Research Article

Advances in Applied Biological Research

Year 2024, Volume-1, Issue-1 (January - June)

Isolation and Identification of Bacteria from the Oral Cavity of Monitor Lizard (*Varanus bengalensis)*

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ARTICLE INFO

Key Words: Varanus, Pathogens, Zoonotic, Virus, Fungi

doi: 10.48165/aabr.2024.1.05

ABSTRACT

The current research was carried out in the Kasur district, Punjab, Pakistan with the duration of July 2021 to June 2022. The purpose of study was to isolate and identify the bacterial community in the oral cavity of monitor lizard (*Varanus bengalensis*). As monitor lizards are ectotherm reptiles so they spent most of their winter season from November to March in hibernation. So, sampling is done before and after the hibernation period. Ten specimens were captured from the study area by the direct encounter and also using hand nets and pitfall traps. The collected samples were transferred to the University of Veterinary Animal Sciences, Ravi Campus, Pattoki at the Postgraduate Lab, Department of Wildlife and Ecology. Specimens were handled with care to aviod injury. The cotton-tipped swab stick was used to collect a saliva sample from the oral cavity by using a sterile mouth gag. At least three cotton swab samples were taken from each specimen.Each swab sample was stored in sterile buffered peptone water in falcon tubes at -4º C. The solution from the tubes was streaked over Nutrient agar (NA), Mannitol Salt (MS) agar, Xylose Lysine Deoxycholate (XLD) agar and Trypticase Soya agar with 5% Sheep Blood agar. All of the plates were cultured at 36±°C in an incubator for 24–48 hours. Gram staining was used to identify descriptive colony morphology. Isolates were identified primarily based on colony morphology on selective media, Gram nature, motility test, and pigment production. For possible identification, the selected colonies were sub-cultured on nutrient agar and Trypticase Soya agar (TSA). After that, isolated biochemical tests were carried out in order to identify them. Three most abundant bacterial species were investigated in present study including *Salmonela typhi, Staphylococcus aureus* and *Pseudomonas aeruginosa* their relative abundance would be statisticaly analyzed. Accordingly Salmonela typhi contained 43.33%, *Staphylococcus aureus* contained 46.67% and *Pseudomonas aeruginosa* contained 13.33%.

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Received 08.08.2024; Accepted 05.09.2024

Introduction

With over 1200 genera and 11000 species, reptiles are one of the most diverse and successful vertebrate families (Roll *et al.* 2017) and with four orders in this class: Squamata, Testudines, Crocodylia and Rhynchocephalia (Pincheira-Donoso *et al.* 2013). Since their first appearance 310 to 320 mya in the Late Carboniferous, reptiles haven't changed much in terms of morphology, biology or ecology (Tucker and Benton 1982; Lepetz *et al.* 2009). Over millions of years, vectors and diseases have co-evolved with them. Wildlife diseases have been highlighted as a threat to domestic animal and human health, as well as a significant threat to global biodiversity conservation (Daszak *et al.* 2000). The role of disease drivers including man-made environmental changes and growing human encroachment into wild regions was investigated (Thompson *et al.* 2010; Brearley *et al.* 2013).

Monitor lizard (*Varanus bengalensis)* is large in size and belongs to the Varanidaeae family, which includes a monophyletic group of Anguimorph lizards stratified in a single genus such as *Varanus* (Zheng and Wiens 2016). These are considered visible faunas and after crocodiles and pythons, they are the world's largest living poikilothermic predators (Koch *et al.* 2013). Varanidaeae monitors are much differentiated, with distinct differences in body size, color, tail anatomy, behavior, and surroundings (Pianka *et al.* 2004). These are often seen on the surface and their progeny are usually observed on plants. *Varanus bengalensis* has a higher proclivity for climbing trees. It has a body length of 70 centimeters and a tail length of roughly 100 centimeters. Water lizards can be found in the wild around riversides, water channels, marshes, and bushy areas (Elmahy and Harras 2016). On the earth, they are widely distributed and have a number of 80 species and 23 subspecies of Varanus. The Indo-Australian Archipelago, South and South-east Asia, Africa, Central Asia the Middle East and the Pacific islands such as the Mariana Islands, Admiralties and the Solomon Islands have all been documented (King *et al.* 1991; Böhme, 2003; Koch *et al.* 2013; Zheng and Wiens 2016; Utez *et al.* 2020). They can be found in the river basins of Pakistan, Afghanistan, eastern Iran, India, Burma, Nepal, Bangladesh and Sri Lanka. They are mostly terrestrial and can be found in agricultural areas below 1500 meters in elevation (Auffenberg, 1994).

