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Identification of Microbial Community in Rock Hyrax, Suspected Reservoir of *Leishmania tropica* in Palestine: A Metagenomic Analysis

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Thesis Approval

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Jerusalem-Palestine
1441 / 2020

Jerusalem – Palestine

1441/ 2020

Dedication

For

The sake of Allah, my Creator and my Master, who always inspires me how to believe in myself and my dreams.

My great teacher and messenger, Mohammed (May peace be upon him), who taught us the purpose of life.

My homeland Palestine and Jerusalem, the city of peace.

To those dearest to me, my family, my mother and my father, who offered me unconditional love constant and source of support and encouragement during the challenges of life.

To my brothers and their kids, who draw a smile and laugh on my face every day, my sister, my unconditional special friends and all people who have always been there to support me

To my role model in life, my uncle Mohammad Al Shab'aan, the petroleum geologist

To my friends and colleagues.

I dedicate this research.

Rana Nayef Mesleh Awaysa

Declaration

I certify that this thesis submitted for the Degree of Master is the result of my own research, except where otherwise acknowledged, and that this study (or any part of the same) has not been submitted for a higher degree to any other university or institution. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed.....

Rana Nayef Mesleh Awaysa

Date: 11.1.2020

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Abstract

Hyrax (*Procavia capensis*) is the only representative of the order Hyracoidea in the Middle East.This African species harbor *Leishmania* in their skin and nematodes in their gut, they are susceptible to viral pneumonia and tuberculosis. The widespread co-distribution of this reservoir, suggests a significant threat from the spread of diseases caused by pathogens accompanying with the body of hyraxes. Hyraxes can live several years in nature and thus comprise a natural reservoir for carrying and transmitting infections. Several studies showed that experimentally infected hyraxes by *Leishmania* can sustain infection, showing no clinical signs but they are infective to sand flies. Therefore, this study aimed to investigate the microbial community structure of tissue samples taken from hyraxes collected from different regions in Palestine which may pose a threat to human health.

Sixteen rock hyraxes have been collected using raccoon traps from different localities in Palestine (A'nabta-Tulkarem, Haifa, Amara near Beer Al-Saba'a, Mas'ha-Salfit and Faqqua-Jenin). The DNA was extracted from hyraxes tissue samples (n=16) including blood (n=3), nose (n=2), ear (n=3) and spleen (n=8), only one sample was taken from each hyrax. All DNA samples were tested for presence of pathogens including parasites, bacteria, viruses and fungi using next generation sequencing (NGS). DNA library were prepared based on Nextera® XT assay and deep sequenced on Illumina MiSeq Machine. Bioinformatics analysis was applied to identify the NGS obtained sequences using the online tool on Galaxy . The retrieved pathogenic sequences were blasted using BLAST search on NCBI . The sequences with >97% identity and >97% query cover were adopted and compared to the available reference nucleotide sequences in the database for species identification.

Among the tested hyraxes samples (n=16), four samples including spleen (n=2), blood (n=1) and nasal tissue (n=1) were excluded from the study. Therefore, the analysis was applied only on twelve hyrax samples, from which *Leishmania* have been detected in four hyrax samples, which were obtained from spleen (n=3) and ear (n=1). Three of them were identified as *L. tropica* while the forth sample was identified only up to the genus level . These hyraxes were collected from A'nabta-Tulkarem and Haifa where cases of human cutaneous leishmanias due to *L. tropica* were reported. Furthermore, at the bacterial level, *Mycobacterium tuberculosis complex* (MTC) has been detected in three hyraxes. These hyraxes were captured from Haifa,

Faqqua-Jenin and Amara near Beer Al-Saba'a. On the other hand, human pathogen such as, *Neisseria meningitides* have been also detected in tow hyraxes in addition to *Bordetella spp.* which was detected in one hyrax. Moreover, two types of viruses in three hyrax samples, the two dominant viruses were *Human betaherpesvirus* and *Procavia capensis gammaherpesvirus*.

In conclusion, our results showed that rock hyraxes can be involved in transmission of serious pathogens which can be harmful to humans as hyraxes have long lifespan and gregarious habits sharing habitats with several vectors. Our results confirmed the presence of *L. tropica* DNA in rock hyraxes from Palestine and supported their potential role as a reservoir for human CL.. Efforts to prevent human leishmaniasis should be focused on interrupting the transmission of infection cycle that includes in addition to the control of sandfly vectors the control of hyrax population as a reservoir.

Key words: **Key words**: Hyrax, reservoir, *Leishmania*, Cutaneous leishmaniasis, *Mycobacterium*, *Human betaherpesvirus*, metagenomics, Galaxy.

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List of Abbreviation

Abbreviation	Full Term
CL	Cutaneous Leishmaniasis
WHO	World Health Organization
MAP	*Mycobacterium avium* subspecies *paratuberculosis*
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
kDNA PCR	kinetoplast DNA- polymerase chain reaction
nPCR	nested polymerase chain reaction
NGS	Next-generation sequencing
EDTA	Ethylenediaminetetraacetic acid
TE	Tris-EDTA
NT	Nucleotide
WGS	Whole Genome Sequencing
BLAST	Basic Local Alignment Search Tool
BLASTN	Nucleotide BLAST
NCBI	National Centre for Biotechnology Information
SNP	Single Nucleotide Polymorphisms
MTC	*Mycobacterium tuberculosis complex*

List of Appendices

Chapter One: Introduction

1.1 Background:

Hyrax (*Procavia capensis*) is the only representative species of the order Hyracoidea in the Middle East. Rock hyrax is mainly distributed in Africa and the Middle East. In Palestine, the rock hyrax has been reported on the mountains of the western side of the Dead Sea and the Jordan Valley, including Wadi Qelt, west of Jericho city and Zubaidat village 50 kms north of Jericho city. The presence also extends to the northern Palestinian districts of Tubas and Jenin on the outskirts of villages close to human activity and even reaching the Lake of Tiberias with populations also found close to Umm Al-Rashrash (Al-Jawabreh et al., 2017; Talmi-Frank et al., 2010). Rock hyraxes harbor *Leishmania* in their skin and nematodes in their gut; they are susceptible to viral pneumonia and tuberculosis. Furthermore different ecto-parasites also infect hyraxes (Lemma, 2008; Rifai et al., 2000). The widespread distribution of this reservoir, suggests a significant threat from the spread of diseases caused by pathogens accompanying with the body of hyraxes in the Middle East, central Asia and southern Europe (Faiman et al., 2013).

1.2 Classification:

The hyraxes, from the Greek word 'hyrak' meaning shrew-mouse, are classified under order hyracoidean, the order Hyracoide consists of one extant family, Procaviidae and one extinct family, Pliohyracidae. The extant family comprises three living genera; *Procavia*, *Heterohyrax* and *Dendrohyrax*. Members of the genera *Procavia* Storr are strictly rock-dwelling and considered to have evolved first in Africa before the Oligocene period 40 million years ago (Teklehaimanot and Balakrishnan, 2018; Walker et al., 1975). *Heterohyrax* Gray is rock-dwelling and partially arboreal bush hyrax, whereas *Dendrohyrax* Gray is strictly arboreal (Stuart and Stuart, 2007; Teklehaimanot and Balakrishnan, 2018), (Figure 1.1). Hyrax belongs to super order Afrotheria which includes elephants, sea cow, elephant shrew, aardvark and dugongs (Springer et al., 1997). Hyraxes and elephants share many of the characteristics of the nature karyotype, this supports the development of African animal that group within the superorder of Paenungulata (Al-Dakan and Al-Saleh, 2015; Pardini et al., 2007).

1.3 Rock hyrax characteristics and behavior

The rock hyraxes *(Procavia capensis*) are small (~3–4 kg) mammals that have short legs, round ears, and a rudimentary tail (Figure 1.2). They have a lifespan of approximately 12 years, and usually live in groups of 10–50 individuals on rocky outcrops. Hyraxes make their dens in the crevices between rocks where they are protected from predators and can avoid the midday heat (Kershenbaum et al., 2010; Naylor, 2015). They share the habitat with the sandflies of the genus *Phelobotomus* and enhance the environment by the accumulation of organic matter in their latrine for sandfly breeding (Lemma et al., 2009)**.** These herbivorous mammals feeds on a wide variety of different plants including shrub leaves, grasses and sometimes they feed on insects and lizards (Al-Dakan and Al-Saleh, 2015; Burton and Burton, 2002).The proximity of hyrax colonies to villages is also of concern**,** and the proliferation of preferred den sites may lead to disease outbreaks (Jacobson et al., 2003; Kershenbaum et al., 2010).

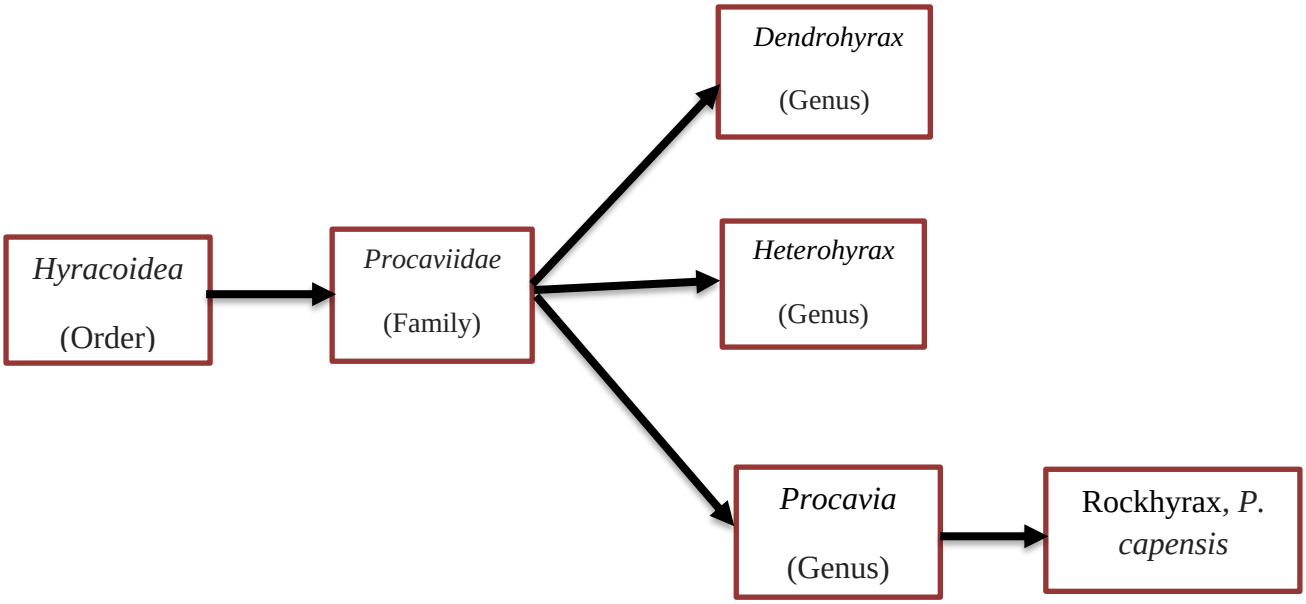


Figure 1.1: The taxonomy of the rock hyrax (*Procavia capensis).*

Figure 1.2: Rock hyrax (*Procavia capensis*) resting on the rocks surrounding Zubaidat village north of the Jordan Valley in Palestine (Courtesy: Amer Al-Jawabreh-Jericho).

1.4 Geographic distribution of rock hyrax:

The rock hyrax is found throughout sub-Saharan Africa and northeast Africa, from Senegal through southern Algeria (discontinuously distributed), Libya and Egypt (east of the Nile River) to central and southern Africa (Butynski et al., 2015; Hoeck, 1989; Olds and Shoshani, 1982; Teklehaimanot and Balakrishnan, 2018). The distribution extends to the Arabian Peninsula, mainly in the west, and to Lebanon, Jordan, it is also present in Palestine (Harrison and Bates, 1991; Olds and Shoshani, 1982; Wilson and Reeder, 2005). (Figure 1.3).

Figure 1.3: A map showing the distribution of *Procavia capansis* in Africa and Asia (Butynski et al., 2015).

1.5 Rock hyrax and human cutaneous leishmaniasis:

Rock hyraxes are the suspected natural reservoir for cutaneous leishmaniasis (CL) (Jaffe et al., 2004; Saliba and Oumeish, 1999; Shani-Adir et al., 2005; Talmi-Frank et al., 2010) Cutaneous leishmaniasis is a parasitic disease caused by *Leishmania* genus with wide range of clinical symptoms ranging from localized skin lesions to disfiguring scars, (de Vries et al., 2015; Zappacosta et al., 2010) Figure (1.4). The causative pathogens of CL in the east Mediterranean region are *Leishmania major* and *Leishmania tropica*. The disease transmitted to humans by the bite of a blood-feeding insect female sand fly (*Lutzomyia* in the new world leishmaniasis and *Phlebotomus* in the old world leishmaniasis) (de Vries et al., 2015; Maroli et al., 2013; Steverding, 2017).

Figure (1.4): Clinical presentations of CL. (A) crusty lesions with a patchy distribution, local edema and color changes.(Rana Awaysa, 2018).

CL is transmitted by phlebotomine sandflies in tropical, subtropical and temperate regions. Currently 350 million person at risk of this disease in 98 countries (Manzano et al., 2011). The overall prevalence of CL disease is 12 million cases and the annual incidence is 2–2.5 million. In most countries, the incidence numbers are probably underestimated because cases are not recognized and reporting is not mandatory (de Vries et al., 2015) (Figure .15).

Thus, the World Health Organization (WHO) considers leishmaniasis to be one of the eighteen neglected tropical diseases (NTDs) (Akhoundi et al., 2016; Faiman et al., 2013; Svobodova et al., 2006; WHO, 2017). Rock hyraxes are the natural reservoir for *Leishmania aethiopica* in Africa in Ethiopian highlands (Ashford et al., 1973). Furthermore, this animal also is a reservoir for human leishmaniasis in kenya, east Africa and Indian subcontinent, (India, Nepal, Bangladesh) (Alemayehu and Alemayehu, 2017).

Because there is no available vaccines or drugs to prevent infection, understanding the reservoir system is important in designing rational control. As rock hyrax (*P. capensis*) is the suspected natural reservoir for leishmanaisis, prevention and control of this disease requires a combination of intervention strategies because transmission occurs in a complex biological system involving the human host, sandfly vector, and parasite. These animals live 8–9 years in nature, and thus comprise an efficient reservoir for carrying infections into the next transmission season. Early diagnosis and proper treatment is required to reduce the prevalence of the disease and to prevent disabilities and death (Gonzalez et al., 2015; WHO, 2018).

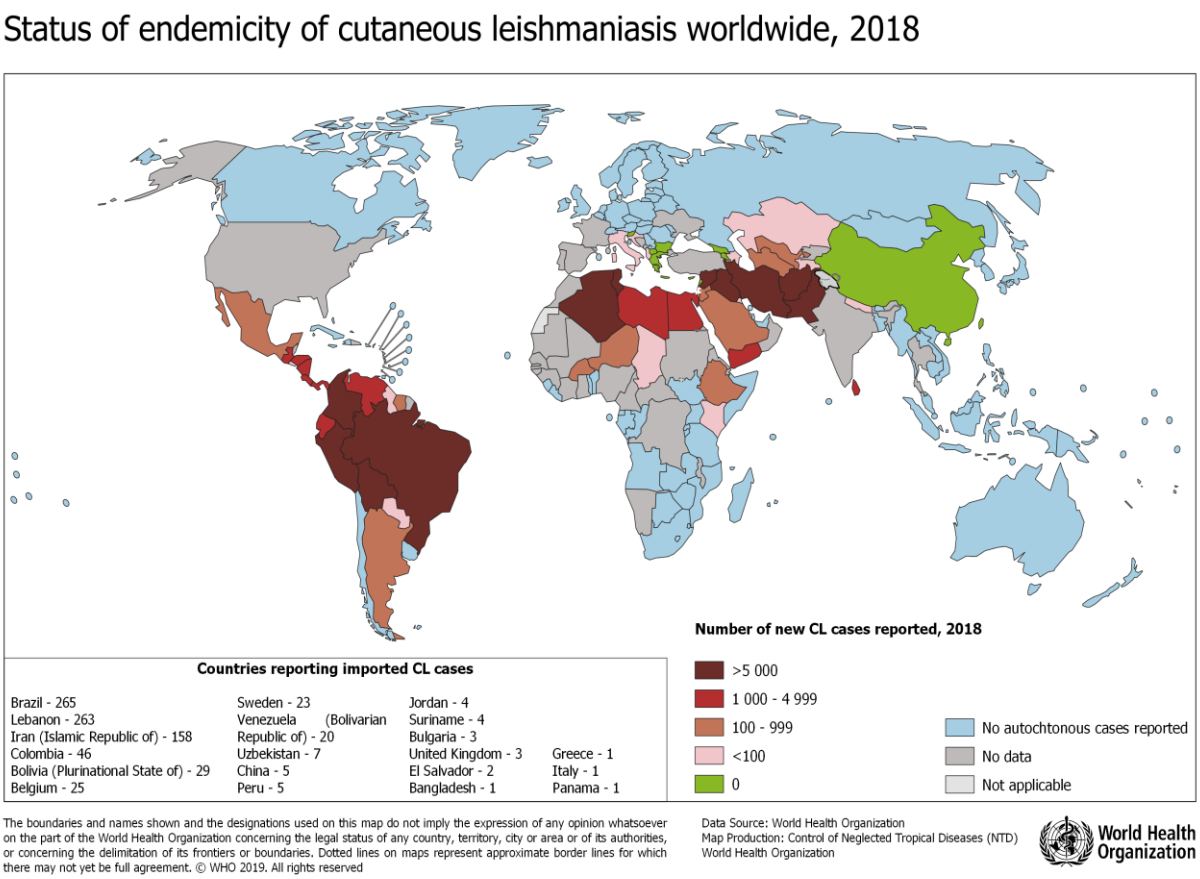


Figure 1.5: Geographical distribution of Cutaneous Leishmaniasis**.** Status of endemicity of cutaneous leishmaniasis worldwide, 2018. (WHO, 2019).

1.5.1 Human cutaneous leishmainasis in Palestine:

In Palestine, human CL was considered hyperendemic in Jericho which is caused by *L. major* and *L. tropica.* Human CL was also reported in the northern districts (Jericho, Bethlehem, Nablus and Tubas) of the West Bank which is caused by *L. tropica* (Al-Jawabreh et al., 2003). Regionally, there are two recognized vectors of *L. tropica*, *Phlebotomus arabicus* and *Phlebotomus sergenti* (Rohoušová et al., 2018). Furthermore, *L. tropica* is considered to be anthroponotic (person- to- person transmission) by female sand flies of the species *Phlebotomus sergenti* (Al-Jawabreh et al., 2017; Al-Jawabreh et al., 2004; Schnur et al., 2004), mainly during the 1994–2015, an annual average incidence of 0.9 to 12.9 per 100,000 within the whole area of the Palestinian West Bank. (Figure 1.6)(Al-Jawabreh et al., 2017).

