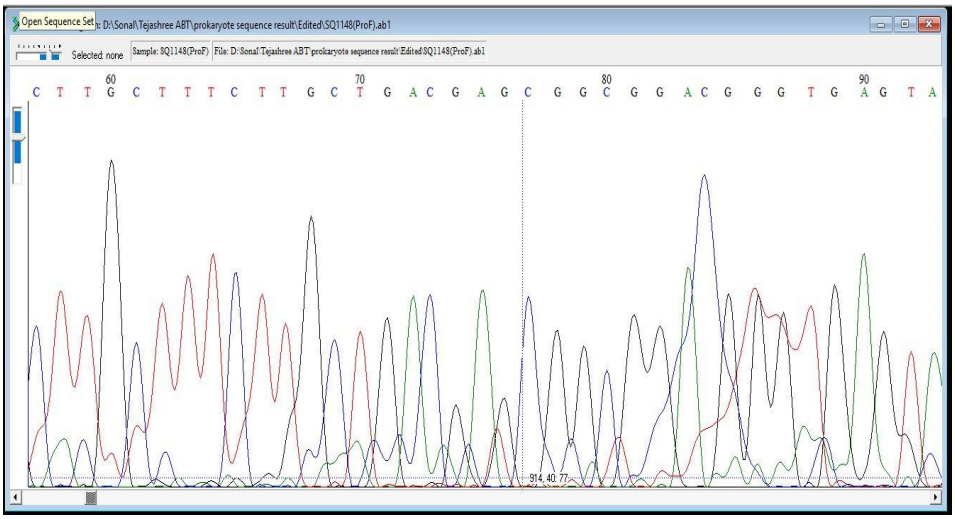

Figure 4.4 Chromatograph of Isolate T17

Table 4.3 Organisms Identified via Sequencing

Sample No.	Organism Identified	Percent Identity	Accession Numbers
T12	*Pseudomonas aeruginosa*	99.28	JQ900531.1
			EF683087.1
		99.04	AB627899.1
			MT640266.1
			MN889007.1
T14	*Citrobacter amalonaticus*	99.76	MN956654.1
			CP041362.1
			MN314590.1
			MG209575.1
			LT899963.1
T15	*Escherichia fergusonii*	99.59	MW832269.1
			MW832260.1
			MW832273.1
		99.18	MW832328.1
			MW832318.1
T17	*Proteus mirabilis*	100.00	MN124173.1
		99.60	MT470980.1
			MH396765.1
			KR967387.1
			KJ950776.1

Application of Polymerase Chain Reaction targeting the 16S rDNA region has been studied and recommended by various workers for precise identification of the bacterial isolates (Brockmann *et al*., 2020; Peng *et al*., 2020). Chung *et al.*, (2017) found that eight different species of bacteria could be identified using 16S rDNA PCR which included four isolates of *Citrobacter fruendii*. Wendt *et al.* (2017) successfully identified 17 isolates of *P. aeruginosa*, from different species of turtles, using 16S rDNA PCR, sequencing of the PCR products and BLAST analysis.

It is to be noted that most pathogens isolated and identified in the present study belong to the family Enterobacteriacae. The presence of enterobacterial organisms in respiratory tract of the turtles can be attributed to the aquatic nature of the animals. However, these pathogens being zoonotic in nature pose a serious threat to the health of the pet owners (Chiacchio *et al*., 2014). Selvarajah and Khairani-Bejo (2019) identified *Aeromonas spp., Klebsiella spp., Escherichia*

spp., *Yersinia spp*. and *Salmonella spp*. from cloacal swabs of red-eared sliders, thus indicating the zoonotic significance of the isolate.

4.3.2 Characterization of *Pseudomonas* Isolates

One of the isolates (T12) was identified as *Pseudomonas aeruginosa* by 16S rDNA PCR. Two more isolates out of thirty, tested positive for *Pseudomonas* spp by biochemical evaluation (table 4.1) and showed characteristic greenish pigmented colonies on BHI agar and MA. The DNA extracted from said isolates was subjected to a PCR targeting the OprF gene for further characterization. The *Pseudomonas* outer membrane porin protein F (OprF) gene may be considered as a diagnostic protein which can be used for identification of organism belonging to *Pseudomonas* spp. The involvement of OprF in virulence is linked to attachment of bacteria to host cells (Azghani *et al.*, 2002).