The Indian or Bengal Monitor is one of three species found in Pakistan as (*V. bengalensis*) Desert or Grey Monitor (*V. griseus*), also known as the Yellow Monitor (*V. flavescens*), has two subspecies: Indo-Pak Desert Monitor (*V. griseus koniecznyi*) and Transcaspian Desert Monitor (*V. griseus caspius*) (Khan, 2006). Except for the Indian desert monitor lizard, all are Least Concern (IUCN, 2020). Large adults will occasionally stem and capture dormant bats by climbing vertically on plant trunks. They are typically fed tiny terrestrial animals such as birds and their eggs, fish, frogs, rodents, lizards and snakes as they grow up. Varanidaeae, with the exception of a few species (e.g., *V. bitatawa, V. olivaceus, V. indicus*), are highly adaptable to their surroundings and opportunistic predators (Shine, 1986; Losos and Greene 1988; Molnar, 2004).

The ecology of the *V. bengalensis* (Indian monitor lizard) is still unknown, and it has not been assessed (IUCN, 2020). Monitor lizards are considered eco and human-friendly. As they possess sharp and serrated teeth which help them to play a vital role to feed dead and decaying matter. Monitor lizards are ectotherm and remain energetic through the year except winter having the diet (fresh and rotten) for foraging (Attenborough, 2008). Their scavenging behavior helps to maintain the ecosystem. In the field, they also help to control pest strength as they like to eat eggs of different species. Monitor lizards also help to control rodents because they give huge damage to the formers by means of damaging their crops. Many people use them as a source of revenue. By harvesting, they build different ways to earn nutrition, skins, pets and also use traditional medication throughout the world (Leader-Williams, 2002). The unsustainable harvesting can cause the failure of the population which has a bad impact on the exploitation of taxon. Particularly for those scavengers who play a dynamic role in the environment, failing to sustain harvests within maintainable limits can cause a huge pouring effect on ecosystem function (Fortin *et al.* 2005; Heithaus *et al.* 2008).

Many incidents are observed that show how people are still not realized the role of varanidaeae in the environment due to lack of awareness. In Pakistan, monitor lizards are not endangered but people still hunt them for their eggs and also for their meat within the different regions of Sind. These are captured by the local hunter and given to the Local Hakim (traditional medicine makers) who makes some oil that is supposed to have medicinal qualities (Khan, 2006). Meanwhile the period, it is observed that the growing human population and the demolition of wildlife habitats occur because of human encroachment on the forest for the development of agricultural land, establishment of tea gardens and construction; purposes can cause a huge decline in wildlife including varanidaeae. In this way, a human-wildlife conflict occurs which results in the killings and hunting of varanidaeae and after that, they use them for meat and oil (Choudhury and Choudhury 2019). Toxins found in toads belonging to the Bufonidae

family can be fatal to monitor lizards (family Varanidaeae) (Pettit *et al.* 2021).

Some studies reveal that they possess a variety of gram-positive and gram-negative pathogenic bacteria that have the ability to give huge damage to human concerns by means of contaminating their environment and food as well. There are also monitor lizard bites occur that cause some diseases in humans such as rhabdomyolysis, intravascular hemolysis, coagulopathy, abscesses (boils), cellulitis, and acute kidney injury (AKI). Also, there are many theories relevant to animals as well bites that result in sepsis, envenomation, and blood loss involving animals like deer, water buffalo and pigs. Captive reptiles are known repositories of key zoonotic pathogens (such as *Salmonella spp*. and *Pseudomonas aeruginosa*), they are considered sources of human exposure via faeces, bite wounds and scratches (Ebani *et al.* 2008). Historically, diseases affecting wildlife populations have gotten a lot of attention if they have an impact on human health or have economic ramifications. Because of their propensity to develop antibiotic resistance and express various virulence characteristics, *Pseudomonas spp.* has been linked to substantial morbidity and death in infected animals under certain conditions and when extremely harmful (Rossolini *et al.* 2005; Shanooba *et al.* 2011).