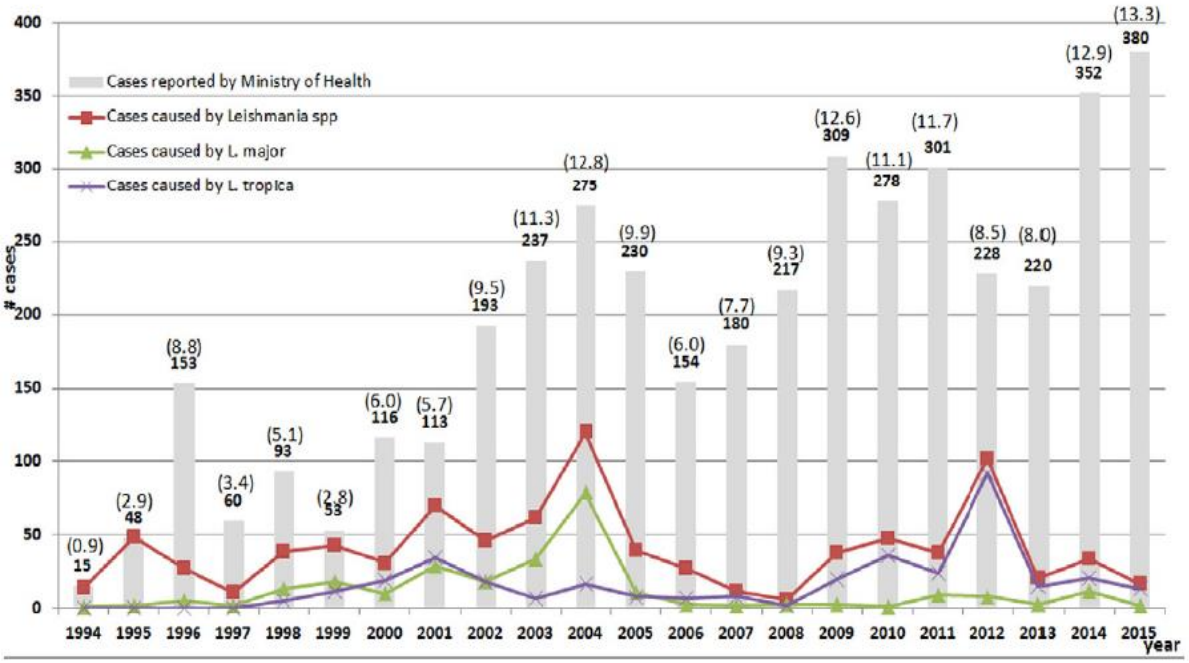


Figure (1.6): Distribution of cases of cutaneous leishmaniasis by year in Palestine between 1994 and 2015. (Al-Jawabreh et al., 2017)

Rock hyraxes are suspected for *Leishmania tropica* in Palestine (Jacobson et al., 2003; Svobodova et al., 2006; Talmi-Frank et al., 2010). In Jordan, the neighboring country, hyraxes were encountered in three areas (Irbid, Salt, and Tafieleh governorates), hyraxes were infected with *Leishmania* parasites that belong to a group close to *Leishmania tropica*, where sporadic cases of CL (Saliba and Oumeish, 1999).

1.5.2 The correlation between hyraxes, sandfly vectors and *leishmania*:

A previous study showed that high density of reservoir population and close proximity of hyrax dens to human houses as well as high density of vector population, with the absence of personal protection against the vector, increased the risk of *Leishmania* transmission. In addition, in more urbanized sites, the effect of living next to the site's edges, facing the wadies and open green areas, and proximity to the reservoir (hyrax) colonies increase the exposure to vector (sand-fly) and thus increase the infection rate (Schlein et al., 1984). On the other hand, the abundance of hyraxes where the localization of the homes of the affected people on a slope suggests that these animals

might have been involved in the transmission of *L. tropica* in this area (Klaus et al., 1994).

In the year 2000, the local population in Palestine (particularly in Jericho city) noted that large numbers of wild animals, presumably hyraxes, invading their homes, farms and even coming close to the city itself on its western side of Wadi Qelt, where many Bedouin inhabitants have acquired CL caused by *L.* tropica (Al-Jawabreh et al., 2017). Moreover, sandflies are present in greater numbers in areas located in the periphery and edges facing the wadies close to hyrax colonies. Sandflies that have been captured next to hyrax colonies were infected with *Leishmania tropica* (Svobodova et al., 2006).

Many reports have indicated that CL disease, caused by *L. tropica*, has become a significant problem in the West Bank, Palestine (Al-Jawabreh et al., 2003; Jaffe et al., 2004; Schnur et al., 2004). From the late1990s onward, epidemiological surveys and field studies have documented a very significant increase in the number of human cases of CL and their wider geographical distribution was observed throughout the central area of the Palestinian- Israeli region (Al-Jawabreh et al., 2004; Azmi et al., 2017; Azmi et al., 2012a; Azmi et al., 2012b; Schnur et al., 2004; Singer et al., 2008). Furthermore, *L. aethiopica* which is endemic in Ethiopia, Kenya and South-west Africa have been isolated from rock hyraxes, trapped in Najran, Saudi Arabia (Morsy, 1997). Moreover, hyraxes are considered to be the suspected reservoir *L. aethiopica* in Ethiopia (van Henten et al., 2019).

1.5. 3 Prevention and control of leishmaniasis

Recent approaches to prevent and decrease transmission of parasitic and bacterial diseases include vector control and thus reducing human contact with infected vectors and reservoir control and thus reducing the number of infected animals (Gonzalez et al., 2015). Knowing the source of the disease greatly helps to get rid of it. The world health organization (WHO) has also suggested *Leishmania* control based on reservoir host and vector control (Lemma, 2008).

Managing reservoirs of pathogens plays a crucial role in effective disease control. Many emerging diseases of human, domestic animal, and wildlife populations are assumed to be maintained in reservoir hosts. The reservoir is the one epidemiologically connected

population in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population (Haydon et al., 2002). Effective reservoir host expected to be long-lived, at least surviving through any non-transmission season. A large proportion of individuals become infected during their lifetime; they remain infected for a long time without acute disease, and the parasites are in the skin or circulation, where they are "presented" to feeding sandfly vectors. Also the reservoir host(s) essential to the maintenance of parasite populations(Ashford, 1996). Several strategies were proposed to prevent leishmaniasis based on reservoir animals including poisonous baits for rodents eating seeds, removal of plants for rodents which feed on them, destruction of burrows, trapping (Gonzalez et al., 2015).

1.6 Microbial infections in rock hyrax:

Mycobacterium tuberculosis complex was isolated from a zoo resident rock hyrax (*Procavia capensis*), which are imported into Canada from South Africa (Lutze-Wallace et al., 2006). A case of extensive necrogranulomatous pneumonia has been reported in a free-living pregnant adult female from South Africa due to infection with the dassie bacillus (*Mycobacterium tuberculosis* complex *spp*.) (Parsons et al., 2008). Furthermore, tuberculosis was diagnosed in an adult female hyrax, and *M. microti* was isolated from the lung of an adult male hyrax in the same colony (Cousins et al., 1994). Tuberculosis due to *Mycobacterium africanum* has been diagnosed in an adult female hyrax, in animals that have been imported from the United Arab Emirates and were held in captivity at the Zagreb Zoo in Croatia (Gudan et al., 2008). Also a recent study has confirmed *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infections in three wild-born rock hyraxes that have been imported from South Africa to Germany (Fechner et al., 2017).

On the other hand, a previous study concluded that rock hyraxes are possible reservoirs for *Borrelia persica* and naturally been infected with this kind of spirochete, as they have gregarious habits, long life spans and share habitats with vector ticks(Kleinerman et al., 2018). Further, *Bartonella rochalimae* DNA was detected from rock hyraxes, which represent the first detection of zoonotic *Bartonella spp.* in rock hyraxes (Marciano et al., 2016).

In addition to diagnosis of hyrax with parasites and dangerous bacteria, they have also been diagnosed with viruses. Novel *alpha herpesviruses* have been identified in rock hyraxes. The molecular characteristics of this *herpesvirus* support its inclusion in the genus Simplex virus. This virus has caused focally extensive ulcerations in periorbital and perioral skin of the infected hyraxes (Galeota et al., 2009). All of previous discussed information indicates the susceptibility of hyraxes to carry many dangerous microorganisms, not only for hyrax itself but also to other animals and humans.

1.7 Rock hyrax infections: Molecular diagnostic approaches:

Previous studies have been done on hyraxes using different molecular techniques, like PCR, Restriction fragment length polymorphism (RFLP), kinetoplast DNA- polymerase chain reaction (kDNA PCR), acid-fast staining and culturing for a certain type of bacteria (Gudan et al., 2008; Jacobson et al., 2003; Lemma et al., 2009; Lutze-Wallace et al., 2006; Svobodova et al., 2006), nested polymerase chain reaction (nPCR) for identification of viruses (Galeota et al., 2009). All of these approaches are generally target-specific and thus lack the ability to detect unsuspected pathogens, also these techniques target only one gene in a single species. Furthermore, these methodologies need a pure isolation of the microorganism of interest, so as different bacteria have different growth requirements, this leads to a complex set of workflows for handling samples in the clinical microbiology laboratory (Pallen, 2014). In addition, the classical diagnostic techniques are unsuitable for several microorganisms presenting fastidious growth characteristics, low physiological and morphological specificity, and require a specific biosafety infrastructure; also these methods rely on microscopic examination with different staining methods, culture isolation.

On the other hand, PCR methods does have its limitations, false negative result which may due to sample inhibitors, and physical limits of detection associated with PCR, also false positive PCR results may be attributed to sample contamination or carryover contamination (Maurer, 2011).

1.8 Recent advances in molecular biological techniques for identification of pathogens:

Infectious diseases remain in the top five causes of mortality worldwide. Despite sophisticated research tools for microbial detection, rapid and accurate molecular diagnostics for identification of infection in humans have not been extensively adopted. Nowadays molecular testing allows for a large number of pathogens highly specific and sensitive identification from clinical isolates and specimens (Srinivasan et al., 2015). The ability of molecular techniques to identify of pathogens directly from clinical samples makes a rapid identification without recourse to culture possible (Barbut et al., 2011; Lehmann et al., 2008; Srinivasan et al., 2015). Metagenomics is a recent and rapidly developing field that attempts to analyze the genetic content of whole communities, without need for isolation and lab cultivation of individual species, it allows study of microbes in their own environments. However traditional techniques are limited in its ability to elucidate the microbial communities, understanding microbial communities require that the traditional techniques of pure culture be supplemented with new approaches. Metagenomics can in principle access 100% of the genetic resources of an environment (Council, 2007).

Metagenomics next-generation sequencing (NGS) is a comprehensive approach for sequence-based identification of pathogenic microbes, culture-independent screening for pathogens with NGS only needs a small amount of DNA directly taken from the sample and a bioinformatics tool, which identifies pathogens by linking sequence reads to an accurate reference genome database (Forbes et al., 2017; Li et al., 2018). Furthermore, sequence data have an important advantage, due to its quality, robustness and low noise (Buermans and Den Dunnen, 2014). Metagenomics sequencing is a promising solution for studying viral communities (Bergner et al., 2019), also, next-generation sequencing (NGS) can be used to identify fungal species (Alonso et al., 2018; Donovan et al., 2018). Shotgun sequencing produces massive amounts of data that already dwarf the data for existing genomic sequences in public databases (Kunin et al., 2008), from which we can detect and characterize pathogens present in clinical samples. In fact NGS permits an open view into the sequenced sample and allows screening for any nucleic acids associated with any organism in the sample (Andrusch et al., 2018).

Shotgun metagenomics is applied to the immediate sequencing of DNA extracted from a sample with no needs for culture or capture or target-specific amplification for the interested pathogens, metagenomics creates the promise of an open-ended, assumption-free one-size fits all workflow that could be applied to any kind of specimen, to identify any type of pathogen (bacteria, viruses, parasites and fungi). In the outbreaks of life-threatening infection with an unknown causes, diagnostic metagenomics help in a research setting and play a role in identifying the causes of unknown outbreaks and illnesses (Pallen, 2014).

1.9 Study aims and objectives:

Aim: To investigate the role of hyrax in transmission of diseases like leishmaniasis or other diseases that pose a threat to human life, i. e tuberculosis, borreliosis and bartonellosis.

Objectives:

1. To identify the parasite content of the hyrax.
2. To investigate the pathogenic bacterial content in the hyrax.
3. To delve into hyrax for the presence of viral and fungal metagenomes.
4. To study the correlation between cutaneous leishmaniasis and rock hyrax distribution in the selected Palestinian regions.
5. To identify possible suggestions on microbial control strategies based on reservoir system.

Chapter Two: Materials and methods

2.1 Study area and hyrax collection:

Rock hyraxes (n=16) were collected using raccoon traps in 2017. The study area included different localities in Palestine. Five hyraxes have been collected from A'nabta-Tulkarem , seven hyraxes from Haifa, two hyraxes from Faqqua-Jenin, one hyrax from areas next to Mas'ha village in Salfit, and one hyrax from Amara near Beer Al-Saba'a (Figure 2.1).

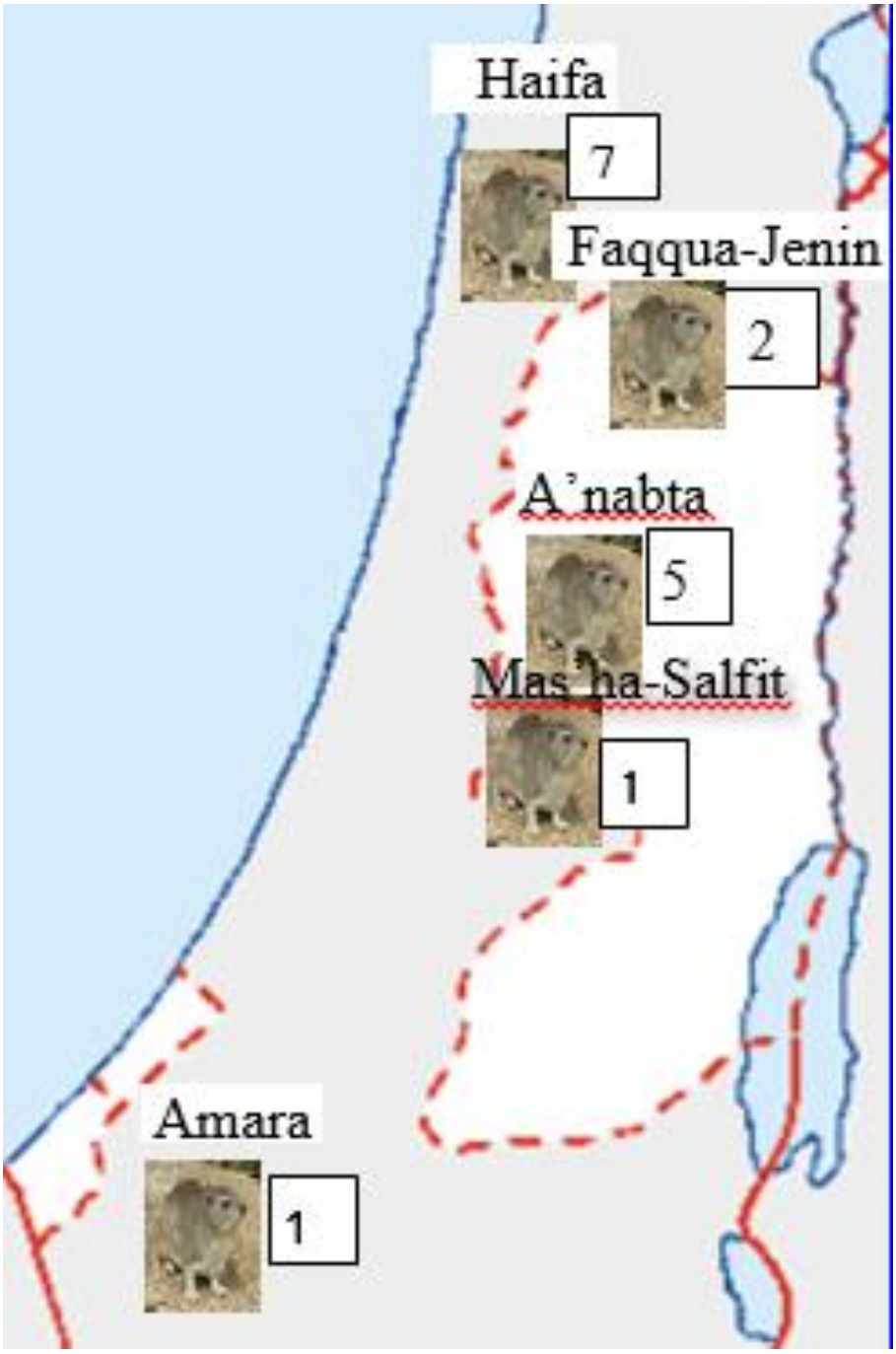


Figure (2.1): The Map of Palestine, showing the study area and the number of samples in the respective region.

2.2 Tissue sampling:

Spleen biopsies were taken from hyraxes after quelling by ministry of health officials. Nose and ear biopsies were taken from hyraxes after were anesthetized (10 mg/kg intramuscularly Ketamine-HCl, Ketaset) using a pole syringe. Different scissors and forceps were used to avoid cross contamination of samples. Spleen samples were dissected from field hyraxes (n=8) while samples of blood (n=3) and skin biopsies (n=5) (nose and ear) were taken from 8 hyraxes that released at the site of capture. Only one sample was taken for each hyrax. All tissue samples were kept at -20°C until use. Prior to DNA extraction, all tissue samples were thawed and homogenized in 1.5 ml microtubes in 50 ml NET-50 (define the buffer components briefly) buffer. Table (2.1) shows the locality and the type of tissue sample used in this study

Table 2.1: The region of hyrax that have been taken from, and the type of the sample that have been analyzed**.**

Hyrax code	Region	Type of sample	Hyrax code	Region	Type of sample
HAS1	A'nabta-Tulkarem	Spleen	**HHB1**	Haifa	Blood
HAS2	A'nabta-Tulkarem	Spleen	**HHB2**	Haifa	Blood
HAS3	A'nabta-Tulkarem	Spleen	**HHB3**	Haifa	Blood
HAS4	A'nabta-Tulkarem	Spleen	**HHE1**	Haifa	Ear
HAS5	A'nabta-Tulkarem	Spleen	**HEE2**	Mas'ha-Salfit	Ear
HHS6	Haifa	Spleen	**HME3**	Amara, Beer Al-Saba'a	Ear
HHS7	Haifa	Spleen	**HGN1**	Faqqua-Jenin	Nose
HHS8	Haifa	Spleen	**HGN2**	Faqqua-Jenin	Nose

2.3 DNA Extraction:

DNA was extracted from 10 mg of tissue samples and from 200µl of whole blood using the DNA extraction kit (QIAamp DNA Mini and blood Mini, Germany) according to the manufacture instructions. The detailed protocols for DNA extraction of both tissue and blood samples were described in appendix (1).

2.4 DNA Quantification:

DNA concentration was measured using spectrophotometer (Nanodrop 2000cThermoscientific). Elution buffer TE (Tris-EDTA buffer) was used as a blank, spectral measurement at 260nm and 280nm were also done and the ratio of (260/280 nm) were calculated to assess the purity of DNA samples. DNA concentration was adjusted to 0.4 ng/µl for DNA library preparation.

2.5 DNA library preparation:

DNA libraries (Process of generating a collection of DNA fragments for sequencing by fragmenting a genomic DNA and ligating specialized adapters to both fragment ends) were prepared using Nextera® XT DNA Library Prep Reference Guide (Illumina), according to the manufacture instruction. Briefly, Tagment DNA Buffer (10µl) was added to 5µl normalized gDNA (0.4 ng/µl), followed by addition of 5 µl of Amplicon Tagment Mix which contains transposomes (Enzymes used to fragment and insert adapters into DNA) to each sample. The reaction mixture was incubated at 55°C for 5 minutes and then kept at 10℃ to stop the action of transposomes. Neutralize Tagment Buffer (5µl) was added to each sample for a total volume 25µl. for barcoding of each sample, 5 µl of 2 indexes (10uM); Index 1 (i7) adapter and 5µl of Index 2 (i5) adapter were added followed by addition of 15µl Nextera PCR Master Mix to a final volume of 50µl, The reaction mixture was subjected to a thermal cycling as followed: 72℃ for 3 minutes, 95℃ for 30 seconds, then 12 cycles of: 95℃ for 10 seconds, 55℃ for 30 seconds, 72℃ for 30 seconds, then 72℃ for 5 minutes, and hold at 10℃. The principle of Metagenomics and nextera assay was shown in the schematic figure 2.2.