The primers targeting the OprF gene were used as mentioned in the table 3.3, which amplified a product of ~511bp (Plate 17). All the isolates identified as *Pseudomonas* spp. by biochemical characterization and 16S rDNA PCR showed amplification of the OprF gene. This confirms that the two isolates which were not characterized by the 16s rDNA PCR also belonged to the *Pseudomonas* spp. Although studies done by Wendt *et al.*, (2017) detected the presence of other virulence genes viz. toxA, lasB and exoS of *Pseudomonas aeruginosa* isolated from pet turtles. Studies conducted by Oliveira *et al.*, (2017) also show detection of *Pseudomonas* spp.by targeting oprL and oprI genes. These studies indicate the presence of common virulence genes in the *Pseudomonas* organisms detected in turtles. Close contact with these organisms through the pet turtles might pose a significant public health risk.

4.3.3 *Mycoplasma* 16S rDNA PCR

Mycoplasmas are reported to be very frequently associated with respiratory infections in Red Eared Slider turtles. Being fastidious in nature and having a long makes it difficult to isolate and detect the presence of this organism from a clinical sample. Therefore, a PCR assay targeting genus specific region of 16S rDNA was employed for detection of *Mycoplasma* spp. from the DNA

Plate 18. *Mycoplasma* 16s rRNA PCR Assay
Lane 1 - 100bp Ladder, Lane 2 – Negative Control, Lane 3-7 - Isolates

extracted directly from oral swabs of the turtles. The primers as mentioned in table 3.4 were used for the PCR assay. These amplified a 710 bp region of the bacterial DNA (Plate 18).

Out of the 31 samples screened for *Mycoplasma* spp, four showed the amplification of genus specific 16S rDNA region of ~710bp. *Mycoplasma* infection was found to be low (12.9 %) amongst the population investigated in this study. Most common species reported to cause respiratory infections in turtles is *M. agassizzii* (Feldman *et al.*, 2006; Silbernagel *et al.*, 2013; Ossiboff *et al.*, 2015a; Palmer *et al.*, 2016). A novel species of *Mycoplasma* which was closely related to but distinct from *M. agassizzii* was reported by Feldman *et al.,* (2006) based on the sequence analysis of 16S rDNA PCR product. Ossiboff *et al.*, (2015b) reported a high incidence as high as 49 % of *Mycoplasma* spp. in apparently healthy *Emydidae* Turtles. Palmer *et al.*, (2016) detected *Mycoplasma* infections in box turtles using a pan-*Mycoplasma* consensus PCR/sequencing assay. However, the species identified was again related to *M. agassizzii*, but also divergent at certain levels, indicating the incidence of a distinct Mycoplasmal species.

5. SUMMARY AND CONCLUSIONS

Trachemys scripta elegans, commonly known as Red Eared Sliders, are a popular species of turtles as pets due to their compact size. However, this is a highly invasive species and poses threat of transmission of diseases to the native population and replacement of the native turtle species. Close association of the humans, especially kids, with these semi aquatic animals exposes to the risk of infection with common enteric and respiratory pathogens. Therefore, precise detection of the pathogens for accurate treatment of the disease condition is essential.

Present study thus aimed to detect the pathogens causing respiratory infections in captive Red Eared Slider Turtles from Mumbai region. A total of 31 turtles presented to different veterinary clinics with typical symptoms viz. nasal discharge, respiratory distress, anorexia and blepharedema were selected in the study. Choanal swabs were collected from each animal in duplicates and further processed using standard protocols. One set of the swabs was collected in nutrient broth and incubated at 37°C for 18 - 24 hrs. Once the turbidity was observed, the growth was streaked on Blood Agar plates for isolation of bacteria. Growth on blood agar showed hemolysis by eight isolates. This can be an indicator of the virulence of the organisms.