People belonging to different regions of the world are consuming their meat because of the taste and belief of the people in healing disease. They have the capacity to transmit zoonosis and are very susceptible to infection by several pathogens, depending on their habitat and food. Animal-human contact has long been known as both allies and foes. While dog bites and snake bites are common, monitor lizard bites are extremely rare. Because they have sharp, serrated teeth but a weak bite, animals may resist the lizard's initial attack; yet, if bitten and not killed right away, prey may suffer from lethargy and shock (Fry *et al.* 2009; Bull *et al.* 2010). Varanidae oral flora has a wide variety of gram-positive and gram-negative disease-causing bacteria. Varanidae bite infections have been discovered to include a diverse range of germs. In the present stuy, the oral microbiota of *V. bengalensis* will be examined, as well as the antibiotic exposure of various bacteria, in order to develop an appropriate treatment.

MATERIALS AND METHODS

Study area

The current research was carried out in the Kasur district, Punjab, Pakistan. The district Kasur is 150 and 200 m above sea level, and the temperature ranges from scorching hot in the summer (April to September) to bitterly cold in the winter (November to February). The annual rainfall averages 500 mm. A substantial section of the district has been damaged by waterlogging and salinity, making the subterranean water brackish. The tanneries, Changa Manga plantations, plant/flower nurseries, and district Head Baloki helped Kasur become increasingly well-known throughout the country (Ali *et al.* 2016).

Samples collection

A total of 10 *V. bengalensis* specimens were captured from the study area by the direct encounter and also using hand nets and pitfall traps. The samples were transferred to the University of Veterinary Animal Sciences, Ravi Campus, Pattoki at the Postgraduate Lab, Department of Wildlife and Ecology. Date, time, habitat, species age, and GPS coordinates were all recorded in the lab book. Each sample was identified by using taxonomic keys (Khan *et al.* 2006).

Collection of oral swabs

To avoid injury, specimens were handled with care (Jessup and David 2008). The cotton-tipped swab stick was used to collect a saliva sample from the oral cavity of the *V. bengalensis* using a sterile mouth gag. At least three cotton swab samples were taken from each specimen. The swab samples were acquired by gently spinning the swab on the *V. bengalensis* oral mucous membrane cavity and mandibular teeth. After the sampling, specimens were set free in their natural habitat (Kikillus *et al.* 2011).

Isolation and identification of bacterial species

Each swab sample was stored in sterile buffered peptone water in falcon tubes at -4° C. Tubes were brought at room temperature and vortexed to uniformly mix the contents before use. The solution from the tubes was streaked over Nutrient agar (NA), Mannitol Salt (MS) agar, Xylose Lysine Deoxycholate (XLD) agar and Trypticase Soya agar with 5% Sheep Blood agar. All of the plates were cultured at 36±°C in an incubator for 24–48 hours. Gram staining was used to identify descriptive colony morphology prior to differential staining (Mataranyika and Percy 2019). Isolates were identified primarily based on colony morphology on selective media, Gram nature, motility test, and pigment production. For possible identification, the selected colonies were sub-cultured on nutrient agar (Kikillus *et al.* 2011). After that, isolated biochemical tests were carried out in order to identify them.

Statistical analysis

The data thus obtained was presented in the form of prevalence in percentage (%) by using MS Excel.

RESULTS

This study was conducted at Kasur from July 2021 to June 2022 at the Postgraduate Laboratory, Department of Wildlife and Ecology, University of Veterinary and Animal Science, Ravi Campus Pattoki.

Collection of Specimens

A total number of 10 specimens of monitor lizard (*Varanus bengalensis*) were collected from the different selected sites of Kasur and brought to the Postgraduate Laboratory, Department of Wildlife and Ecology, the University of Veterinary and Animal Science, Ravi Campus Pattoki following the institutional rules for the use of animals in research by using clothes bag. During the study period observation of the selected sites was carried out. As monitor lizards are ectotherm reptiles so they spent most of their winter season from November to March in hibernation. So, sampling is done before and after the hibernation period. All the specimens were handled with care. Demographic data and coordinates were also taken by using GPS Essential.

Collection of Oral Swab

To avoid injuries physical method was used to collect oral swabs from the tongue, roof and cheek of the monitor lizard (*V. bengalensis*) by using an oral cotton swab stick and a mouth gag. Three swab samples were collected from a single specimen. The swab samples were acquired by gently spinning the swab on the *V. bengalensis* oral mucous membrane cavity and mandibular teeth. These swab samples were stored in falcon tube by adding buffered peptone water at 4°C. samples were brought at room temperature and vortex to uniform and mixed all content before use. After the collection of oral swabs, specimens were set free in their natural habitat.