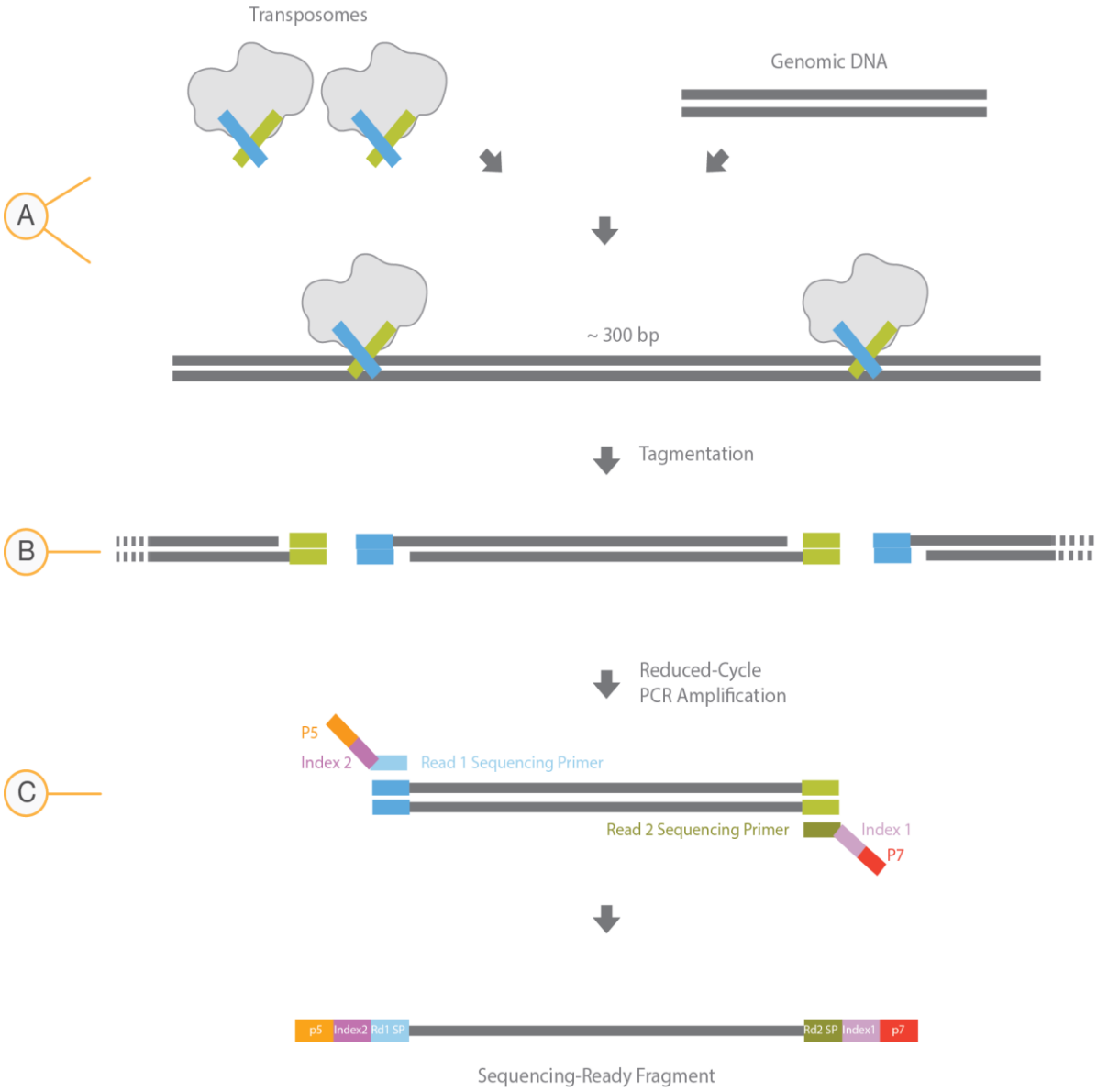


Figure 2.2: Principle of Metagenomics and nextera assay. (**A)** Nextera XT transposome with adapters combined with template DNA, (**B)** Tagmentation to fragment and add adapters, (**C)** Limited-cycle PCR to add index adapter sequences. (Nextera® XT DNA Library Prep Reference Guide) (Illumina).

2.6 Clean-up of DNA libraries:

DNA library clean-up (Removeal of contaminants and shorter DNA fragments from a sample) was done as follows:

25µl of AMPure XP beads were added to 50µl of each samples and incubated at room temperature for 5 minutes. The samples were placed on a magnetic stand until the liquid cleared (~2 minutes), the supernatant was transferred to a new tube. Another 35µl of AMPure XP beads were added to the supernatant. The samples were placed on a magnetic stand until the liquid cleared, all supernatant were removed and discarded from tubes. Freshly prepared 80% ethanol (180µl) was used to wash the pellets two times.

The supernatants were discarded again and the pellets were left on the magnetic stand for 15 minutes for air drying. The samples were removed from the magnetic stand and 25µl of elution buffer were appended to each sample and incubated at room temperature for 2 minutes, subsequently tubes were placed on a magnetic stand until the liquid become clear (~2 minutes), then 22µl of the supernatant (DNA) were transferred to new tubes and stored at -20 °C until sequencing. The flowchart for DNA library preparation was shown in Figure 2.3.

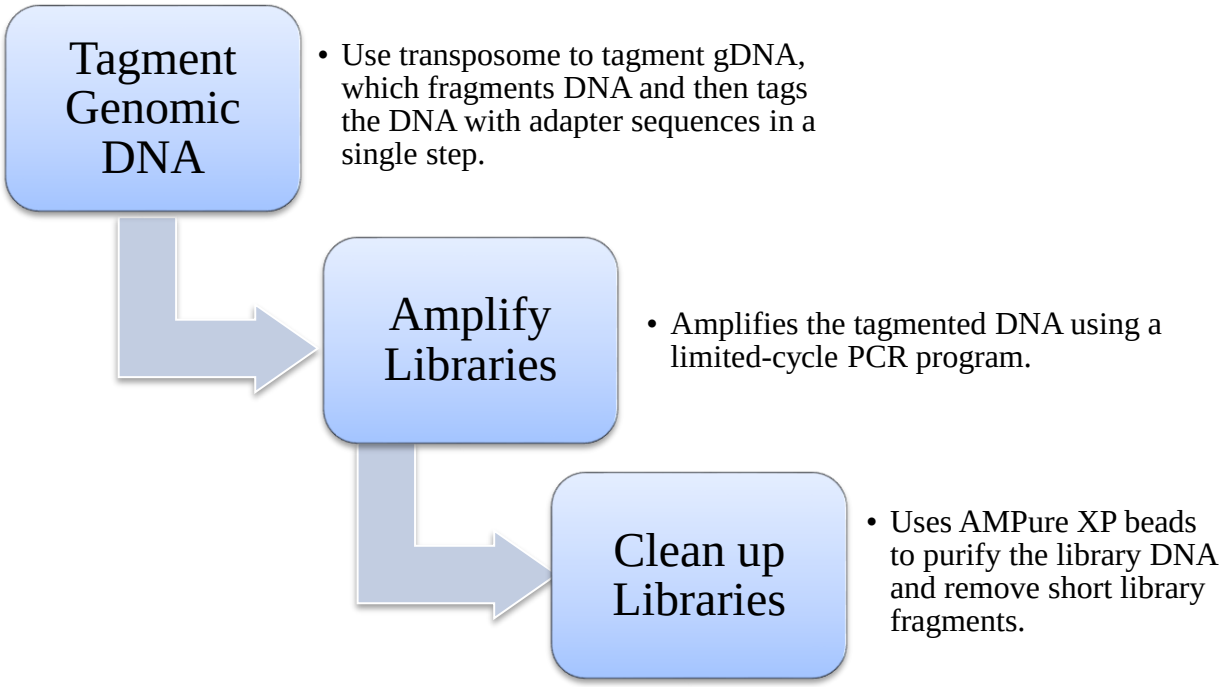


Figure (2.3): Library Preparation flowchart.

2.7 DNA Sequencing:

All DNA libraries were deeply sequencing. DNA deep sequencing was performed at the Hylabs sequencing service- Jerusalem. DNA libraries were normalized, pooled at equimolar concentration, denatured using 0.2 NaOH, and mixed with PhiX control. DNA library was loaded on MiSeq machine using 150 cycle miseq kit (Illumina).

2.8 Metagenomics data and Bioinformatics:

The analysis of hyrax DNA sequences was performed by Galaxy software (https://usegalaxy.org/).The bioinformatics analysis consisted of 19 steps (input dataset) was outlined in Figure 2.4. The workflow built by Galaxy software (https://usegalaxy.org/) was described in appendix 2 . The analysis was initialized by uploading of sequence files to the website, the sequences were subjected to processing follows:

1. We started with a typical output of miseq (Illumina), a read file in FASTQ format and reads produced by sequencing were subjected to basic trimming, to remove unidentified nucleotides designated as 'N' in the sequence from the 3' end of read 1 after adapter (data sets 1 and 2).
2. High-quality segments were selected from each read (data set 3). One is primarily concerned with the overall base quality and the extent of read segmentation due to low quality base calls. A high-quality segment is defined as a run of nucleotides of length L where all bases have the phred quality value above Q. Phred means the quality score of a base and representing the estimated probability of an error. The minimum quality for selected sequences was 20 Phred, while the minimum size was 50 NT.
3. Next, sequence artifact were removed, this tool filtered sequencing artifacts. Then, sequence reads were collapsed, which allowed confirmation of identical sequences in a FASTA file into a single sequence (data sets 4 and 5).
4. Sequences in a FASTA format were converted to Tabular format (data set 6), this convert to tabular format so that column for additional metadata were added.
5. A column were added, this column for storing where the data came from (data set 7).
6. Sequences in Tabular format were converted back to FASTA format for more analysis (data set 8).

7. The sequences were renamed and a Galaxy MegaBLAST tool was used to compare the reads with nucleotide (NT) and whole genome sequencing (WGS) databases (data sets 9 and 10), hits above 97% identity were selected.

8. While MegaBLAST searches are running, the length of sequences were computed (data set 11), this data were used later for filtering suboptimal sequences.

9. Results of the two MegaBLAST runs (NT and WGS) were merged (data set 12), this merge MegaBlast was run to produce a single dataset for reads compared to both WGS and NT. Sequences were joined with data set 13 which contains sequences length to combine sequence length data with results from MegaBlast runs.

10. The resulting data set (14) contained all columns from data sets 12 and 13, including alignment length (column 5) and sequencing read length (column 15). From this data set, it is simple to filter suboptimal hits by retaining those reads that satisfy the $c5/c15 \leq 0.5$ condition (value in column 5, alignment length divided by the value in column 15, read length) to data set 14. Herein, we defined the suboptimal hits as those in which an alignment between metagenomics read and a database entry covers less than 50% of the reads.

11. Taxonomic representation for filtered and aligned sequences using the "fetch taxonomic representation tool" (data set 15) was obtained. An application of the "find lowest diagnostic rank" tool give reads specific to ranks below Kingdom level produced data set 16.

12. Finally, Krona pie chart was built and a phylogenetic tree from the list of ranks (data set 17 and 19) and tabulate a list of taxonomic groups was obtained (data set 18).

All data sets and tools described in this manuscript are available at the test Galaxy server at http://usegalaxy.org.

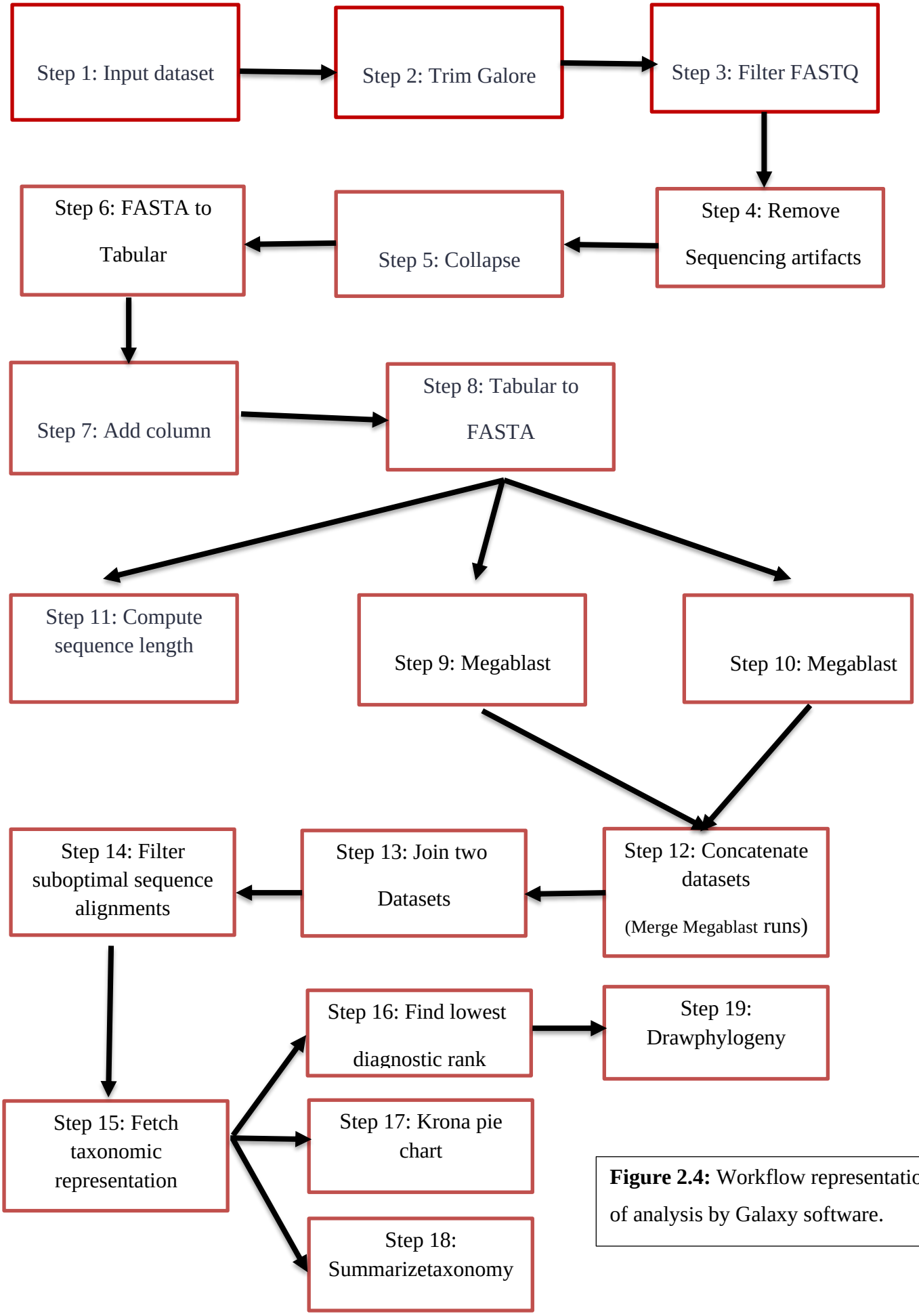


Figure 2.4: Workflow representation of analysis by Galaxy software.

2.9 Retrieval of DNA Sequences:

To retrieve the sequences of each pathogenic microorganism from Galaxy, which is an important step to verify and increase credibility of the workflow of analysis. The Galaxy tool was also used to retrieve these sequences. The first step was to select lines that match an expression, then the name of microorganism was entered in the pattern with regular expression, the selection was done on fetch taxonomic representation on data 13 (the step 15 in the workflow).After that the results were used for next step, also selection of lines that match an expression was applied. The number of reads were entered in the pattern we search for. , The selection was done on add column on data 5 (the step 6 in the workflow). Finally, Tabular file was converted to FASTA format which is ready to use in blast analysis. Tools described in this manuscript are available at the test Galaxy server at (https://usegalaxy.org/). Appendix (2).

2.10 BLAST analysis:

BLASTn search was performed for the sequences of interest compared to the reference genome databases using https://blast.ncbi.nlm.nih.gov/Blast.cgi. The identity, (the percentage for a set of aligned segments to the same subject sequence) was ≥97%. The query coverage (the percent of the query length that is included in the aligned segments) was ≥97%, which has been marked for reference.

For species identification, the sequences obtained from DNA library were compared to the different species present in the BLASTn. The maximum number of aligned sequences to display were 20,000 sequences. Multalin Multiple sequence alignment tool (http://multalin.toulouse.inra.fr/multalin/) was used for screening of DNA sequence variation and single nucleotide polymorphisms (SNPs).

In this study, we focused on the pathogenic microorganisms, therefore, all sequences for each pathogenic organism were extracted, blasted on https://blast.ncbi.nlm.nih.gov/Blast.cgi sequence similarity tools, to identify homologs in reference sequences and to compare each read with all sequences in GenBank. For every sequence : the query cover, identity and the accession number were acquired to confirm the presence of these organisms based on database.

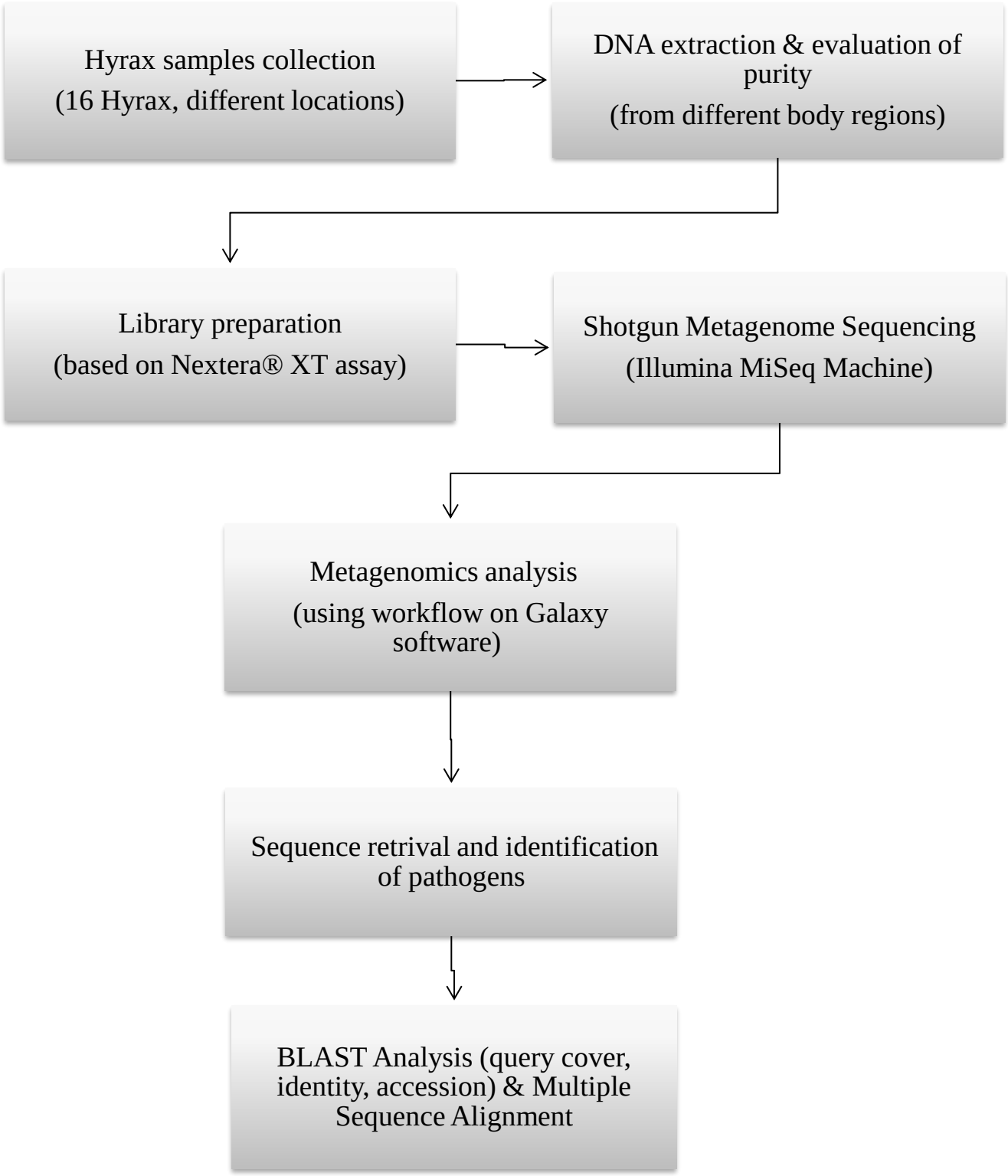


Figure 2.55: Analysis procedures for metagenomics data.

2.11 Ethical considerations:

The study procedures was approved by the research ethics committee of Al-Quds public health society- East Jerusalem, Palestine.

Chapter Three: Result

3.1 Distribution of hyraxes by locality and sample types

In 2017, 16 hyraxes were collected from five different Palestinian regions (Haifa, A'nabta-Tulkarem, Amara near Beer Al-Saba'a, Faqqua-Jenin and Mas'ha-Salfit). Seven hyraxes were collected from Haifa (44%), five hyraxes from A'nabta-Tulkarem (31%), two hyraxes were collected from Faqqua-Jenin (13%), one hyrax from Amara near Beer Al-Saba'a (6%), and one hyrax Mas'ha-Salfit (6%), as shown in figure 3.1.