Pure isolates thus obtained were subjected to Gram's staining, and further biochemical characterization for identification of the organism. Staining reaction revealed that 30 isolates were Gram negative (rods and coccobacilli) while only one was Gram positive bacillus. Gram negative isolates were further inoculated on MacConkey's agar and EMB agar. Out of 30 isolates, seven exhibited lactose fermentation on MacConkey's agar. Only three isolates showed growth on EMB agar with black, circular colonies. Thus, the staining reaction, cultural characters and biochemical assays aided in identification of eight different genera of bacteria among 30 isolates viz, *Proteus* spp. (12/30), *Aeromonas* spp. (4/30), *Pseudomonas* spp (3/30), *Klebsiella* spp (3/30), *Escherichia* spp. (3/30), *Serratia* spp (2/30), *Citrobacter* spp (2/30) and *Moraxella* spp (1/30).

DNA was extracted from the pure isolates and used for Polymerase Chain Reaction targeting 16S rDNA region of the prokaryotes. All the 30 isolates showed amplification of ~1400 bp fragment. Sequences of PCR products of four isolates were obtained and identified using BLAST algorithm of NCBI. These isolates were identified as *Pseudomonas aeruginosa, Citrobacter amalonaticus, Escherichia fergusonii* and *Proteus mirabilis*. The results of molecular screening for these four samples corresponded with those of their biochemical assays. The *Pseudomonas spp*. isolates were screened for the presence of OprF gene. All three isolates showed the specific amplification of ~511bp fragment of OprF, thus further confirming the presence of *Pseudomonas* spp.

The other set of swabs was collected in MEM with 2% FBS and directly subjected to extraction of DNA. The DNA was further subjected to PCR for identification of *Mycoplasma* spp. by targeting the genus specific 16S rDNA fragment. Out of 31 samples screened, four were found to be positive for *Mycoplasma* spp. showing and amplification of ~710bp fragment of the DNA.

All the pathogens identified by conventional and molecular techniques are common bacterial species infecting both humans and animals. Aquatic nature of these pets results in presence of the enteric bacteria in their environment and also the oral and respiratory tracts. Close association of humans with these pets and improper, unhygienic handling poses a risk to the health of pet owners, especially children. Therefore, accurate detection of the pathogens and the screening of pathogens for potential virulence factors and antibiotic resistance is essential for precise diagnosis and treatment of the condition.

Thus, following conclusions can be drawn from the present study:

1. Amongst the 30 organisms isolated, eight different genera of bacteria could be identified based on staining reactions, differential cultural characteristics and biochemical test.
2. The identified genera included *Proteus* spp., *Aeromonas* spp., *Pseudomonas* spp, *Klebsiella* spp, *Escherichia* spp., *Serratia* spp, *Citrobacter* spp and *Moraxella* spp.

3. PCR assay targeting 16S rDNA region and BLAST analysis of the sequences successfully identified four species of bacteria viz. *Pseudomonas aeruginosa*, *Citrobacter amalonaticus*, *Escherichia fergusonii* and *Proteus mirabilis*.
4. Amplification of 511 bp fragment of OprF gene from the three suspected *Pseudomonas* spp. isolates confirmed the presence of *Pseudomonas* spp.
5. Mycoplasma infection could be successfully detected by employing the genus specific 16S rDNA PCR assay on the DNA extracted directly from the choanal swabs.
6. Further screening of the isolates for antibiotic resistance and virulence markers will help in accurate treatment of the pet turtles and prevent the spread of infection to the owners.

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APPENDIX - I

List of equipment

The major important equipment used during the study is as shown below.

Sr. No.	Name	Manufacture
1.	Mastercycler nexus gradient Thermal cycler	Eppendorf and Bio-Rad
2	Gel documentation system (Gel Doc)	Bio-Rad
3	Micropipettes	Eppendorf research plus
4	Ice maker	Sanyo, Japan
5	Power pack	Tarsons, Consort
6	Water bath	Biotechnics India
7	Microcentrifuge	Genei
8	pH meter	Toshniwal Instruments, India
9	Weighing balance	OHAUS
10	Micrometer	Hp
11	CO_2 Incubator	Thermo Scientific
12	Microwave oven	LG
13	Deep freeze (– 20oc)	Voltas TATA
14	Cooling centrifuge	REMI
15	Vortex	Spinix

APPENDIX – II

Media and Stains

Media:

1. **MacConkey Agar (MA) (Dehydrated, HiMedia)**

Ingredients	Grams / liter
Peptic digest of animal tissue	20.00
Lactose	10.00
Bile salts	5.00
Sodium chloride	5.00
Neutral red	0.07
Final pH (at 25^0 C)	7 + 0.2

Suspended 55.07 gm of dehydrated MCA in 1000ml distilled water sterilize by autoclaving at 15lbs pressure (121^0C) for 15mins. The molten medium was cooled to about 50^0C temperature and poured into sterile petri plate.