Microorganisms and Cultures

Before the preparation of cultures, there must be a need to sterilize the lab equipment to avoid contamination.

Sterilization

Lab equipment such as flask, Petri plates, test tubes and falcon tubes were washed with detergent and then autoclaved. The sterilization through autoclave was done for 15mints at 121°C (200kPa). After that, Petri plates and test tubes were wrapped in paper and placed in the dry oven at 170°C (figure 3). After that, these types of equipment were unwrapped in a biosafety cabinet. UV radiations were also passed to avoid contamination.

Detail of Media

Different media were used to culture different bacterial species such as Nutrient Agar, Mannitol Salt Agar, Hektoen enteric agar (HEA), Cetrimide agar, Blood Agar and Trypticase Soya agar (TSA).

Streaking on Media

The media were prepared and autoclaved as per the description. After that, the sample was brought to room temperature and vortex to mix all the contents. The media was poured into the Petri dishes and allow solidifying. With the help of sterilizing a wire loop sample was streaked over agar and allowed to incubate at 36±°C for 24 to 36h. Different colonies were formed on the Nutrient agar (figure 2). After that selected colonies were pure culture on Nutrient agar and Trypticase Soya agar (TSA).

Identification of Bacteria

Gram staining

After the incubation period gram staining was performed. On the basis of morphological characters, different bacterial species were identified on media i.e (shape, color, roughness, and smoothness). Microscopic identification was done by gram staining. Gram staining allows differentiating between gram-positive and gram-negative bacteria. A blue to purple color shows gram-positive and pink to reddish shows gram-negative bacteria (figure 3). Biochemical tests were also performed to confirm the bacterial species.

Biochemical test

Different biochemical tests were performed to identify the different bacterial species within the samples.

A total number of 30 swabs were taken from the oral cavity of the monitor lizard (*Varanus bengalensis)*. These swabs were grown on different agar and selected media also. After that, these biochemical tests were performed which confirm the stain of the bacterial species.

Statistical Analysis

To check the relative abundance of the Bactria the data passed through MS Excel in the form of prevalence in percentage (%).

Table 1: Biochemical tests

Sr No.	Name of Bacteria	Gram staining	Catalase test	Oxidase test	Motility test	Methyl red test
1	*Salmonella typhi*	Negative	Positive	Negative	Motile	Positive
2	*Staphylococcus aureus*	Positive	Positive	Negative	Negative	Positive
3	*Pseudomonas aeruginosa*	Negative	Positive	Positive	Motile	Negative

Table 2. Prevalence of bacteria isolated from monitor lizard.

Sr. No	Bacteria Isolate	Prevalence in percentage (%)
1	*Salmonella typhi*	43.33
2	*Staphylococcus aureus*	46.67
3	*Pseudomonas aeruginosa*	13.33