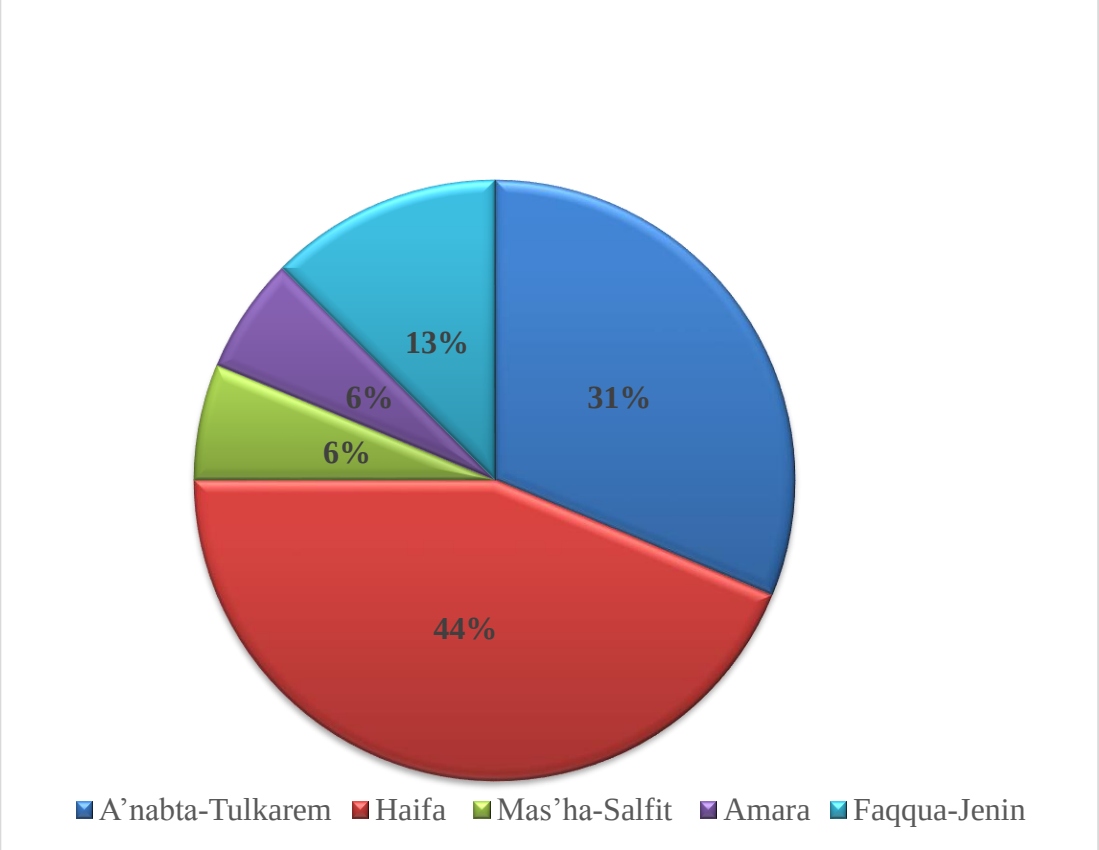


Figure 3.1: The distribution of hyrax by locality.

Samples were collected from four different body regions. However, the majority of samples were obtained from the spleen (n=8), followed by blood (n=3), ear tissue (n=3), and nasal tissue (n=2). Only one sample was obtained from each hyrax.

3.2 Bioinformatic analysis and BLAST search result

The microbial community composition in hyrax samples was assessed using two strategies: metagenomics and BLAST analysis. Each sample was tested for the presence of the pathogenic parasites, bacteria, viruses and fungi.

Among the collected 16 hyraxes, two samples failed to give any sequence after sequencing procedure and another two samples had only eukaryotic sequences belonging to hyrax DNA. These four samples were excluded from the analysis process. The excluded hyraxes were two samples from A'nabta-Tulkarem (Spleen), one sample from Haifa (blood) and one sample from Faqqua-Jenin (nasal tissue). Thus, 12 samples were included in this study.

3.3.1 Sequencing reads

The number of reads generated were 33,515,321 reads, with 2.8 million reads being sequenced per sample on average. Around 92% of the reads were mapped to the hyrax genome, while 2.28 % of the reads were unmapped. Microbial reads consisted 5.75% of the total reads. Most of the taxonomically classified sequence reads corresponded to hyrax genome sequences and hyrax DNA exceeded microbial DNA. Most of identified microbes are part of the normal flora associated with hyrax body. Furthermore, in total, 634290 reads were shared between all hyrax samples analyzed and Elephantidae family (Appendix 4).

3.3.2 Parasitic finding

In this study, *Leishmania* parasites were identified in four hyrax samples (33.3%). The remaining eight hyraxes (66.6%) were negative for the presence of *Leishmania* parasites as revealed by metagenomics and BLAST analysis. No other pathogenic parasites were found in all hyraxes samples collected in this study.

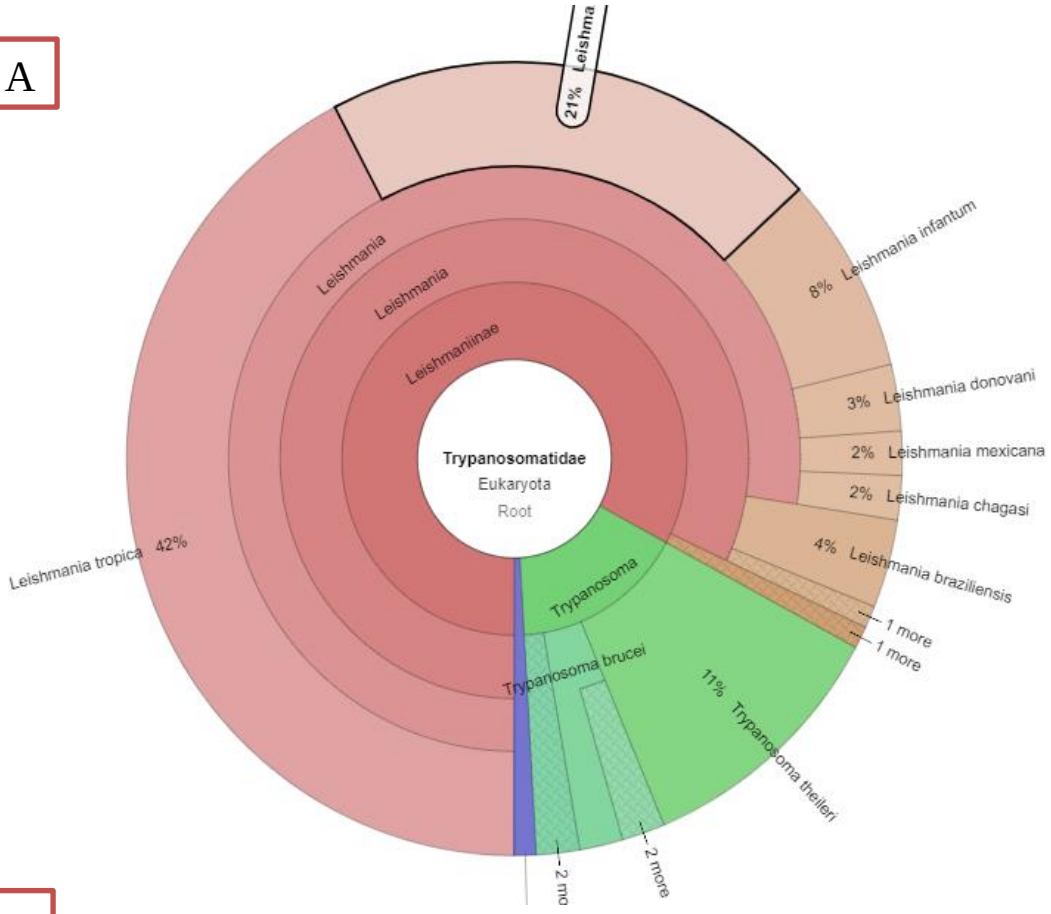
Leishmania parasites were detected with different number of reads ranging from 40 reads to 516 reads as shown in Table 3.1). All sequences identified by metagenomics analysis were confirmed by BLAST analysis. Despite the low number of *Leishmania* reads in the four samples, the BLAST search showed high sequence identity (>97%) and high query cover (>97%) confirming their identity (Figure 3.2).

These *Leishmaina* positive samples (n=4) were collected from two different localities A'nabta-Tulkarem and Haifa. The samples from three hyraxes from A'nabta-Tulkarem were obtained from spleen, the sample from the fourth hyrax from Haifa was obtained from ear (Table 3.1). The sequences obtained from deep sequencing were aligned to the reference sequences obtained by BLAST analysis using the multiple alignment tool (http://multalin.toulouse.inra.fr/multalin/). The species of *Leishmania* in the positive sample were identified as *Leishmania tropica* in three hyraxes collected from A'nabta-Tulkarem, while *Leishmania spp* was identified in the fourth positive hyrax collected from Haifa (Figure 3.3). Identification of *Leishmania spp*. was based on the highest scoring BLAST hit in the GenBank with 97–100% sequence identity and 100% coverage as shown in table 3.2.

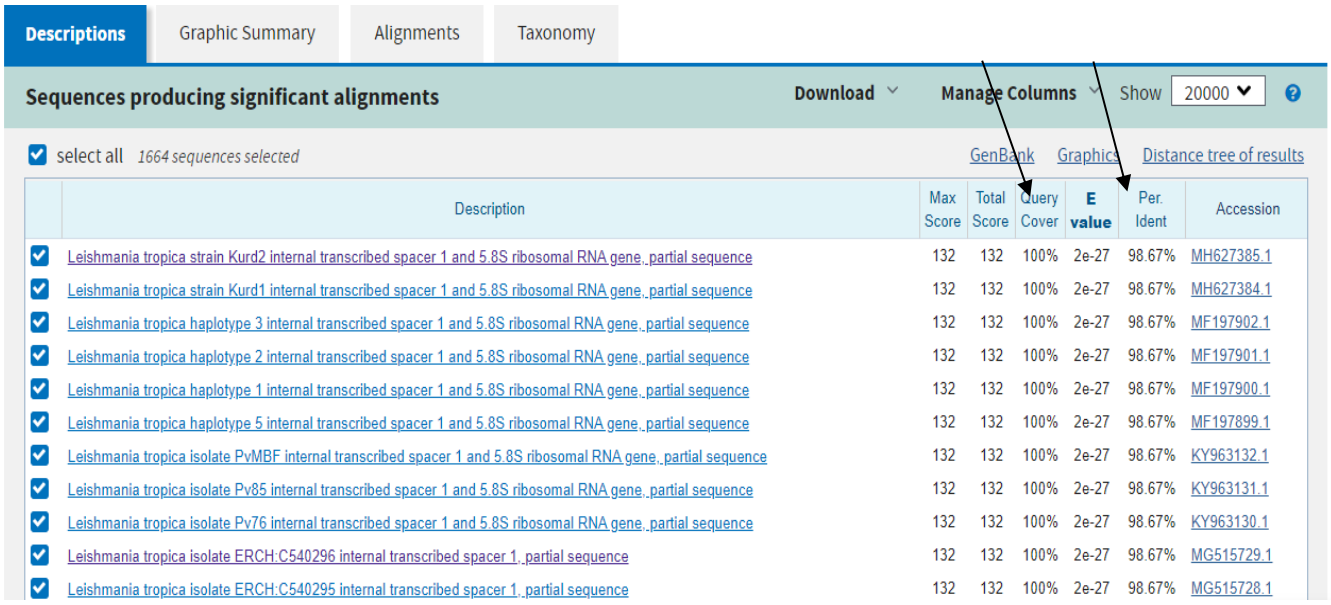
Table 3.1: Detection of *Leishmania* in hyrax samples.

Hyrax code	**Region (locality)**	***Leishmania* species**	**Type of sample**	**Number of reads**
HHE1	Haifa	*Leishmania spp.*	Ear	40
HAS3	A'nabta-Tulkarem	*Leishmania tropica*	Spleen	516
HAS4	A'nabta-Tulkarem	*Leishmania tropica*	Spleen	156
HAS5	A'nabta-Tulkarem	*Leishmania tropica*	Spleen	184

A



B



Descriptions | Graphic Summary | Alignments | Taxonomy

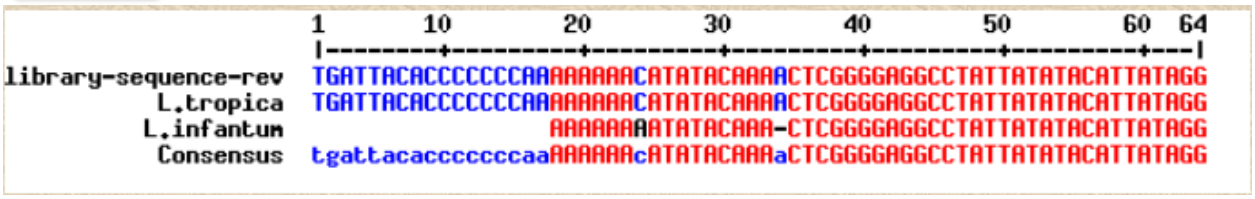
Sequences producing significant alignments Download Manage Columns Show 20000

select all *1664 sequences selected* GenBank Graphics Distance tree of results

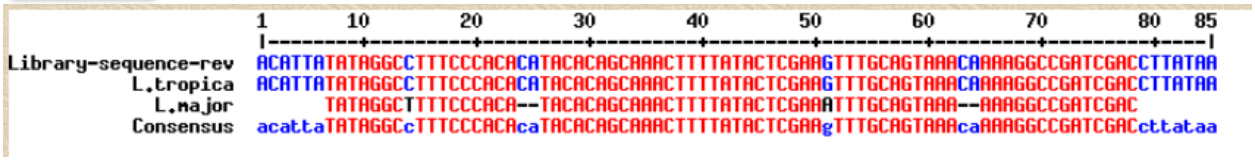
Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Leishmania tropica strain Kurd2 internal transcribed spacer 1 and 5.8S ribosomal RNA gene, partial sequence	132	132	100%	2e-27	98.67%	MH627385.1
Leishmania tropica strain Kurd1 internal transcribed spacer 1 and 5.8S ribosomal RNA gene, partial sequence	132	132	100%	2e-27	98.67%	MH627384.1
Leishmania tropica haplotype 3 internal transcribed spacer 1 and 5.8S ribosomal RNA gene, partial sequence	132	132	100%	2e-27	98.67%	MF197902.1
Leishmania tropica haplotype 2 internal transcribed spacer 1 and 5.8S ribosomal RNA gene, partial sequence	132	132	100%	2e-27	98.67%	MF197901.1
Leishmania tropica haplotype 1 internal transcribed spacer 1 and 5.8S ribosomal RNA gene, partial sequence	132	132	100%	2e-27	98.67%	MF197900.1
Leishmania tropica haplotype 5 internal transcribed spacer 1 and 5.8S ribosomal RNA gene, partial sequence	132	132	100%	2e-27	98.67%	MF197899.1
Leishmania tropica isolate PvMBF internal transcribed spacer 1 and 5.8S ribosomal RNA gene, partial sequence	132	132	100%	2e-27	98.67%	KY963132.1
Leishmania tropica isolate Pv85 internal transcribed spacer 1 and 5.8S ribosomal RNA gene, partial sequence	132	132	100%	2e-27	98.67%	KY963131.1
Leishmania tropica isolate Pv76 internal transcribed spacer 1 and 5.8S ribosomal RNA gene, partial sequence	132	132	100%	2e-27	98.67%	KY963130.1
Leishmania tropica isolate ERCH:C540296 internal transcribed spacer 1, partial sequence	132	132	100%	2e-27	98.67%	MG515729.1
Leishmania tropica isolate ERCH:C540295 internal transcribed spacer 1, partial sequence	132	132	100%	2e-27	98.67%	MG515728.1

Figure 3.2: Bioinformatics analysis of *Leishmania*. **(A)** A Krona pie chart showing the abundance of taxa. The higher taxonomic levels are in the center and the lower taxonomic levels are in the outer circle. This pie chart showing the presence of *Leishmania* in a hyrax samples as revealed by Galaxy software.**(B)** An example of positive *Leishmania tropica* sequence on BLAST tool based on identity and query coverage cover**.**

HAS3



HAS4



HAS5

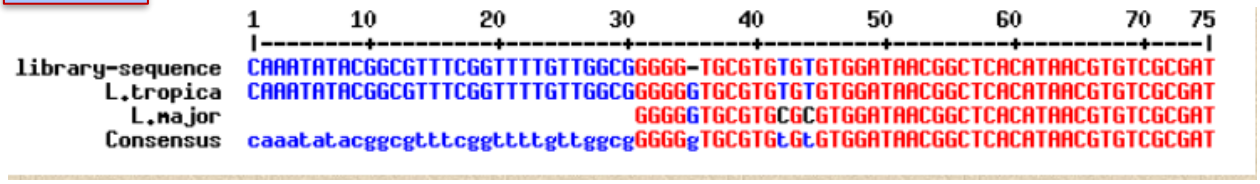


Figure 3.3: Species identification results. Multiple alignments showed the species of *Leishmania* in the positive samples: *L.tropica* was identified in three hyraxes (HAS3, HAS4 and HAS5). Red color represents highly conserved residue; Blue color represents weakly conserved residue.

Table 3.2: The nucleotide sequences, % query cover, % identity, and accession number of the detected *Leishmania* in the hyrax samples as revealed by BLAST analysis.

Hyrax code	Query cover	Identity	Accession number/ gene /locus	Sequence
HHE1	100%	98%	>CP027811.*1 Leishmania infantum* strain TR01 isolate Lin_TR01 chromosome 13	CCCGCACAACGGTGTGAAGC CGATCCCGTGTAAGACGTGC GGTCAGCCGATCTTCATTGG CGACCGACACGTGTGCAAGC GCCAGCTGGCGGTCTGCCCC CACTGTAGGCAGGATG
	98%	97%	>CP027825.1 *Leishmania infantum* strain TR01 isolate Lin_TR01 chromosome 26	CCCTAAGGTGACGTACTAGA CGCCGTTCAATCGTCAAGGG AGCAGAGCTCACAAGCAGAG AGTGTGCT
	100%	100%	>CP027827.*1 Leishmania infantum* strain TR01 isolate Lin_TR01 chromosome 28	CGGATGGACGGAGGAAGGG AAAGCTGTGGGGTACGTGTG TGTGTGTGGTGAG
	100%	98%	>FR796426.1 *Leishmania major* strain Friedlin complete genome, chromosome 30	CTCCGCTTCCCCTCTCGTGGT GCCGCCTGTTGTTGCCCTCTA CAGCTTTATCCTTGTG
	100%	100%	>CP027827.1 *Leishmania infantum* strain TR01 isolate Lin_TR01 chromosome 28	CGCCACGCTTCCTTTGTCCTC GGAAGTTGCCGAGACGAGTG AGCCGCCAACCAGGAAGAGC AACACCTCCCTGGCCCTCTCA TCCCAAGCGCGAGATA
	100%	97%	>FR796426.1 *Leishmania major* strain Friedlin complete genome, chromosome 30	GACCTGCACAGAGGGGCGAG GGCGGAAAAGAGCAAGGAA GGGATTCGGCAGAAACGCCA CGCGCCGTGGACAGAGA

HAS3	100%	100%	>MH347928.1 *Leishmania tropica* isolate Sanliurfa_MHOM/SR/2015/HRURFA016 internal transcribed spacer 1 and 5.8S ribosomal RNA gene	CCTATAATGTATATAATAGGCCTCCCCGAGTTTTGTATATGTTTTTTTTGGGGGGGTGTAATCA
HAS4	100%	100%	>MG515729.1 *Leishmania tropica* isolate ERCH:C540296 internal transcribed spacer 1	TTATAAGGTCGATCGGCCTTTTGTTTACTGCAAACTTCGAGTATAAAAGTTTGCTGTGTATGTGTGGGAAAGGCCTATATAATGT
HAS5	100%	99%	>MH627385.1 *Leishmania tropica* strain Kurd2 internal transcribed spacer 1 and 5.8S ribosomal RNA gene	CAAATATACGGCGTTTCGGTTTTGTTGGCGGGGGTGCGTGTGTGTGGATAACGGCTCACATAACGTGTCGCGAT

3.3.3 Bacterial findings:

In this study, we focused on pathogens that may lead to disease outbreaks or can lead to serious illness to the human. The study detected the presence of pathogenic bacteria in six hyrax samples (50%) by metagenomics and BLAST analysis, while the remaining six hyraxes (50%) were free from presence of pathogenic bacteria.