2. **Nutrient Agar (NA) (Dehydrated, HiMedia)**

Ingredients	Grams / liter
Peptone	5.000
HM peptone B	1.500
Sodium chloride	5.000
Yeast extract	1.500
Agar	15.000
Final pH (at 25^0 C)	7 + 0.2

Suspend 28 grams in 1000ml distilled water. Sterilize by autoclaving at 15lbs pressure (121^0C) for 15mins. Mix well and pour into sterile Petri plates.

3. **Brain Heart Infusion Agar (BHI) (Dehydrated, HiMedia)**

Ingredients	Grams / liter
HM infusion powder	12.500
BHI powder	5.000
Proteose peptone	10.000
Dextrose (Glucose)	2.000
Sodium chloride	5.000
Disodium hydrogen	2.500

phosphate	
Agar	15.000
Final pH (at 25^0 C)	7.4±0.2

Suspend 52.0 grams in 1000 ml purified/distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Mix well and pour into sterile Petri plates.

4. **Eosin Methylene Blue Agar (EMB) (Dehydrated, HiMedia)**

Ingredients	**Grams / liter**
Peptone	10.000
Dipotassium hydrogen phosphate	2.000
Lactose	5.000
Saccharose (Sucrose)	5.000
Eosin - Y	0.400
Methylene blue	0.065
Agar	13.500
Final pH (at 25^0 C)	7.2±0.2

Suspend 35.96 grams in 1000 ml purified / distilled water. Mix until suspension is uniform. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C and shake the medium in order to oxidize the methylene blue (i.e. to restore its blue colour) and to suspend the flocculent precipitate.

5. **Simmons Citrate Agar (Dehydrated, HiMedia)**

Ingredients	**Grams / liter**
Magnesium sulphate	0.200
Ammonium dihydrogen phosphate	1.000
Dipotassium phosphate	1.000
Sodium citrate	2.000
Sodium chloride	5.000
Bromothymol blue	0.080
Agar	15.000
Final pH (at 25^0 C)	6.8±0.2

Suspend 24.28 grams in 1000 ml purified/ distilled water. Heat, to boiling, to dissolve the medium completely. Mix well and distribute in tubes or flasks. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

6. Christensen's Urea Agar (Dehydrated, HiMedia)

Ingredients	Grams / liter
Peptone	1.000
Dextrose (Glucose)	1.000
Sodium chloride	5.000
Disodium hydrogen phosphate	1.200
Potassium dihydrogen phosphate	0.800
Phenol red	0.012
Agar	15.000
Final pH (at 25^0 C)	6.8±0.2

Suspend 24.01 grams in 950 ml purified / distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 10 lbs pressure (115°C) for 20 minutes. Cool to 45-50°C and aseptically add 50 ml of sterile 40% Urea Solution (FD048) and mix well. Dispense into sterile tubes and allow to set in the slanting position.

7. Brain Heart Infusion Broth (HiMedia)

Ingredients	Grams / liter
HM infusion powder	12.500
BHI powder	5.000
Proteose peptone	10.000
Dextrose (Glucose)	2.000
Sodium chloride	5.000
Disodium hydrogen phosphate	2.500
Final pH (at 25^0 C)	7.4±0.2

Suspend 37.0 grams in 1000 ml purified/distilled water. Dispense into bottles or tubes and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

8. Nutrient Broth (NB) (HiMedia)

Ingredients	Grams / liter
Peptone	5.000
HM peptone B	1.500
Sodium chloride	5.000
Yeast extract	1.500
Final pH (at 25^0 C)	7 + 0.2

Suspend 13.0 grams in 1000 ml purified / distilled water. Heat, if necessary, to dissolve the medium completely. Dispense into tubes or flasks as desired. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Stains