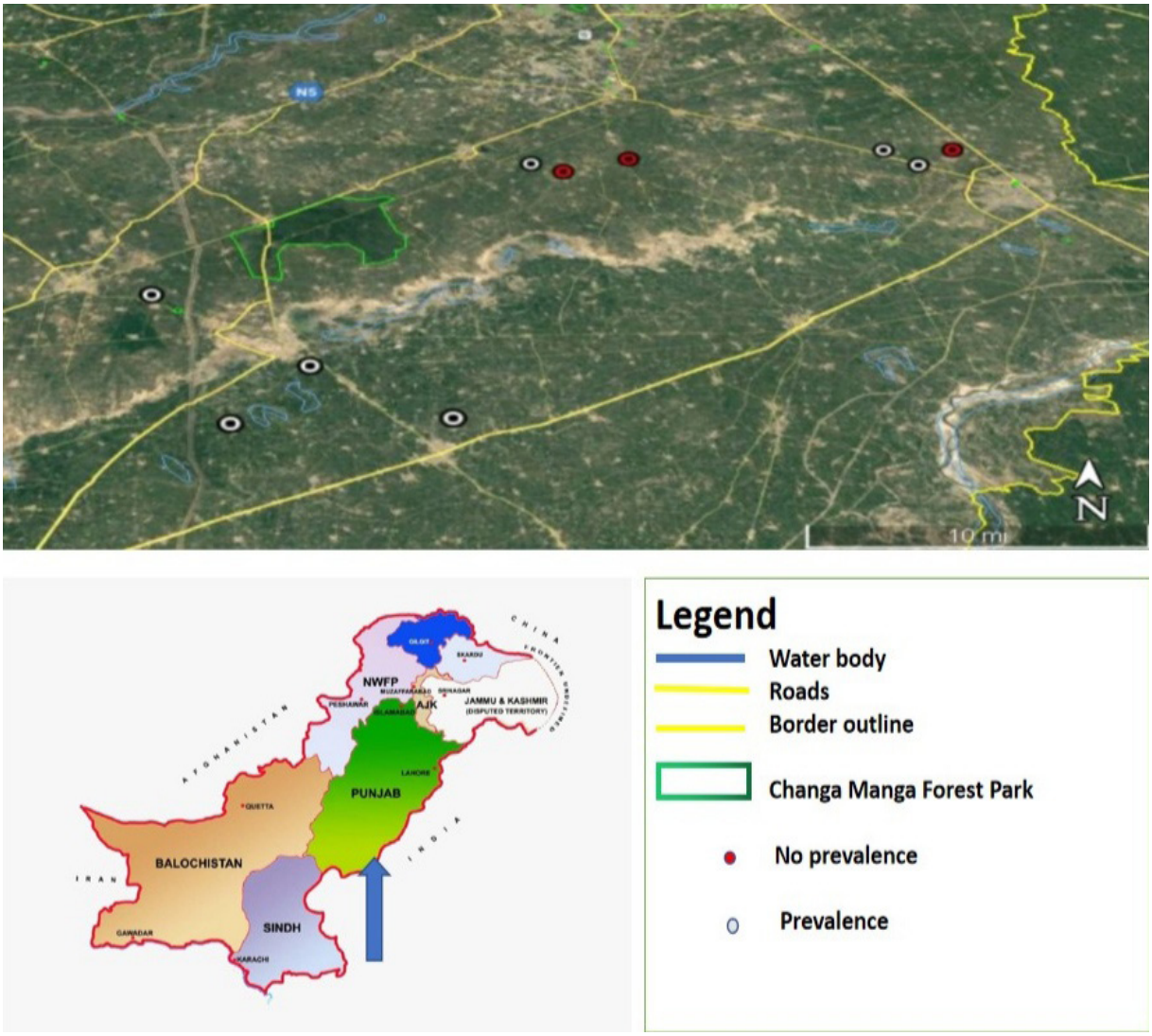


Fig. 1: A map presenting the research site of district Kasur

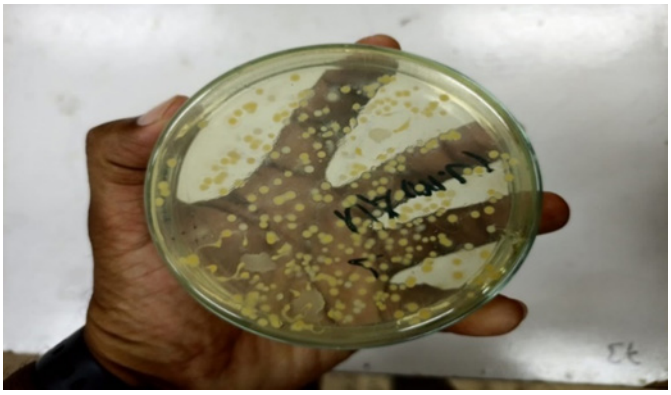
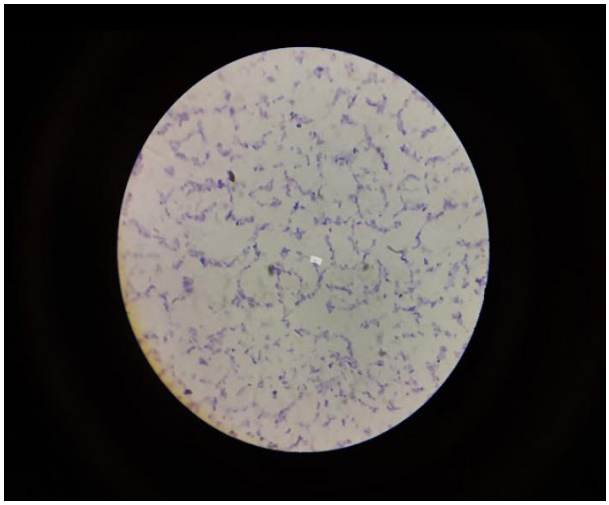