The analysis results indicated the detection of three pathogenic bacteria: *Mycobacterium tuberculosis complex, Neisseria meningitides* and *Bordetella pertussis*. *Mycobacterium tuberculosis complex* were the most commonly identified pathogenic bacteria in hyrax samples, which have been detected in three hyraxes (25%). The positive samples were collected from three different localities; Haifa, Faqqua-Jenin and Amara near Beer Al-Saba'a (Table 3.3). The samples were obtained from spleen, nose and ear. The number of reads ranged from 84 to 332. (Appendix 5).

Bordetella spp. has been detected in the spleen of one hyrax (8.3%) captured from A'nabta-Tulkarem with high number of reads (75704). (Appendix 6). *Neisseria meningitides* have been also detected in the spleen of two hyraxes (16.6%) in A'nabta-Tulkarem with number of reads 16572 and 1488 respectively. (Appendix 7). Identification of bacteria spp. was based on the

highest scoring BLAST hit in the GenBank, 97–100% sequence identity and 100% coverage as shown in Figure 3.4 and Table 3.4. Multiple alignment of representative bacterial sequences that obtained by deep sequences against reference sequences obtained by Blast search are shown in Appendix 10.

Table (3.3): Detection of *Mycobacterium tuberculosis complex spp, Bordetella spp, Neisseria spp* in hyrax samples

Hyrax code	Pathogenic bacteria	Region (locality)	Type of sample	Number of reads
HHS8	*Mycobacterium tuberculosis complex*	Haifa	Spleen	230
HGN2	*Mycobacterium tuberculosis complex*	Faqqua-Jenin	Nose	332
HME3	*Mycobacterium tuberculosis complex*	Amara near Beer Al-Saba'a	Ear	84
HAS5	*Bordetella spp.*	A'nabta-Tulkarem	Spleen	75704
HAS3	*Neisseria meningitides*	A'nabta-Tulkarem	Spleen	16572
HAS4	*Neisseria meningitides*	A'nabta-Tulkarem	Spleen	1488

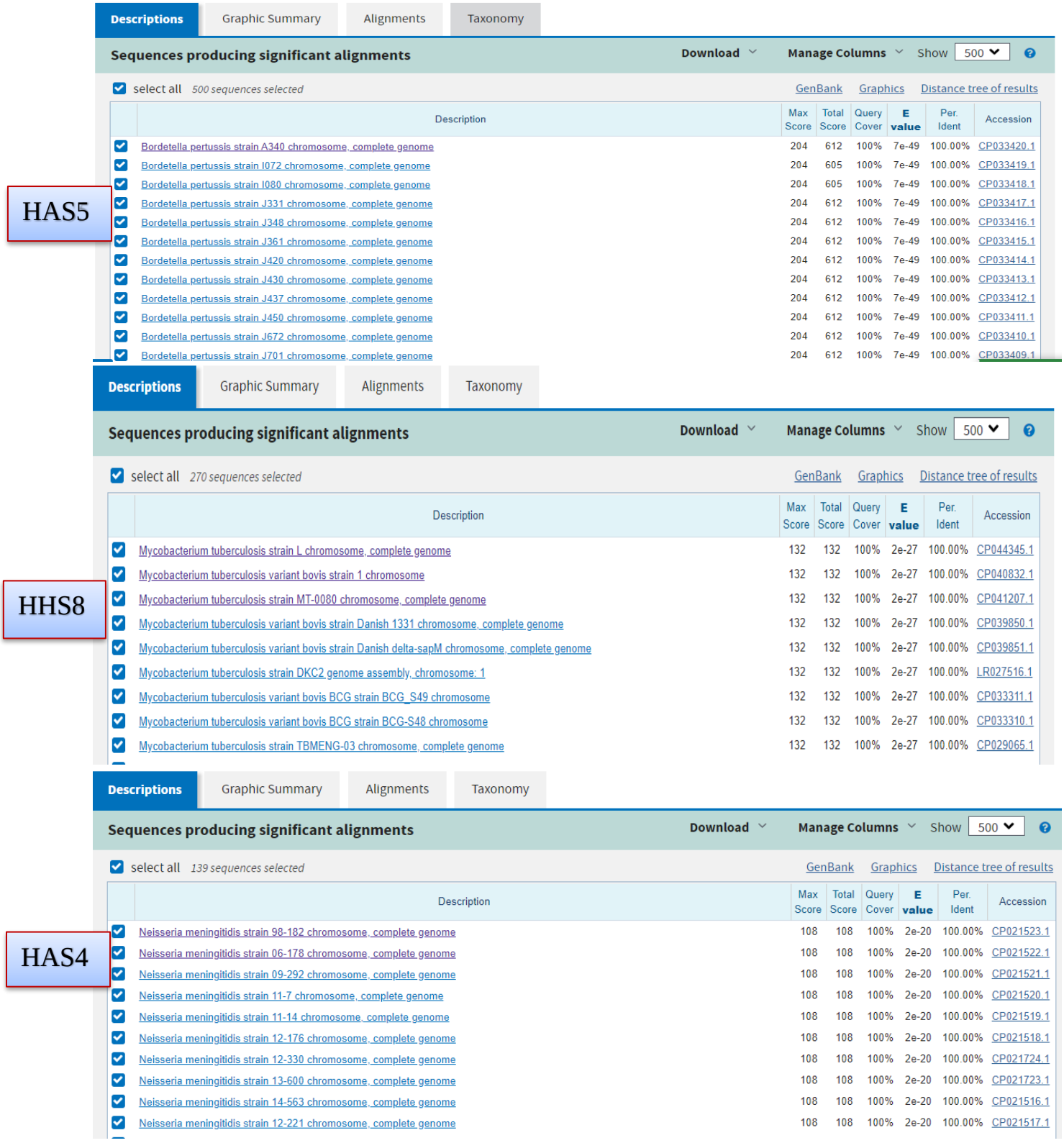


Figure 3.4: *Bordetella spp.*, *Mycobacterium tuberculosis complex* and *Neisseria meningitides* results of sequence search on BLAST tool based on identity and query coverage cover**.**

Table 3.4: The nucleotide sequences, % query cover, % identity, and accession number with probable species identification as revealed by BLAST search.

Hyrax code	Pathogenic Bacteria	Query cover	Identity	Accession number/gene /locus	Sequence
HHS8	*Mycobacterium tuberculosis complex*	100%	100%	>CP029065.1 *Mycobacterium tuberculosis* strain TBMENG-03 chromosome	GCCGATGGATGCGCCGGATGGTCGACGTCAAAGCCGCGAGTGCGAGAAGCGAGCCCGGGGAGCCGGTAGGC
		100%	100%	>CP029065.1 *Mycobacterium tuberculosis* strain TBMENG-03 chromosome	GGTTCCGCTATTAGAATTCGCGGTTGTCTTGGAACTGGCGATACTCAGTATTGGTTGGATTATTTCGAAT
		100%	100%	>CP029065.1 *Mycobacterium tuberculosis* strain TBMENG-03 chromosome	CTTTTGGACAAAGTCCTACGGGTGATTCGCCAGATGTGGTGGCGGTGTGGCTTGGGGACCTGGTCGCTCATACC
HGN2	*Mycobacterium tuberculosis complex*	100%	100%	>CP029065.1 *Mycobacterium tuberculosis* strain TBMENG-03 chromosome	GGGTCGGTTCATCGGTAGTCCGCCAGCTTCAGCCGTGAGGAAGACATCACAGTGGTTACTC
HME3	*Mycobacterium tuberculosis complex*	100%	100%	>CP029065.1 *Mycobacterium tuberculosis* strain TBMENG-03 chromosome	CATCCGAGAATGGGGTTGAAGCCGTGCTCGCGTTGCAGCTTTTGCATTTCGAGCGCCATCCGCTGACG
HAS5	*Bordetella spp.*	100%	100%	>CP031114.1 *Bordetella pertussis* strain UK76 chromosome	GGCTTGTAAAGGCTGCTGGAGGTATCAGAAGTGCGAATGCTGACATGAGT
		100%	98%	>CP021111.1 *Bordetella* genomo sp. 13 strain AU7206	GCTTCGACGCCTTCAAGGAAGCCTTCAACAAGTCGGCCGCCGGCAACTTCGGTTCGGGC
		100%	100%	>CP031114.1 *Bordetella pertussis* strain UK76 chromosome	GAAGTAGTTAGCCTAACCGCAAGGGGGGCGATTACCACGGTAGGATTCATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTT

		100%	99%	>CP031114.1 *Bordetella pertussis* strain UK76 chromosome	CCTAAGGCGAGGCAGAG ATGCGTAGCTGATGGGA AGCTGGTTAATATTCCA GCACCGTCGTACAGTGC GATGGTGGGACGGATCG CGGAAG
		100%	97%	>CP028901.1 *Bordetella* sp. HZ20 chromosome	TCCTTGTGGGGTGACTG CGTACCTTTTGTATAATG GGTCAGCGACTTACATT CAGTGGCAAGGTTAACC GAATAG
		100%	100%	>CP031114.1 *Bordetella pertussis* strain UK76 chromosome	GCGTAACGATGGCCACA CTGTCTCCTCCTGAGACT CAGCGAAGTTGAAGTGT TTGTGAT
		100%	96%	>CP012334.1 *Bordetella* sp. H567	ACCAGGAACACCAGGTT CGGGTCATAGGTGAACA GGGCGGTGCCGCGCAGC GGCTTCATCTGCACCAT GTTCAGGTTGC
		100%	100%	>CP021111.1 Bordetella genome spp. 13 strain AU7206 chromosome	CCTGCGCGCTTAGCATG ATGGGGTCGCAGAGAAT CGGTGGCTGCGACTGTT
		100%	100%	>CP031114.1 *Bordetella pertussis* strain UK76 chromosome	CCTTCCGATACGGCTAC CTTGTTACGACTTCACCC CAGTCATGAATCCTACC GTGGTAATCGC
		100%	100%	>CP031114.1 *Bordetella pertussis* strain UK76 chromosome	GGCTCGAACCAGCGACC TGCGGATTAACAGTCCG TCGCTCTACCGACTGAG CTATCGGGG
		100%	98%	>CP033420.1 *Bordetella pertussis* strain A340 chromosome	GACCTGCGGATTAACAG TCCGTCGCTCTACCGAC TGAGCTATCGGGGAACA GGTAGAG
		100%	100%	>CP031114.1 *Bordetella pertussis* strain UK76 chromosome	TTGCTATACTGGCCCCG TTTTCCTGATATGCGGC GGGGGTTCGCTGCGAGT CAC
		98%	100%	>CP021110.1 *Bordetella* genome sp. 9 strain AU14267	GTCGGTTAGAGCACCGT CTTGATAAGGCGGGGGT CGTTGGTTCGAATCCAA CCAGACCCACCAAGTA
		100%	100%	>CP031114.1 *Bordetella pertussis* strain UK76 chromosome	GTTAGCCTAACCGCAAG GGGGGCGATTACCACGG TAGGATTCATGACTGGG GTGAAGTCGTAACAAGG TAGCCGTATCGGAAGG
HAS3	*Neisseria meningitides*	100%	100%	>CP031327.1 *Neisseria meningitides* strain M27477 chromosome	ATCTGTACTGTCTGCGG CTTCGTCGCCTTGTCCTG ATTTTTGTTAATCCACTA TAAAGC

		100%	98%	>CP021725.1 *Neisseria meningitides* strain 95-134 chromosome	CTTTAAATCAGGACAAG GCGACGAAGCCGCAGAC AGTACAAATAGTACGGC AAGGCGAGGCAACGCC GTACTGGTTTAAAGTTA ATCCACTATATAT
		98%	100%	>CP016883.1 *Neisseria meningitides* strain M22790 genome	CCCACTGTTTGTATGCAT CAGGTTTCAGGTTCTATT TCACTCCCCTCCCGGGG TTCTTTTCGCCTTTCCCT CACGGTAC
		100%	98%	>CP016883.1 *Neisseria meningitides* strain M22790 genome	GCTATGATCCCACGATG TCCGAATGGGGAAACCC ACTGCATTCTGTGCAGT ATCCTAAGT
		100%	100%	>CP031327.1 *Neisseria meningitides* strain M27477 chromosome	TAGTTTTAGGTTGAACTT TCAAGCATACGCCAAGA GAATTAACGATGCAAAG GCAATG
		100%	100%	>CP031327.1 *Neisseria meningitides* strain M27477 chromosome	ATCTGTACTGTCTGCGG CTTCATCGCCTTGTCCTG ATTTTTGTTAATCCACTA TAAC
		100%	100%	>CP031327.1 *Neisseria meningitides* strain M27477 chromosome	GGTGCACCTGTTCTTAG CTTCCATAGTATGGCTTC CATCACGGTACGGTCGT TCTTCCATTGATGACAG C
		100%	100%	>CP031327.1 *Neisseria meningitidis* strain M27477 chromosome	ATCCACAGCTCATCCCC GCATTTTGCAACATGCG TGGGTTCGGTCCTCCAG TAC
HAS4	*Neisseria meningitides*	100%	100%	>CP031327.1 *Neisseria meningitides* strain M27477 chromosome	AAATCAGAGATAAAGCG ATAGAACACGGTATGAA AGCTATTATACCAAAG
		100%	100%	>CP031327.1 *Neisseria meningitides* strain M27477 chromosome	ATCTGTACTGTCTGCGG CTTCGTCGCCTTGTCCTG ATTTAAATTTAATCCACT ATAT
		100%	100%	>CP031324.1 *Neisseria meningitides* strain M23347 chromosome	ATAATGTAATGGGTTCT CTTACTGTGGGTTATTCA TAATCAATCTAACGT
		100%	98%	>AM889136.1 *Neisseria meningitides* alpha14 complete genome	ATCCACAGGTAAAGCGT GTTTCTTGACAGGTTAA ACGTTGCTGCGGTTTGG CTGATGTTTTTGCATTGT TCGTAATAGTTTAAAGC TTTGTTCCTTAAGTC

3.3.4 Viral findings:

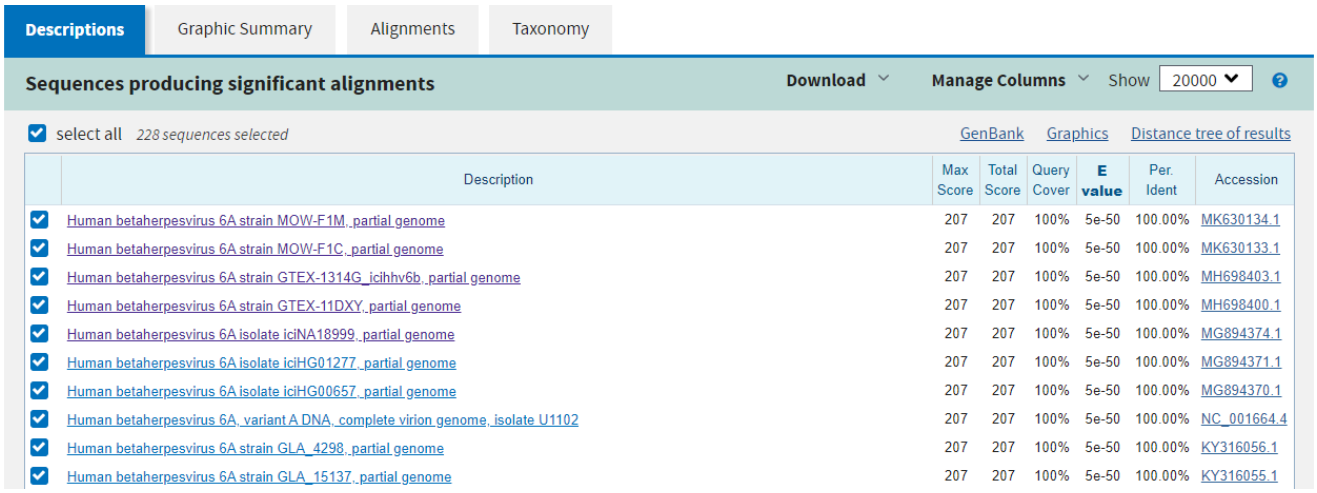
Metagenomics have provided information on the identification of viruses from hyrax samples. Viruses were detected in three hyrax samples (25%) using metagenomics and BLAST analysis, the remaining nine hyraxes (75%) did not show presence of any type of pathogenic viruses.

The two dominant viral families within the detected viruses were *Human betaherpesvirus 6A* and *Procavia capensis gammaherpesvirus*, (Table 3.5). *Procavia capensis gammaherpesvirus* was detected in ear sample of one hyrax sample captured in Haifa with 8 reads. Appendix 8. *Human betaherpesvirus 6A* has been detected in two hyraxes from A'nabta-Tulkarem and Faqqoua-Jenin. The virus were detected in spleen and nose of the hyraxes with the number of reads of 24 and 142, respectively, Appendix 9. Identification of viruses was based on the highest scoring BLAST hit on GenBank, 97–100% sequence identity and 100% coverage were adopted as shown in figure 3.5 and table 3.6. Multiple alignment of representative viral sequences that obtained by deep sequences against reference sequences obtained by Blast search are shown in Appendix 11.

Table (3.5): Detection of *Procavia capensis gammaherpesvirus* and *Human betaherpesvirus 6A* in the hyrax samples.

Hyrax code	Pathogenic virus	Region (locality)	Type of sample	Number of reads
HHE1	*Procavia capensis gammaherpesvirus*	Haifa	Ear	8
HAS3	*Human betaherpesvirus 6A strain*	A'nabta-Tulkarem	Spleen	24
HGN2	*Human betaherpesvirus 6A strain*	Faqqua-Jenin	Nose	142

HGN2



HHE1

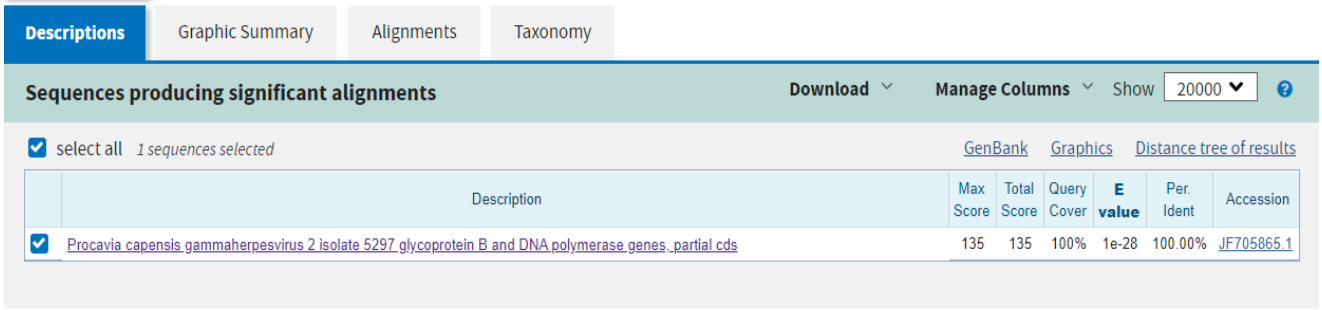


Figure 3.5: *Human betaherpesvirus 6A strain* and *Procavia capensis gammaherpesvirus* results of sequence search on BLAST tool based on identity and query coverage cover**.**

Table 3.6: The nucleotide sequences, % query cover, % identity, and accession number with probable species identification of the hyrax viruses as revealed by BLAST search.