Gram's Stain

a) Crystal Violet (HiMedia)

b) Gram's Iodine

Iodine	1 gm
Potassium iodide	2 gm
Distilled water	300 ml

c) Dilute Carbol Fuchsin (counter stain)

Strong carbol fuschin	50ml
Distilled water	300 ml

APPENDIX – III

Buffers and Reagents

Reagents used for DNA extraction

1. **1 M Tris HCL (pH 7.65)**

   Dissolve 24.288 g Tris buffer in 120 ml distilled water and adjust pH to 7.65 with concentrated HCL. Adjust the final volume to 200 ml with distilled water. Filter through Whatman filter paper no. 1 and sterilize by autoclaving at 15 lbs fo 15min. Store at refrigeration temperature.

2. **10 % SDS**

   Dissolve 5 g SDS in 40ml distilled water at 68^0 C and adjust final volume to 50 ml with distilled water. Filter through Whatman filter paper no. 1 and store at room temperature.

3. **1 M EDTA**

   Stir 186.1 g disodiumethylenediaminetetraacetate $2H_2O$ into 800 ml of distilled water.Stir the solution vigorously using a magnetic stirrer. Add NaOH to adjust the pH to 8.0. Dilute the solution to 1 L with distilled water. Filter the solution through Whatman filter paper no. 1 and sterilize by autoclaving at 15 lbs fo 15min. Store at refrigeration temperature.

4. **DNA Extraction Buffer (pH 7.5) for extraction from swabs**

50mM Tris-HCl (pH 7.5) : 0.1 ml
1 mM EDTA : 0.05 ml
0.25% SDS : 0.125 ml
Distilled Water : 49.725 ml

5. **Chloroform: Isoamyl alcohol (24 : 1)**

Chloroform : 24 ml
Isoamyl alcohol : 1ml

6. Absolute ethanol
7. 70% Ethanol
Absolute ethanol : 70 ml

Distilled water : 30 ml

8. Phenol
Equiliberated phenol was used.

9. Autoclaved Distilled Water
10. Protenase K (10mg/ml)
Protenase K : 0.010 gm

Distilled water

Reagents used for Agarose Gel Electrophoresis (AGE)

1) Ethidium bromide (10 mg/ml)
Ethidium bromide : 50 mg

Autoclaved DW: 5 ml

Stored the solution in amber colored vial at 4°C.

2) 0.5 M EDTA (pH 8.0)
EDTA $2H_2O$: 18.61 g

Autoclaved DW to make : 100 ml

pH was adjusted to 8.0 with solid Sodium hydroxide pellets. The solution was filtered through Whatman filter paper no.1 and stored at room temperature.

3) Tris – Acetate – EDTA (TAE) stock solution (50 X)

Tris base : 24.2 g

Glacial acetic acid : 5.7 ml

0.5 M EDTA, pH 8.0 : 10 ml

Distilled water to make : 100 ml

(For preparing the working solution (1X), dilute the stock TAE in distilled water)

3) 6X Gel loading dye

Sucrose : 40 % w/v in autoclaved DW

Bromophenol blue : 0.25 % w/v in autoclaved DW

The solution was stored at 4ºC.

Appendix - G

THESIS ABSTRACT

a)	Title of the thesis (in Capital letters)	**:**	MOLECULAR DETECTION OF RESPIRATORY PATHOGENS IN RED EARED SLIDER TURTLES
b)	Full name of student	**:**	MISSAL TEJASHREE SURENDRA
c)	Name and address of Major Advisor	**:**	Dr. S.A.Ingle Assistant Professor, Biotechnology, Department of Microbiology Mumbai Veterinary College, Parel, Mumbai-400012
d)	Degree to be awarded	**:**	M.V.Sc.
e)	Year of award of degree	**:**	2021
f)	Major subject	**:**	Animal Biotechnology
g)	Total number of pages in the thesis	**:**	
h)	Number of words in the abstract	**:**	
i)	Signature of Student	**:**	
j)	Signature, Name and address of forwarding authority (HOD / SH)	**:**	Dr.(Mrs.) S. B. Majee Professor, Department of Microbiology, Mumbai Veterinary College, Mumbai
k)	Signature of the Associate Dean		