Fig. 2: Colonies formation on Nutrient agar



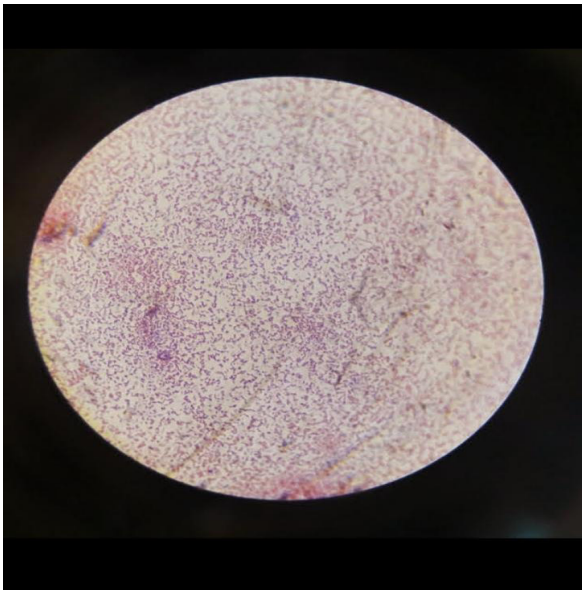


Fig. 3: Gram staining was performed to differentiate between gram-positive and gram-negative bacteria

DISSCUSSION

Monitor lizard (*Varanus bengalensis)* is large in size and belongs to the Varanidaeae family, which includes a monophyletic group of Anguimorph lizards stratified in a single genus such as Varanus (Zheng and Wiens 2016). These are considered visible faunas and after crocodiles and pythons, they are the world's largest living poikilothermic predators (Koch *et al.* 2013). Varanidaeae monitors are much differentiated, with distinct differences in body size, color, tail anatomy, behavior, and surroundings (Pianka *et al.* 2004). They are extensively spread throughout the old world. These are often seen on the surface and their progeny are usually observed on plants. *Varanus bengalensis* has a higher proclivity for climbing trees. It has a body length of 70 centimeters and a tail length of roughly 100 centimeters. Water lizards can be found in the wild around riversides, water channels, marshes, and bushy areas (Elmahy and Harras 2016). *V. salvator* favors locations near water bodies, although *V. bengalensis* replaces it in plains areas, and *V. flavescens* inhabits mountainous terrains at heights greater than one hundred and sixty meters (Khatiwada and Ghimire 2009).The ecology of the *V. bengalensis* (Indian monitor lizard) is still unknown, and it has not been assessed (IUCN, 2020). Monitor lizards are considered eco and human-friendly. As they possess sharp and serrated teeth which help them to play a vital role to feed dead and decaying matter. Monitor lizards are ectotherm and remain energetic through the year except winter having the diet (fresh and rotten) for foraging (Attenborough, 2008). Their scavenging behavior help to maintain the ecosystem. In the field, they also help to control pest strength as they like to eat eggs of different species. Monitor lizards also help to control rodents because they give huge damage to the formers by means of damaging their crops. Many people use them as a source of revenue. By harvesting, they build different ways to earn nutrition, skins, pets and also use traditional medication throughout the world (Leader-Williams, 2002). Unsustainable harvesting can cause the failure of the population which has a bad impact on the exploitation of taxon. Particularly for those scavengers who play a dynamic role in the environment, failing to sustain harvests within maintainable limits can cause a huge pouring effect on ecosystem function (Fortin *et al.* 2005; Heithaus *et al.* 2008). *S. typhi* was prevalent in the current data at a rate of 43.33%. According to earlier research, there are wide variations in the percentage of lizards that are infected with different strains of Salmonella. According to various studies, the numbers are as follows: 12% by (Hinshaw and McNeil 1947), 62% by (Lee and Mackerras 1955), 77% by (Iveson *et al.* 1969), 2 and 9% by (Hamel and McInnes 1971), and 8% by (Hoff and White 1977). Even though *Salmonella spp.* were a topic of discussion in each of these investigations, they are not directly comparable. The variations in Salmonella infection rates could be caused by a number of factors. While some of this research

used captive or zoo species, other studies used wild or local lizards. *S. aureus* was prevalent in the findings until this point at 46.67%. Previous research on *S. aureus* revealed precisely the opposite results. In contrast to the results of the current study, 46 (70.8%) isolates were found in earlier research (Foti *et al*. 2013). 13.33% *P. aeruginosa* prevalence was found in the current investigation.

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