Hyrax code	Pathogenic Virus	Query cover	Identity	Accession number/gene /locus	Sequence
HHE1	*Procavia capensis gammaherpes virus 2*	100%	100%	>JF705865.1 *Procavia capensis gammaherpesvirus 2* isolate 5297 glycoprotein B and DNA polymerase genes, partial cds	GTTTATTACTGGCTACAATAT AGCCAACTTTGATTGGCCCT ATCTCATCGACCGGGCCACC AACGTATACAAT
		100%	100%	>JF705865.1 *Procavia capensis gammaherpesvirus 2* isolate 5297 glycoprotein B and DNA polymerase genes, partial cds	ATTCCAACAAGGAAAAGAGG CTCTCTAATGTCTTTGACATC GAGACCATGTTTAGGG
		100%	98%	>JF705865.1 *Procavia capensis gammaherpesvirus 2* isolate 5297 glycoprotein B and DNA polymerase genes, partial cds	TTCTTGTGCTCTACAGAGGTA AAGTAAGAAAAGGGGAGTC GGGTTCTGGAGGCTCCCAGG ATTTTTTGGAGGAGAAAGTC TAACTTTAGGCCCTCGGGAA CACGAACGTAGAAATAAACC C
		100%	100%	>JF705865.*1 Procavia capensis gammaherpesvirus 2* isolate 5297 glycoprotein B and DNA polymerase genes, partial cds	TTCTTGACCAGCTTGAAAAT AATATTCAGCACCATTCTGA CAGGTTTCAATT
HAS3	*Human betaherpesvirus 6A*	100%	100%	>MG894374.1 *Human betaherpesvirus* 6A isolate iciNA18999	CAATAATATTAGGGAGGGTC TGCCAGCTTTATTCTCCGCTT GAGCGTACCACTTTGCAGGG GTGGGGACGGCAGT
		100%	100%	>MG894374.1 *Human betaherpesvirus* 6A isolate iciNA18999	AAACTACGGAGAGCATTTCA AACGGAGAGTTGTTCAGCTT CATTAAAAAGGATGTTGAA
		100%	100%	>MG894374.1 *Human betaherpesvirus* 6A isolate iciNA18999	GTTTTTCATAGATGTTTTGTA GAGTGTTGGCAATGTCAGTC CACTTGTACTTGGAAATC
HGN2	*Human betaherpesvirus*	100%	100%	>MG894374.1 *Human betaherpesvirus* 6A isolate iciNA18999	GTCTGTGCTTGGGAGTCTACT AAAGGACTGGCTGGCCAAGA GACGAGAAGTGAAGGCGGA GATGCAGAACTGTTCGGATC CGATGATGAAACTTCTTCTG GATAAAAAGCAG
		100	100	>MG894374.1 *Human betaherpesvirus* 6A isolate iciNA18999	CGTAGAGGTCACCATAAAAG ACTGATTTAAATATGTCTCA GAGTCTATAG

		100%	100%	>MG894374.1 *Human betaherpesvirus* 6A isolate iciNA18999	AAATTAGAGTCGTAAGAAGT GAGATTGCAGTAATTGACGA ATTTTGTTGTCTCTAGGGCCG TGTTGTAAGCGATATATATG

3.3.5 Fungal findings:

The metagenomics analysis identified fungal reads in nine hyraxes. In total, 22590 reads were obtained for fungal species in all hyrax samples. The fungal species identified in hyrax samples are normally present in plant and soil. The most prevalent fungal species were Saccharomycetales, Agaricomycetes, Dothideomycetidae and others. No pathogenic fungi were identified in the all hyrax samples.

3.3.6 Sum of all pathogenic findings:

In this study, table 3.7 summarizes the overall pathogenic findings in each hyrax. Our results found that pathogenic microorganisms were detected in seven hyrax samples (58.3%). The remaining five hyraxes were free of any pathogenic microorganisms (41.7%). According to locality, three of the seven infected hyraxes were from A'nabta-Tulkarem (42.8%), followed by two hyraxes collected from Haifa (28.5%), and one hyrax each collected from Beer Al-Saba'a from Faqqua-Jenin.

Table (3.7): Parasitic, bacterial and viral pathogens found in each rock hyrax.

Hyrax code	locality	sample	Pathogenic Parasites	Pathogenic Bacteria	Pathogenic Viruses
HAS3	A'nabta-Tulkarem	Spleen	*Leishmania tropica*	*Neisseria meningitides*	*Human betaherpesvirus*
HAS4	A'nabta-Tulkarem	Spleen	*Leishmania tropica*	*Neisseria meningitides*	****
HAS5	A'nabta-Tulkarem	Spleen	*Leishmania tropica*	*Bordetella*	****
HHS8	Haifa	Spleen	****	*Mycobacterium tuberculosis complex*	****
HHE1	Haifa	Ear	*Leishmania spp.*	*****	*Procavia capensis gammaherpesvirus*
HME3	Amara near Beer Al-Saba'a	Ear	****	*Mycobacterium tuberculosis complex*	****
HGN2	Faqqua-Jenin	Nose	****	*Mycobacterium tuberculosis complex*	*Human betaherpesvirus*

****: No infection detected

On the other hand, of all seven infected rock hyraxes, the most frequent sample of origin of pathogens was Spleen (57.1%), ear tissue (28.5%), and then nasal tissue (14.3) %. In three blood samples collected, no pathogens were detected.

In this study, all of the seven infected rock hyraxes have at least one or more type of pathogen, two hyraxes were infected by *Mycobacterium spp.*, four hyraxes infected by two types of pathogens (parasite, bacteria, or viruses), one rock hyrax showed multiple infection with three types of pathogens (*Leishmania tropica*, *Neisseria meningitides* , and *Human betaherpesvirus*).

Chapter Four: Discussion

4.1 Discussion:

Research on rock hyrax (*Procavia capensis*) has developed considerably in recent years, several studies showed that a hyrax serve as reservoir for many diseases that affect human. These diseases include different species of bacteria, viruses and parasites (Cousins et al., 1994; Galeota et al., 2009; Kleinerman et al., 2018; Talmi-Frank et al., 2010). Rock hyrax is the suspected reservoir of *leishmania tropica* that resides in the Middle East countries and may have a major role in cutaneous leishmaniasis transmission in Palestine (Jaffe et al., 2004).

The importance of this study comes with growing evidence that rock hyrax populations are expanding rapidly and encroaching into human habitation (Jacobson et al., 2003; Svobodova et al., 2006). Presence of hyraxes close to the houses was significantly associated with CL (Bsrat et al., 2015). If hyraxes play a role as reservoirs for pathogens in Palestine, their proximity to human habitat may be perceived as a risk factor for human and companion animal infection, and control programs for infections should consider this risk (Kleinerman et al., 2018). Herein, we described the use of shotgun metagenome sequencing which is a culture-independent approach allowing to capture the entire spectrum of organisms and provides rapid pathogen identification.

Previous methods that have been used to study the role of hyraxes in disease transmission and their microbial content were culture-based and or PCR based methods for detection and identification of pathogens, which is generally target-specific and thus lack the ability to detect unsuspected pathogens (Maurer, 2011). Also these techniques targeting only one gene in a single species (Cousins et al., 1994; Gudan et al., 2008; Jacobson et al., 2003; Lemma et al., 2009; Lutze-Wallace et al., 2006; Svobodova et al., 2006). Furthermore, some of these methods depend on the pure isolation for the microorganism of interest. In comparison, shotgun sequencing performed as a single diagnostic test and overcome the limitations of conventional methods (Buermans and Den Dunnen, 2014; Forbes et al., 2017).

In an attempt to investigate the microbial contents of rock hyraxes in Palestine, 16 hyraxes were captured and one tissue sample was taken from each hyrax. However, different tissue samples (spleen, nose and ear) were examined, the majority of the obtained samples were from spleen. The spleen plays supporting roles in the body, which acts as a filter for blood as part of the immune system, also the spleen fights certain kinds of bacteria (Tarantino et al., 2013). Unlike other organs, the spleen maintains the infection during the entire course of infectious diseases such as visceral leishmaniasis (Carrion et al., 2006; Santana et al., 2008). It is reported that the presence of pathogens in tissues rich in small blood vessels such as the spleen possibly functions as a reservoir for the pathogen from which it can spread back to the blood circulation (Barbour and Hayes, 1986; Fuchs and Oyama, 1969; Larsson et al., 2006; Nordstrand et al., 2001). DNA libraries were prepared from all tissue samples (n=16) and subjected to shotgun metagenome analysis. Two hyrax samples failed during sequencing process which may be due to poor DNA library preparation. Another two samples revealed only eukaryotic sequences belonging to hyrax DNA. These samples were sequenced in low coverage which means that the unique and less abundant sequences may not be detected. However, we could not repeat the analysis due to limited volume of these DNA samples. Our findings agreed with previous studies that proved the relationship between rock hyraxes and elephants (Al-Dakan and Al-Saleh, 2015; Kleinschmidt et al., 1986), our finding based on DNA sequencing indicated that hyraxes and elephants evolved from a single common ancestor.

In the present study, *Leishmania* DNA was detected in 4 hyraxes (4/12, 33%), three of them were identified as *L. tropica* (which causes human CL in Palestine) while the fourth sample was identified only up to the genus *Leishmania*. Detection of *Leishmania* in spleen hyraxes obtained from two regions (A'nabta-Tulkarem and Haifa) may strongly supports the role of the rock hyrax as a reservoir for human disease in these regions. Further, *Leishmania* was detected in ear (one tissue sample), the predilection sites of sandfly bite may differ in the same reservoir host. To the best of our knowledge, this is the first time that *L. tropica* DNA has been detected in rock hyraxes in Palestine. However, *L. tropica* has been identified in naturally infected hyraxes in different regions. In Ethiopia, previously reported natural infection rates of *Leishmania* in hyraxes ranged between 3.5% and 27% (Ashford et al., 1973; Lemma et al., 2009). Our study showed higher infection rate compared to previous study conducted Galilee region , rural foci of *L. tropica*, which showed that 10–13% of the hyraxes tested by ITS1 PCR were positive for parasite

DNA(Svobodova et al., 2006). However a higher rate (58 %) have been found in the eastern hills of Jerusalem (Al Quds) when using kinetoplast DNA PCR and serological test (Talmi-Frank et al., 2010). The last study depended on. Conventional serological techniques are limited by cross-reactivity with other parasitic diseases (Paiva-Cavalcanti et al., 2010). Moreover, kDNA-PCR present some limitations and can provide false positive, therefore reduce the accuracy of the test, also PCR assays are highly susceptible to cross contamination (Galluzzi et al., 2018), This is a possible cause of the conflict between our study and this study.

Furthermore, *Mycobacterium tuberculosis complex* (MTC) (a group of Mycobacterium species that causes tuberculosis in humans and animals) was detected in tissue from nose, ears and spleen of 3 hyraxes. This complex include 5 species: *M. tuberculosis, M. bovis* (including the attenuated BCG vaccine strains), *M. africanum, M. microti* and *M. canetti.* Transmission of the MTC in rock hyraxes appears to be primarily via the respiratory tract, infection of the lungs is often accompanied by secondary spread to other organs, including the liver, kidneys and spleen (Clarke et al., 2016; Cousins et al., 1994; Parsons et al., 2008; Wagner and Bokkenheuser, 1961). Our findings are similar to a previous study that isolated a MTC member i.e *M. microtti* from a rock hyrax that imported into Canada from South Africa (Lutze-Wallace et al., 2006). Human *M. microti* infections were described in immunocompromised and immunocompetent individuals (van Soolingen et al., 1998).

In this study, MTC was identified in three hyraxes captured from three different localities including Haifa, Faqqua-Jenin and Amara near Beer Al-Saba'a which may indicate the widespread of this bacterium in hyrax colonies. Hyraxes are reported as the host species for MTC, which are readily habituated to human activity and this trait carries a zoonotic risk and risk to livestock (Clarke et al., 2016). Close contact with animals is suggested as the main factor for transmission of *Mycobacterium spp.* between human and animal which may have impact on the tuberculosis control program in human (Romha et al., 2018).

Three tissues (two ear samples and one nose sample) were infected with two type of pathogens (*Leishmania* and MTC). These body regions, nose and ear, are exposed to the disease vector, which injects the parasites into the host and picks it up from the host (Hart, 2013) This may lead to spreading diseases among the hyrax population and to humans . Nose and ear are typical sites of sand fly bites(Svobodova et al., 2006)

Our study confirmed the presence of P*rocavia capensis gammaherpesviruses* and *Human betaherpesvirus 6A* in ear and nose lesions of rock hyraxes. In 2008, six *gammaherpesviruses* were detected and identified in four species in the superorder Afrotheria by PCR targeting DNA polymerase genes (Wellehan et al., 2008).

Human betaherpesvirus 6A strains infect a wide variety of mammals (Davison and Bhella, 2007). *Human β-herpesvirus* subfamily consists of human *cytomegalovirus*, belong to the *Roseolovirus* genus of the *β-herpesviruses*. These viruses establish a lifelong infection of their host, reactivated frequently. Some evidence suggests that latent infection in the brain may be involved in the pathology of certain neurological diseases, such as recurrent febrile, multiple sclerosis and encephalitis (Kondo and Yamanishi, 2007).

In this study, *Neisseria meningitides* and *Bordetella spp.,* were detected in spleen of rock hyraxes. No previous study reported the presence of these pathogens in hyrax samples. *Bordetella spp.* are respiratory pathogens of humans and animals (Stevenson and Roberts, 2003). The pathogen normally lives in the upper respiratory tract of the mammals and is transmitted to humans by aerosol (Ghasemzadeh and Namazi, 2015). Pertussis caused by *Bordetella pertussis* is a very contagious disease only found in humans and transmitted to another person by coughing or sneezing or when sharing the same breathing space. Similarly, the presence of *Neisseria meningitides* in the rock hyrax which is also known as human pathogen needs further investigation as humans are the only reservoir for these pathogens. However, zoonotic bacterial meningitis have been reported in patients who have recreational or professional contact with animals and in patients living in regions endemic for specific zoonotic pathogens (van Samkar et al., 2016).

The presence of more than one pathogen in the same hyrax may due to polymicrobial diseases, which are being recognized with increasing frequency. It is well known that one microorganism predisposes the host to colonization by other microorganisms or several non-pathogenic microorganisms are clustered together and can be harmful causing diseases (opportunistic infections) (Brogden, 2002; Brogden et al., 2005). Moreover, candidiasis have been documented in a rock hyrax supporting that it is a primary pathogen in this species (Hubbard and Fletcher, 1984). However, fungal genera that have been identified in our study samples were not pathogenic, but rather species of plants, soil and air fungi (Hibbett et al., 2014; Ohm et al., 2012; Suh et al., 2006). Moreover, no pathogens were detected in the three blood samples collected, since blood in healthy organisms is

seen as an entirely sterile environment, it lacks proliferating microbes so detection of pathogens in the blood constantly is abnormal (Potgieter et al., 2015).

Understanding the reservoir system will help in control measures and disease eradication. The inclusion of animal disease data supports a more rapid recognition of the extent and spread of disease. Research into and responses to zoonosis have substantial benefits and contributes to many great advances in public health.

4.2 Limitations:

Our approach has several limitations. First, we utilized Galaxy software for analysis of metagenome sequences which incorporates the MEGABLAST program for a homology search against known nucleotide sequences registered in the NCBI nucleotide database. Therefore, microorganisms whose sequences are unknown or not registered in the database cannot be detected. Secondly, detection of parasitic, viral or bacterial reads by NGS does not always indicate the presence of viable or pathogenic microorganisms. Thirdly, our method is designed to detect DNA viruses, we are unable to detect RNA viruses. Furthermore the size of sample was not large enough which weakens the ability for solid statistical inference . Studies with larger sample size from different districts are needed to identify pathogenic bacteria, parasite and viruses of rock hyraxes.

4.3 Conclusion:

To the best of our knowledge, this study is the first in Palestine that used shotgun metagenome sequencing to investigate the parasitic and microbial contents of hyrax tissue samples. The study sheds light on hyraxes, which is considered a natural reservoir for leishmaniasis. Taking into account the results obtained in this study, as well as the results of previous studies, we concluded that rock hyraxes are possible hosts for serious pathogens which can be harmful to humans as hyraxes have long lifespan and gregarious habits, share habitats with vector. Our results confirmed the presence of *L. tropica* DNA in rock hyraxes from Palestine and supported their potential role as a reservoir for human CL. Moreover, this study was able to detect disease-associated microorganisms using metagenome sequences directly from rock hyrax samples without the need of culture or

several molecular steps. Advanced genome analysis based on assembly of DNA sequences from the whole chromosome should be applied. Thus, the complete genome of human pathogens such as *Neisseria meningitides* and *Human betaherpesvirus* detected in the rock hyrax samples can be obtained, analyzed and compared with other worldwide strains.

4.4 Recommendation:

Further studies should be conducted in the future to evaluate the competence of hyraxes as reservoirs for diseases caused by pathogens harbored in the body of hyraxes like MTC, *Neisseria meningitides, Bordetella spp.* and *Human betaherpesvirus*. Efforts to prevent human leishmaniasis should be focused on interrupting the transmission of infection cycle that includes in addition to the control of sandfly vectors, animal reservoirs including hyrax. Based on our results, attention should be given to hyraxes and hyrax- transmitted diseases in Palestine.

Transmission of zoonotic Cutaneous Leishmaniasis due to *L. tropica* or other diseases caused by pathogens accompanying with the body of hyraxes could be reduced by controlling hyraxes. We suggest shooting of hyraxes present within 1 km of human habitation although this may be possible in some countries, these animals are protected and their control is prohibited and illegal in others. The destruction of the burrows in endemic areas by ploughing followed by planting, this applied in many areas such as Tunisia. Spraying hyrax's dens and eliminating infected animals through fumigation with phosphine-producing tablets, an alternative to the baiting technique. Use of the traditional fast-acting rodenticide like zinc phosphide. The removal of chenopods from areas close to inhabitants would deprive the animal from its food leading to reduction in numbers and would help drive it to migrate to other areas. Furthermore increase community participation and educational health programmes in endemic areas, this may effective in reducing transmission of CL disease.

On the other hand, this study suggests the need for efficient future surveillance system and infection control strategies. As human populations are growing and expanding, joined efforts from ministry of health, ministry of environment, and ministry of agriculture are required for proper planning to ensure a balance between modernization and environmental conservation to avoid destruction of key wildlife habitat

Chapter Five: References

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Appendix 1: DNA Extraction

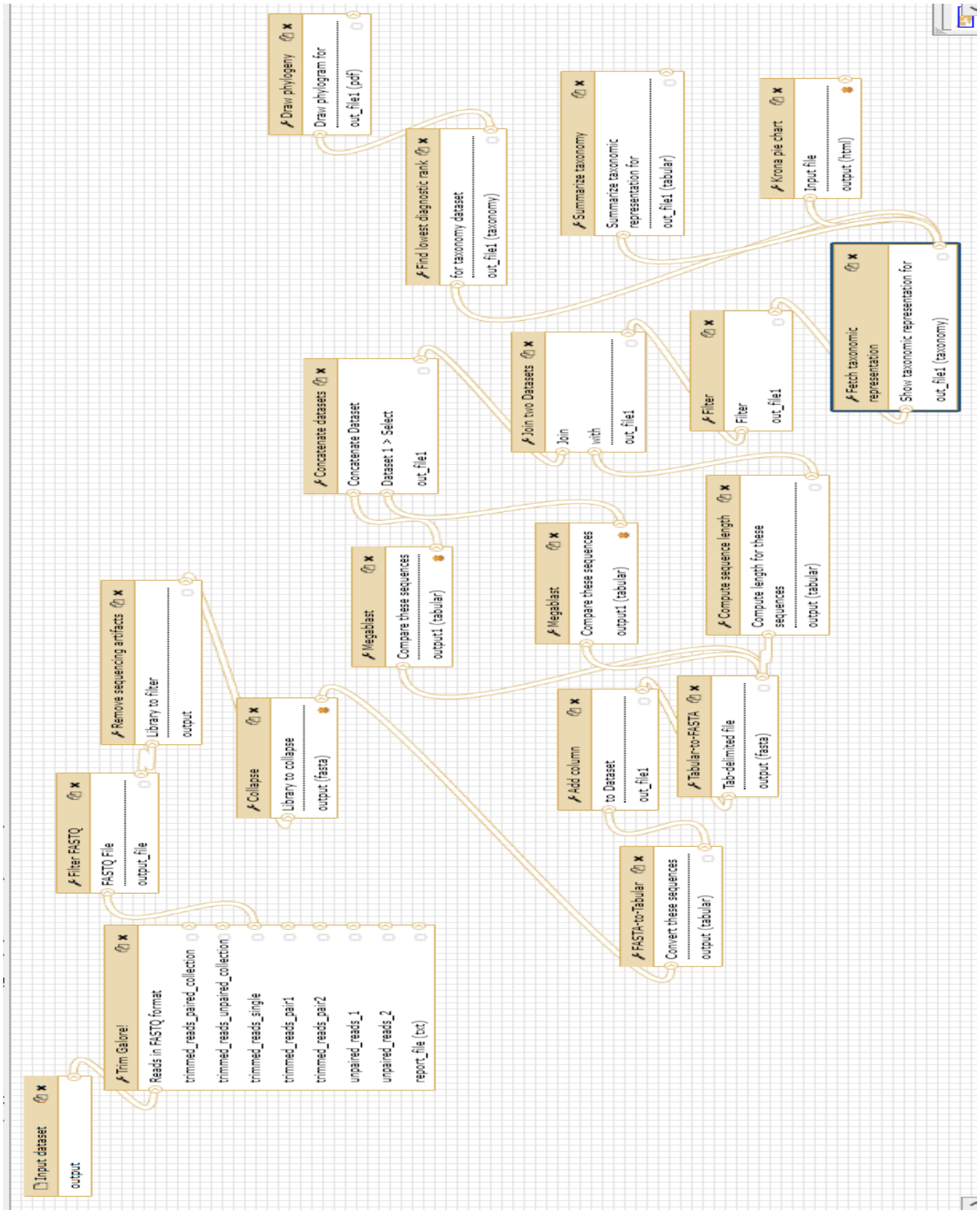
1. 10 mg of tissues were cut into small pieces, placed in a 1.5 ml microcentrifuge tube and 180µl of tissue lysis buffer were added.
2. 20µl proteinase K (600 mAU/ml, solution) were added and incubated at 56°C until the tissue was completely lysed. Samples were centrifuged to remove supernatant.
3. 200µl lysis bufferto were added to the sample, then mixed by vortexing for 15 s, incubated at 70°C for 10 min, samples were centrifuged to remove the supernatant.
4. 200µl ethanol (96–100%) were added to the sample, mixed by vortexing for 15 second, samples were centrifuged to remove supernatant.
5. The mixture from previous step were applied to the QIAamp Mini spin column, after that centrifuged at 8000 rpm for 1 min, the QIAamp Mini spin column were placed in a clean 2 ml collection tube.
6. For washing, 500µl of first washing buffer (contain chaotropic salt) were added and centrifuged for 1 min at 8000×g, then 500µl of second washing buffer were added and centrifuged for 3 minutes at 14000×g.
7. The last step, addition of 200µl elution buffer (AE) (elution) to the QIAamp Mini spin column, samples were incubated at room temperature for 1 min and then centrifuged at 8000 rpm for 1 min, samples were kept at -30°C for long-term storage of DNA.