ABSTRACT

Red Eared Sliders are freshwater turtles, commonly kept as household pets. Respiratory tract infections in captive turtles are a common problem observed in Veterinary practice. Treating these infections can be challenging due to scarce diagnostic approaches. This study aims to determine the etiological components behind these respiratory infections. A total of 31 Red Eared Sliders showing signs of respiratory infections were sampled. Oral and choanal swabs were acquired in duplicates for bacterial isolation and DNA extraction. The swabs collected for bacterial isolation were subjected to various biochemical tests for species identification. Gram negative isolates were obtained with a majority (n=30) and a single Gram positive isolate was found. The organisms identified via biochemical tests were *Pseudomonas* spp (3), *Aeromonas* spp (4), *Proteus* spp (12), *Serratia* spp (2), *Citrobacter* spp (2), *Klebsiella* spp (3), *Escherichia* spp. (3) and *Moraxella* spp (1). Molecular identification of the bacterial isolates was done by targeting a ~1400bp region of 16S rDNA. The PCR products were subjected to sequencing by Sanger's method. Bacterial species of four sequences obtained were confirmed after a BLAST analysis as *Pseudomonas aeruginosa, Citrobacter amalonaticus, Escherichia fergusonii* and *Proteus mirabilis*. A *Pseudomonas* spp specific PCR assay targeting the OprF gene was performed resulting in a 511bp PCR product from the *Pseudomonas* spp isolates. A PCR assay for detection of *Mycoplasma* infection was conducted by targeting the genus specific 16S rDNA region of the *Mycoplasma* genome, using the DNA extracted directly from the choanal swabs. A 710 bp of PCR product was acquired from 4 out of 31 cases, indicating *Mycoplasma* spp. infection in these animals. Although biochemical tests identified the genera of pathogens, identification up to species level could be achieved by molecular techniques targeting specific regions of the DNA. All the pathogens identified have zoonotic significance posing a serious health risk

to the pet owners.

Appendix - G

प्रबंध सारांश

१	प्रबंधाचे नाव	:	रेड इअर स्लाइडसे कासवातील श्वसनमार्गाच्या संक्रमणाची आण्विक चाचणी
२	विद्यार्थ्याचे नाव	:	मिसाळ तेजश्री सुरेंद्र
३	मार्गदर्शकाचे नाव	:	डॉ. एस. ए. इंगळे सहाय्यक प्राध्यापक, पशु जैवतंत्रज्ञान, पशुवैद्यकीय सूक्ष्मजीवशास्त्र विभाग मुंबई पशुवैद्यकीय महाविद्यालय, परळ, मुंबई ४०००१२
४	पदवी	:	पद्व्युत्तर पदवी
५	पदवी प्रदान करण्याचे वर्ष	:	२०२१
६	मुख्य विषय	:	पशु जैवतंत्रज्ञान
७	प्रबंधातील एकूण पाने	:	
८	सारांशाने एकूण शब्द	:	
९	विद्यार्थ्याची सही	:	
१०	विभाग प्रमुखाचे नाव	:	डॉ. सौ. एस. बी. माजी प्राध्यापक, पशुवैद्यकीय सूक्ष्मजीवशास्त्र विभाग, मुंबई पशुवैद्यकीय महाविद्यालय, परळ, मुंबई ४०००१२
११	सहयोगी अधिष्ठाता		