For DNA extraction from the blood samples, DNA was prepared from whole blood usingthe DNA extraction kit (QIAamp DNA Blood, Germany) as following:

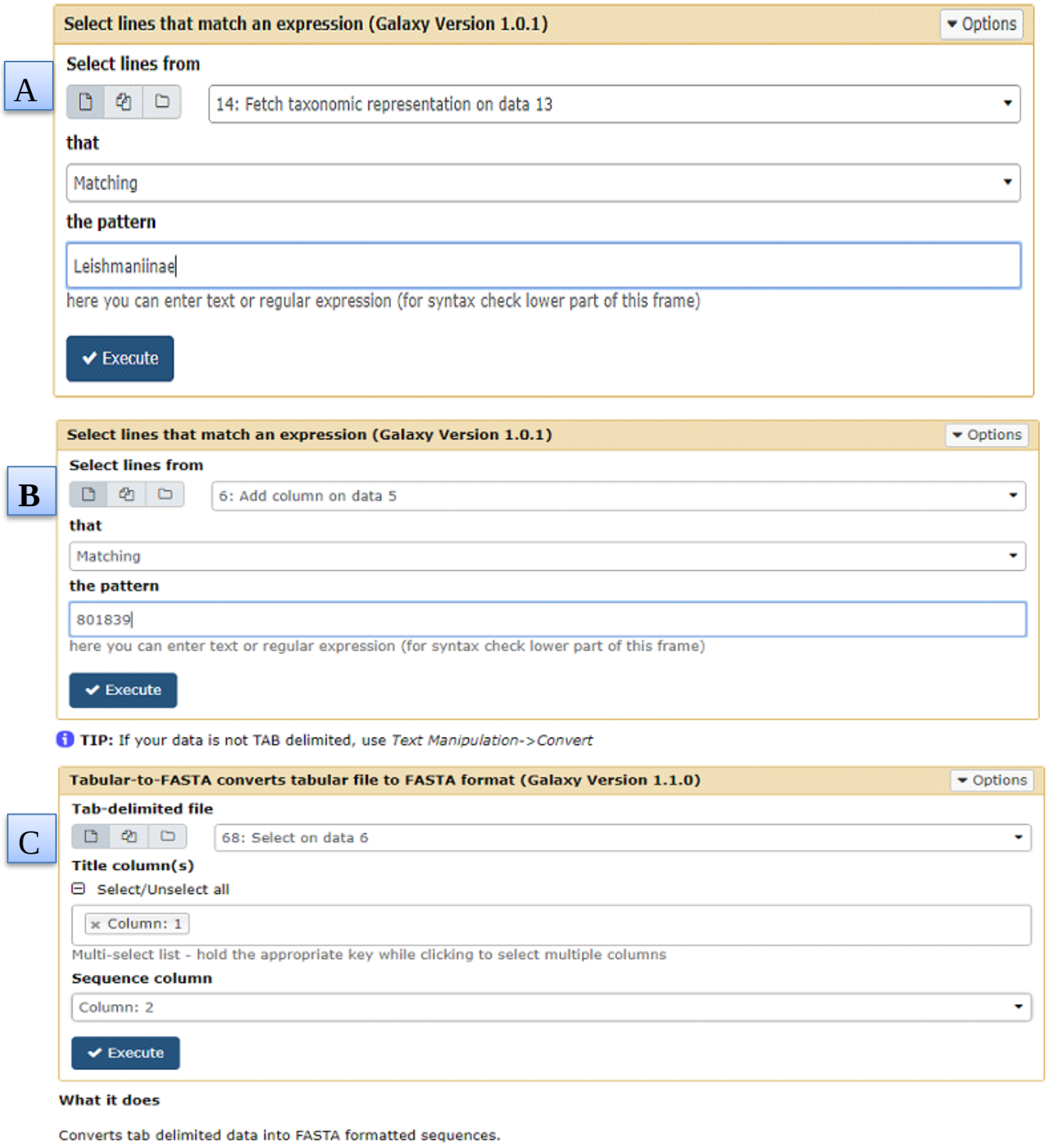
1. 200µl of whole blood were added, 20µl of Proteinase K were added to the tube with 200µl AL buffer. Samples were vortexed vigorously, samples were incubated at 56°C for 10 minutes then were centrifuged to remove the supernatant.
2. To remove the insoluble particles, 200µl absolute ethanol were added then all the amount were transferred into spin column (filter tube), centrifuged for 1 min at 8000×g.
3. For washing, 500µl of first washing buffer were added and centrifuged for 1 min at 8000×g, then 500µl of second washing buffer were added and centrifuged for 3 minutes at 14000×g.

4. Finally, spin column were transferred into a new 1.5 ml micro-tube, 200µl of elution buffer were added and centrifuged for 1 min at 8000×g. The eluted DNA were kept at -30C until use.

Appendix 2: Workflow as shown by Galaxy software

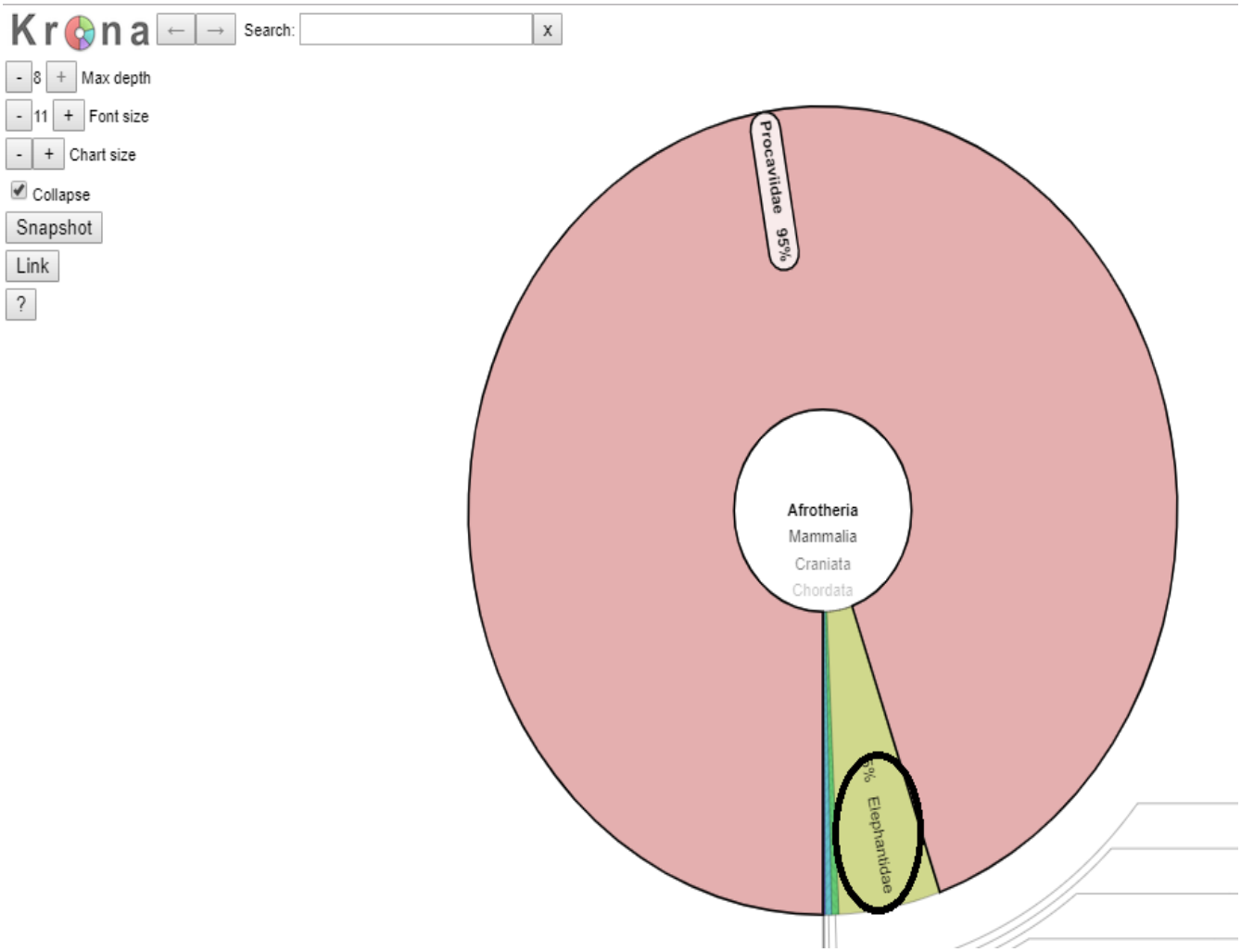


Appendix 3: Retrieval of DNA Sequences Methodology

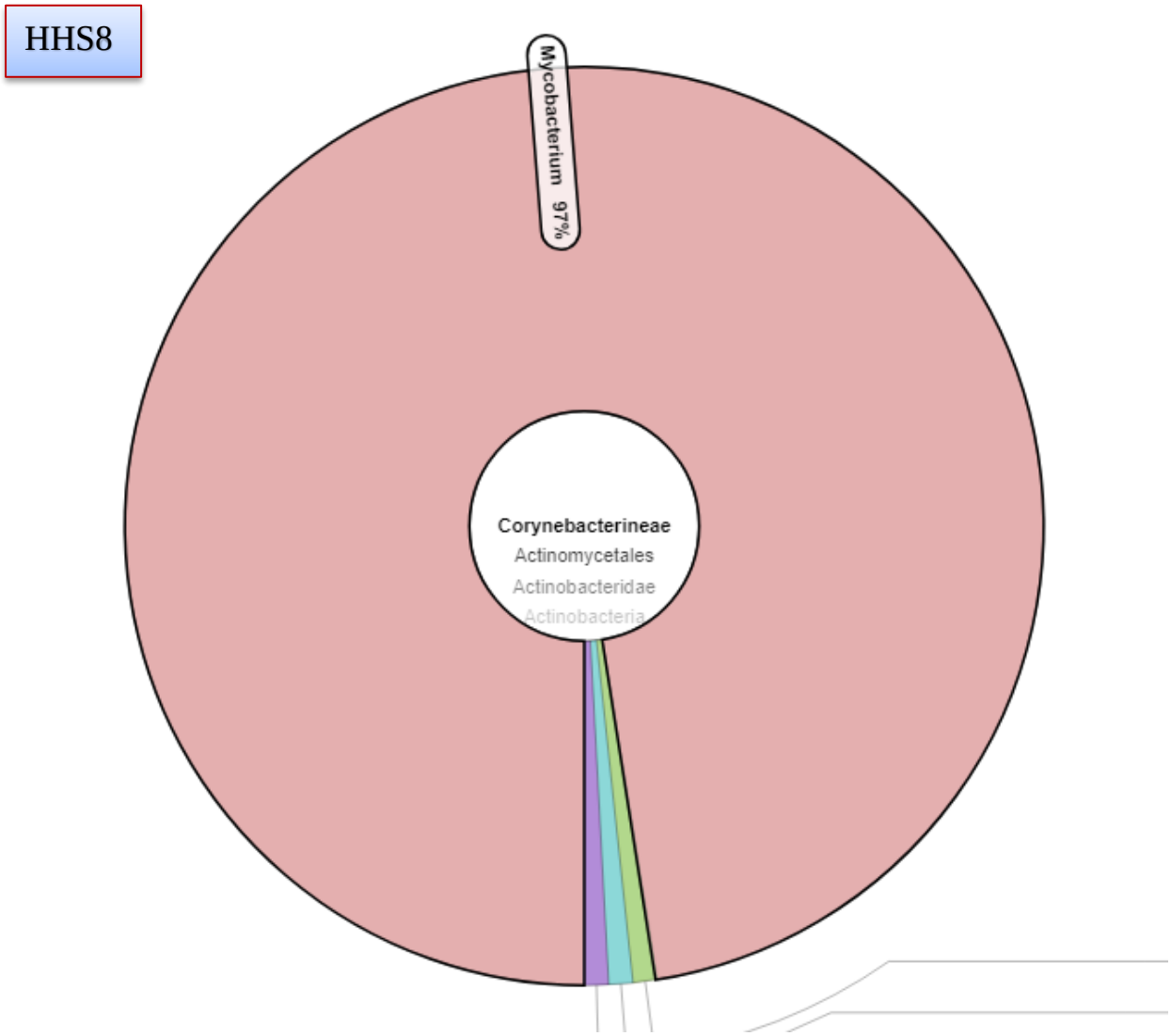


Sequence retrieves: (A) Select lines that match an expression. The selection was done on fetch taxonomic representation on data 13, (B) Select lines that match an expression. The selection was done on add column on data 5, (C) Conversion of Tabular file to FASTA format.

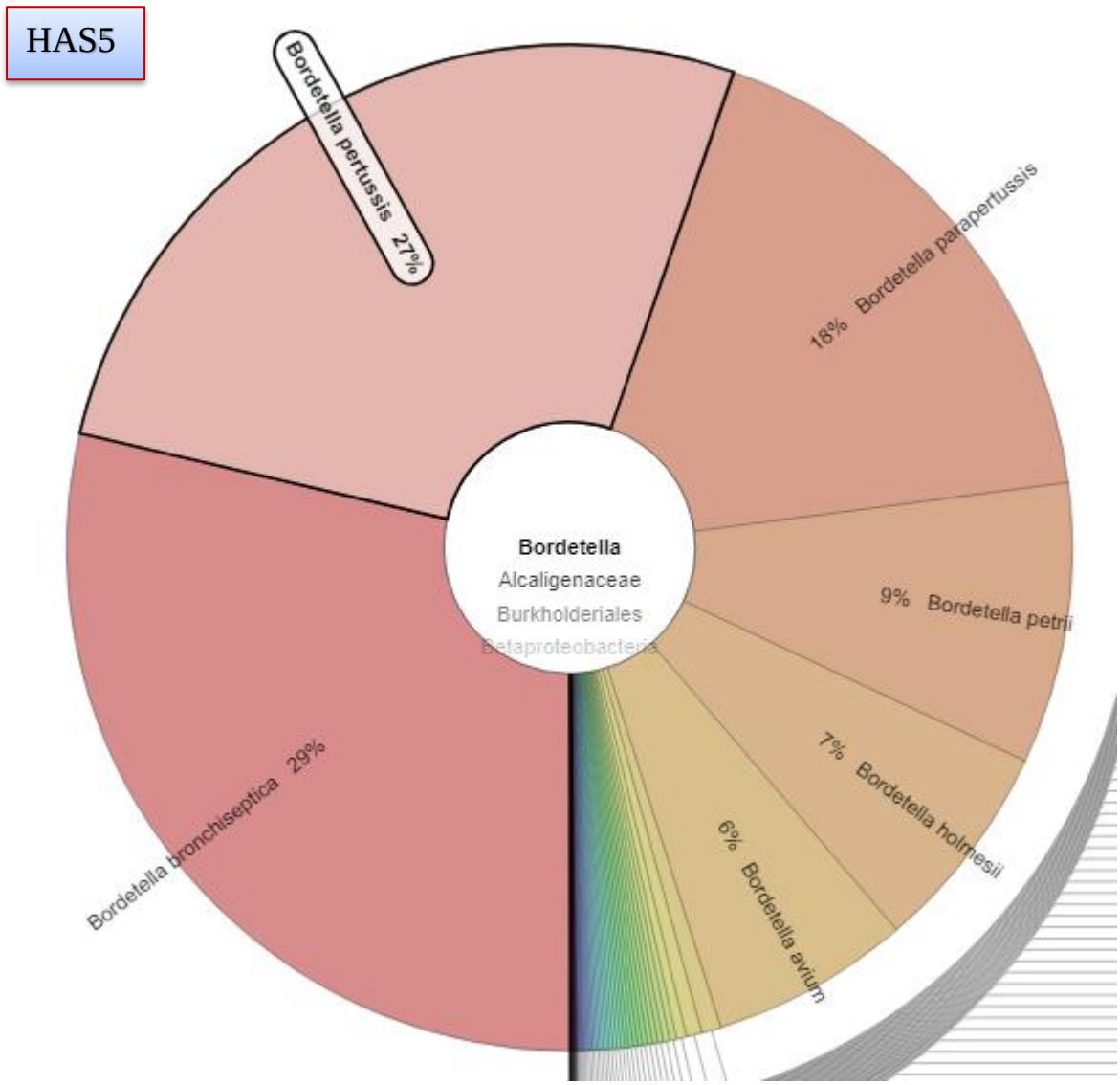
Appendix 4: Krona Pie Chart proved that rock hyrax related to the Elephantidea