प्रबंध सारांश

रेड इअर्ड स्लाइडर्स गोड्या पाण्याचे कासव असतात, सामान्यत: घरातील पाळीव प्राणी म्हणून ठेवले जातात. या कासवांमध्ये श्वसनमार्गाचे संक्रमण ही पशुवैद्यकीय सरावामध्ये आढळणारी सामान्य समस्या आहे. दुर्लभ निदान पद्धतींमुळे या संक्रमणांवर उपचार करणे आव्हानात्मक असू शकते. या अभ्यासाचे उद्दीष्ट या श्वसन संसर्गामागील करणीभूत घटक निश्चित करणे आहे. एकूण ३१ रेड एअर स्लाइडर्स श्वसन संसर्गाची लक्षणे दर्शविणारे नमुने घेतले. जीवाणूचे पृथक्करण आणि डीएनए काढण्यासाठी तोंडी आणि कोएनल स्वबचे नमुने जोडींमध्ये घेतले होते. जिवाणूच्या पृथक्करणासाठी संकलित केलेल्या स्वाब्स वर प्रजातींची ओळख पटविण्यासाठी विविध जैवरासायनिक चाचण्या केल्या गेल्या. सर्वात जास्त (एन = ३०) सह ग्रॅम नेगेटिव्ह आयसोलेट्सचे पृथक्करण प्राप्त केले गेले आणि एक ग्राम पॉझिटिव्ह आयसोलेट आढळला. जैवरासायनिक पद्धतीने स्युडोमोनल प्रजाती (३) असे ओळखले गेले. एरोमोनस प्रजाती (४), प्रोटीयस प्रजाती (१२), शेऱ्याशिया प्रजाती (२), सायट्रोबॅक्टर प्रजाती (२), क्लेबिसीला प्रजाती (३), एसचेरिशिया प्रजाती (३) आणि मोरॅक्सेला प्रजाती (१) १६ एस आरडीएनएच्या १४०० बीपी क्षेत्राला लक्ष्य बनवून जिवाणूंची आण्विक पद्धतीने तपासणी करण्यात आली. पीसीआर उत्पादनांना सेन्जरच्या पद्धतीने अनुक्रमित केले गेले. प्राप्त झालेल्या चार क्रमांकाच्या जीवाणूंच्या प्रजातीची ओळख स्यूडोमोनस एरुगिनोसा, सिट्रोबॅक्टर अमॅलोनाटिकस, एसचेरिशिया फुर्गुसोनाय आणि प्रोटीयस मिराबिलिस म्हणून झालेल्या ब्लास्ट विश्लेषणानंतर झाली. ओपीआरएफ जनुक लक्ष्यित करणारे स्यूडोमोनस प्रजाती विशिष्ट पीसीआर परख निष्पन्न झाले, परिणामी स्यूडोमोनस प्रजाती आयसोलेट्समधील ५११ बीपी पीसीआर प्रॉडक्ट उत्पादन झाले. मायकोप्लाझ्मा संसर्गाची तपासणी करण्यासाठी पीसीआर परख हे मायकोप्लाझ्मा जनुकाच्या विशिष्ट १६ एस आरडीएनए प्रदेशास लक्ष्य करुन, कोएनल स्वॅब्जमधून थेट काढलेल्या डीएनएचा वापर करून आयोजित केले गेले. पीसीआर उत्पादनाचे ७१० बीपी ३१ पैकी ४ प्रकरणांमधून आढळले, जे

मायकोप्लाज्मा प्रजाती दर्शवते. या प्राण्यांमध्ये संसर्ग, जैवरासायनिक चाचण्यांद्वारे रोगजनकांच्या उत्पत्तीची ओळख पटली असली तरी डीएनएच्या विशिष्ट प्रदेशांना लक्ष्य करून आण्विक तंत्राद्वारे प्रजाती पातळीपर्यंत ओळख पटविली जाऊ शकते. ओळखल्या गेलेल्या सर्व रोगजनकांना झुनोटिक महत्त्व आहे म्हणून पाळीव प्राण्यांच्या मालकांना आरोग्याचा एक गंभीर धोका असू शकतो.

VITA

Dr. MISSAL TEJASHREE SURENDRA was born on 10th September, 1994 at Andheri in Mumbai, Maharashtra. She completed her schooling from Vidyanidhi V. P. High School, Juhu, Mumbai and her higher secondary school education from Sathaye College, Vile Parle, Mumbai. Her interest, enthusiasm and immense love for animals made her pursue a career in Veterinary Sciences. After completing her higher secondary education she joined B. V. Sc. & A.H. at the Mumbai Veterinary College in year 2012. She has completed her B. V. Sc. & A. H. with second class in the year 2018.

Besides academics she is a good dancer and singer. She has actively participated every year in the college festivals and performed in various singing and dance competitions. She has actively participated in various volunteer based programs for vaccination, sterilization and adoptions throughout her undergraduate years.

Further being interested in Paraclinical subject she joined the Department of Animal Biotechnology, Mumbai Veterinary College, Parel, Mumbai for pursuing her Post-graduation education. During her academic years, she has actively participated in 'National Service Scheme' (NSS) unit of Mumbai Veterinary College.

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