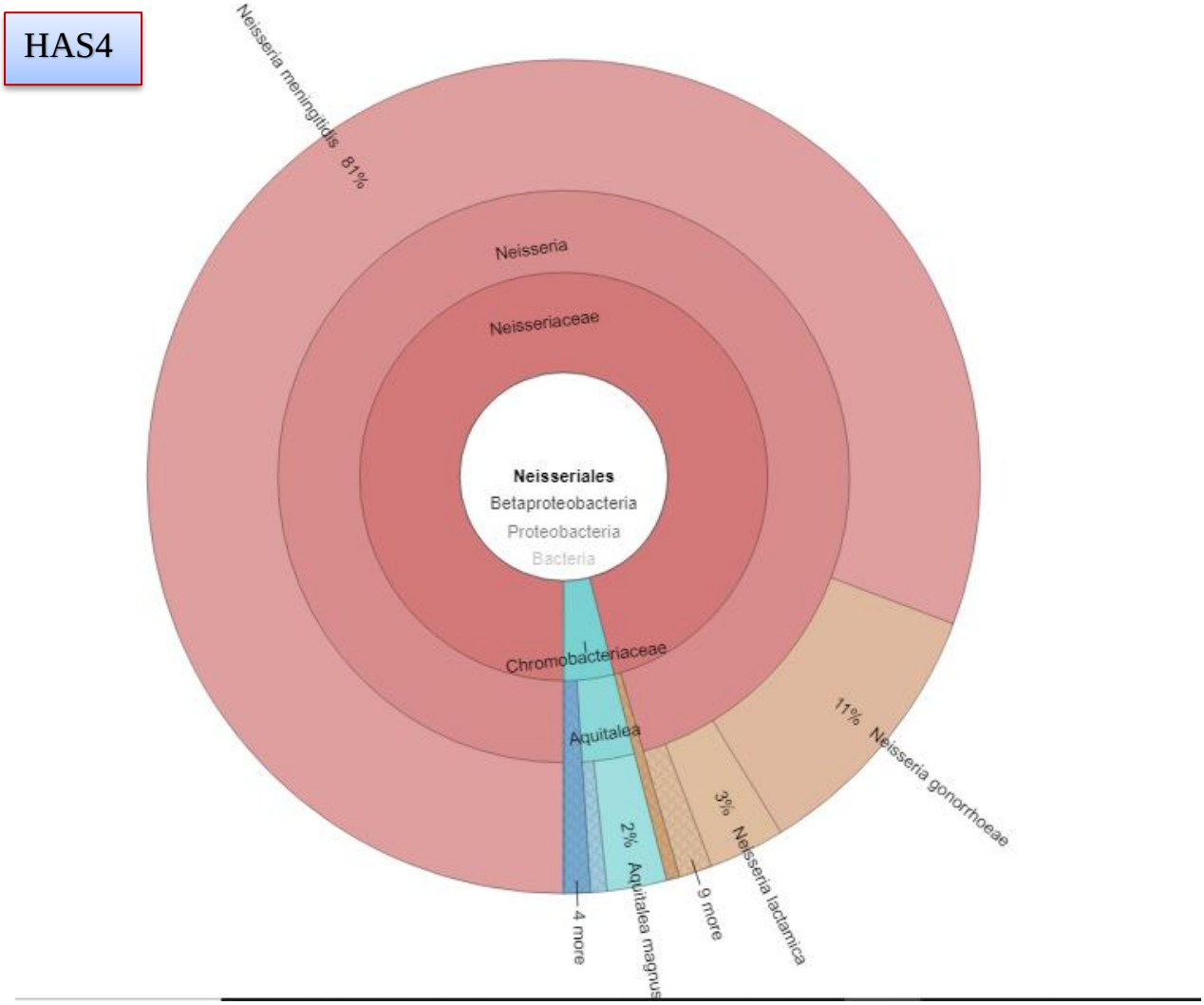
Appendix 5: Krona Pie Chart for *Mycobacterium tuberculosis complex* (MTC) as detected by Galaxy software



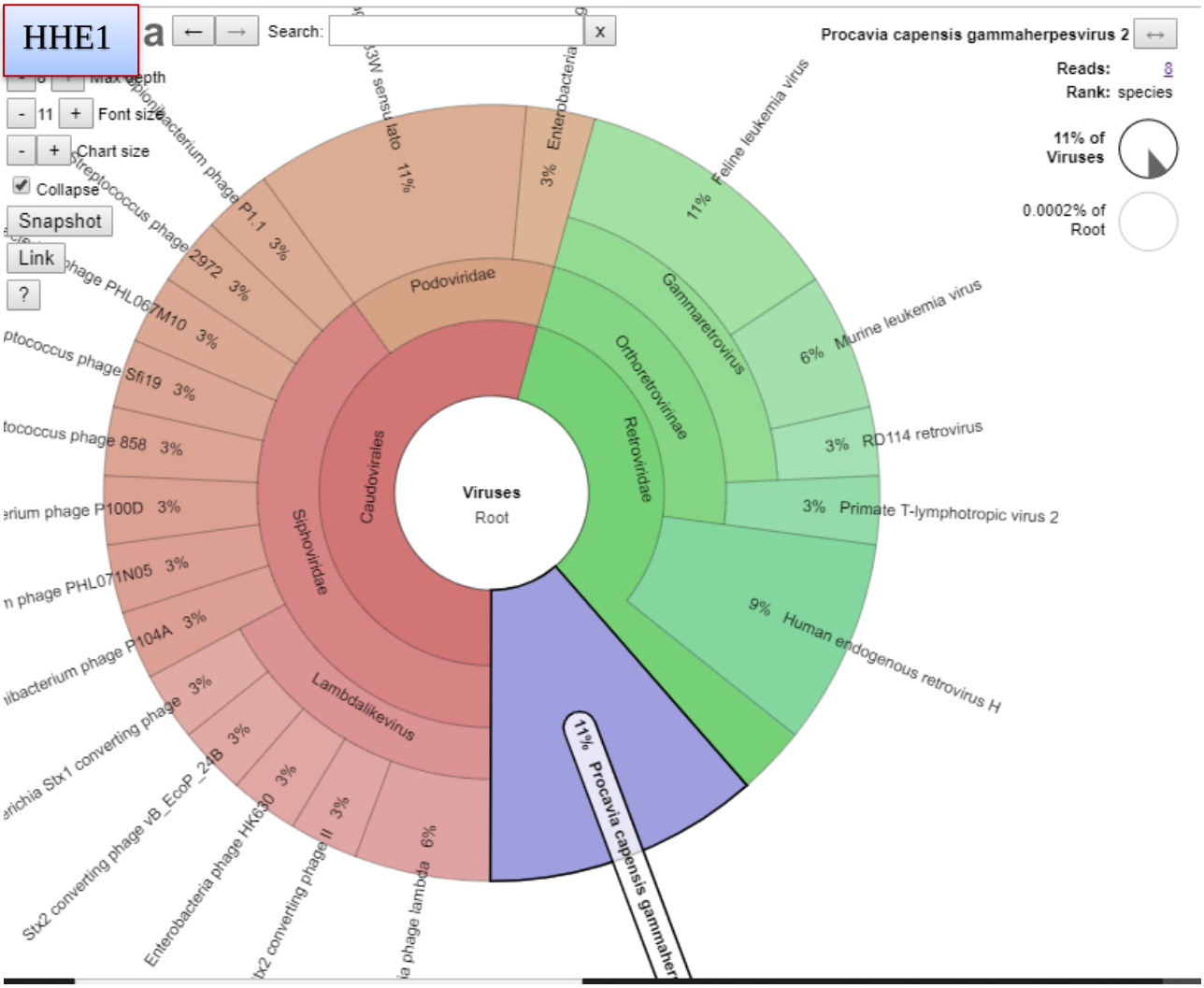
Appendix 6: Krona Pie Chart for *Bordetella* spp. as detected by Galaxy software



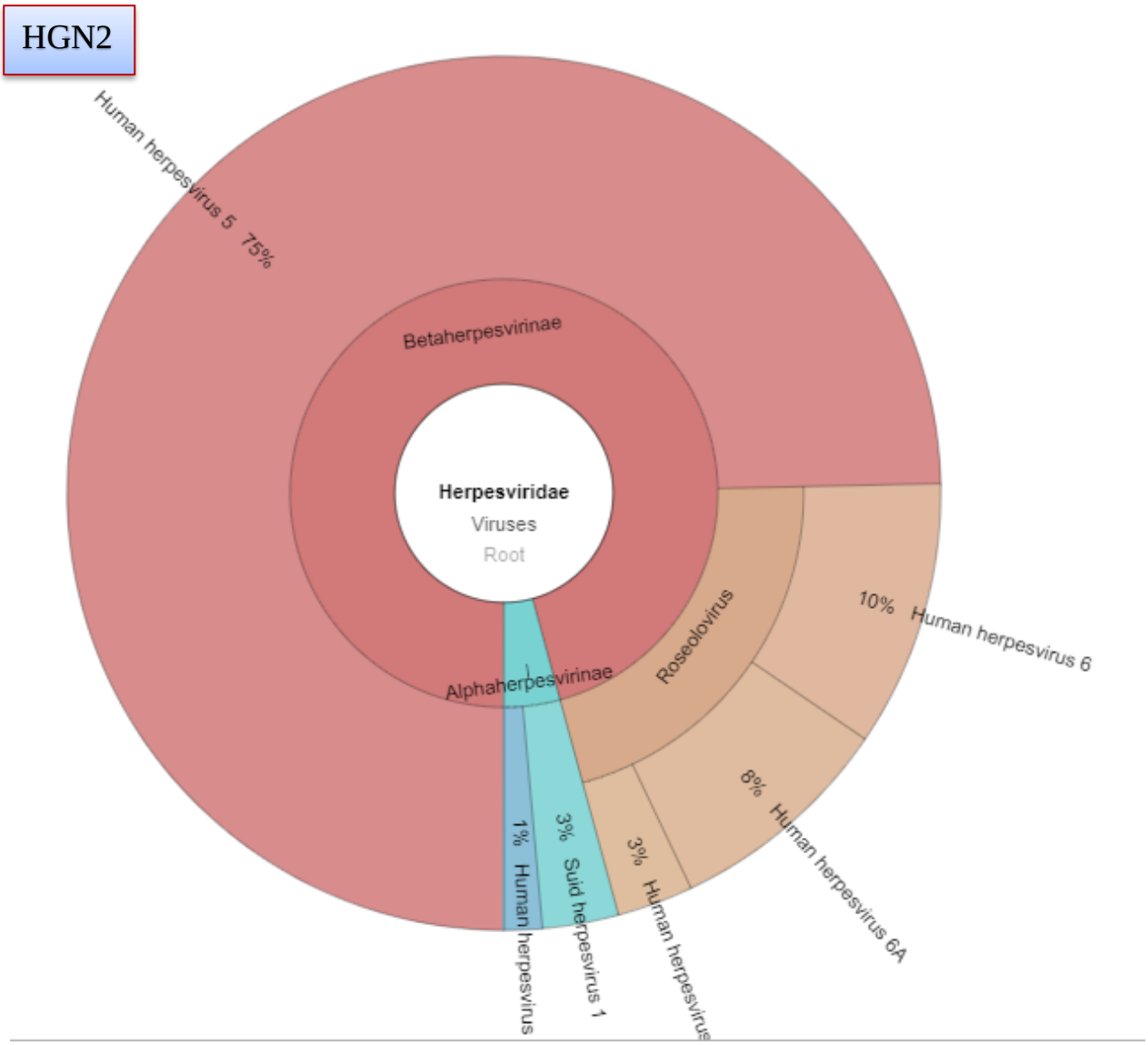
Appendix 7: Krona Pie Chart for *Neisseria meningitides* as detected by Galaxy software



Appendix 8: Krona Pie Chart for *Procavia capensis gammaherpesvirus 2* as detected by Galaxy software

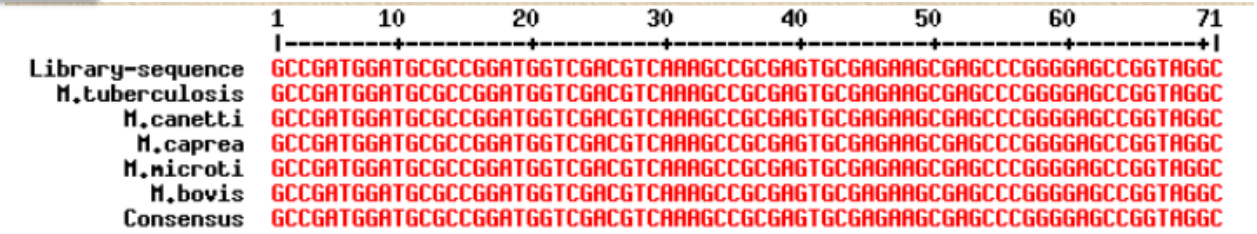


Appendix 9: Krona Pie Chart for *Human betaherpesvirus 6A strain* as detected by Galaxy software

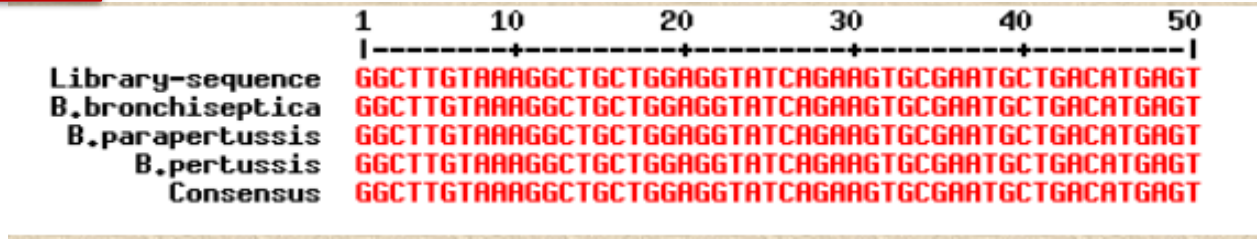


Appendix 10: Multiple alignment of representative bacterial sequences obtained by deep sequencing against reference sequences obtained by Blast search

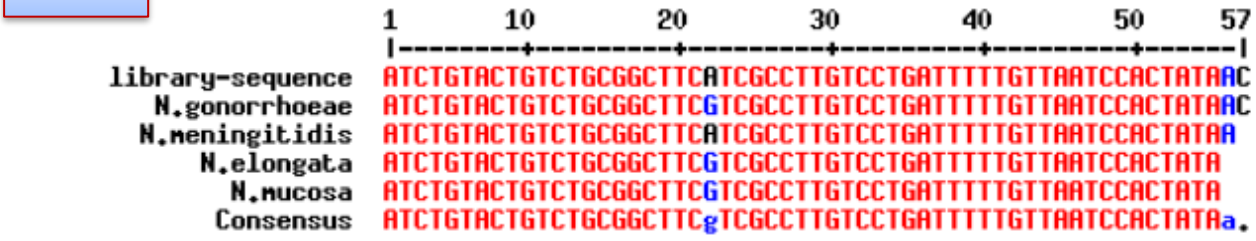
HHS8



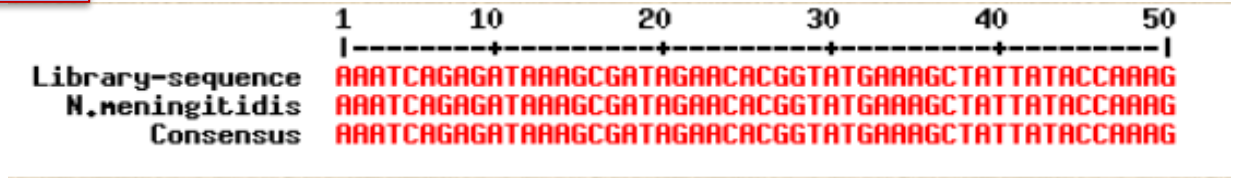
HAS5



HAS3

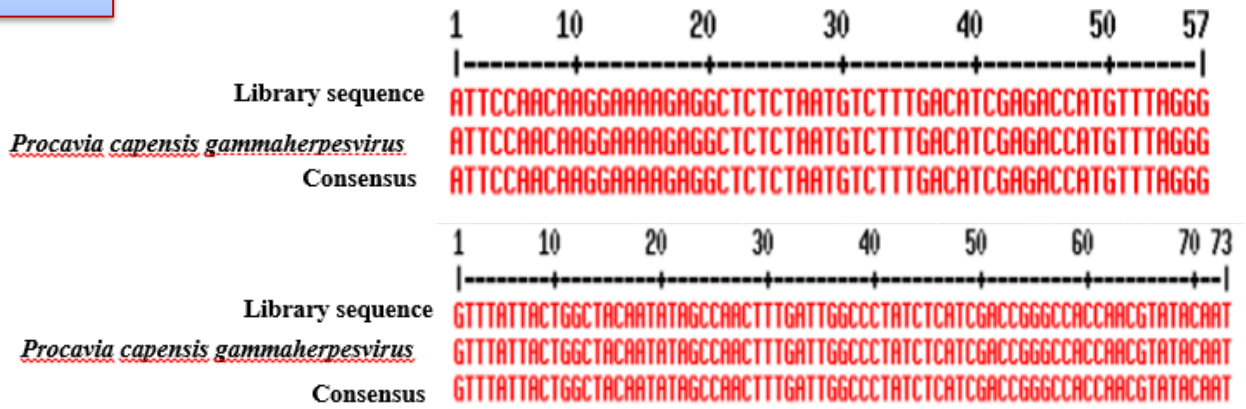


HAS4

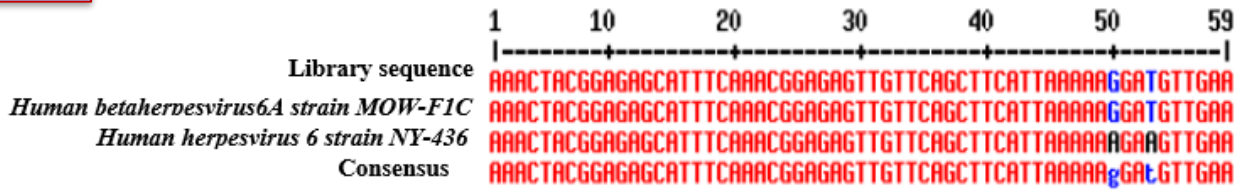


Appendix 11: Multiple alignment of representative viral sequences obtained by deep sequencing against reference sequences obtained by Blast search

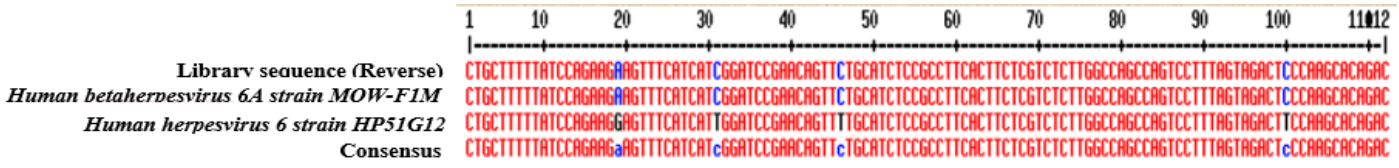
HHE1



HAS3



HGN2



التعرف على مجتمع الميكروبات في الوبر الصخري، المستودع المشتبه به لداء الليشمانيات في فلسطين: تحليل الميتاجينوم

إعداد: رنا نايف مصلح عوايصة
إشراف: د. سهير عريقات

الملخص:

الوبر الصخري هو الممثل الوحيد للترتيب الدمانيات (Hyracoidea) في الشرق الأوسط. هذه الأنواع الأفريقية تؤوي الليشمانيا في بشرتها والديدان الخيطية في أحشاءها وهي كذلك عرضة للالتهاب الرئوي الفيروسي والسل. يشير التوزيع المكاني الواسع لهذا الخازن إلى تهديد كبير من انتشار الأمراض التي تسببها مسببات الأمراض المصاحبة لجسم الوبر الصخري. يمكن أن يعيش الوبر الصخري عدة سنوات في الطبيعة ، وبالتالي فهو يضم خزانًا طبيعيًا لحمل العدوى ونقلها. أظهرت العديد من الدراسات أن الوبر الصخري المصاب تجريبياً من داء الليشمانيات يمكن أن يصاب بالعدوى ، ولا تظهر عليه أية علامات سريرية ولكنها معدية لذباب الرمل الناقل للمرض. هدفت هذه الدراسة إلى دراسة التركيب المجتمعي الميكروبي لعينات الأنسجة المأخوذة من عدد من الوبر الصخري المجمعة من مناطق مختلفة في فلسطين والتي قد تشكل تهديداً لصحة الإنسان.

تم جمع ستة عشر وبر صخري باستخدام مصائد الراكون من مواقع مختلفة في فلسطين (عنبتا - طولكرم ، حيفا ، عمارة بالقرب من بئر السبع ، مسحة - سلفيت ، وفقوعة - جنين). تم استخراج الحمض النووي من عينات أنسجة الوبر الصخري (عدد = 16) بما في ذلك الدم (عدد = 3) والأنف (عدد = 2) والأذن (عدد = 3) والطحال (عدد = 8) ، تم أخذ عينة واحدة فقط من كل وبر صخري. تم اختبار جميع عينات الحمض النووي لوجود مسببات الأمراض بما في ذلك الطفيليات والبكتيريا والفيروسات والفطريات باستخدام تسلسل الجيل القادم (NGS). إعدت مكتبة الحامض النووي الرايبوزي اللاوكسجيني بناءً على اختبار Nextera® XT وتسلسلها على جهاز Illumina MiSeq i طبق تحليل المعلومات البيولوجية لتحديد سلاسل الجيل القادم باستخدام الأداة Galaxy عبر الإنترنت (https://usegalaxy.org/). بحث عن المتسلسلات المسترجعة المسببة للأمراض بواسطة الية البحث BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) ، اعتمدت التسلسلات ذات التماثل الذي يزيد عن 97٪ و غطاء الاستعلام الاكبر من 97٪ ومقارنة التسلسلات النيوكليوتايدية المرجعية المتاحة مع التسلسلات المخزنة في قاعدة البيانات لتحديد الأنواع.

من بين عينات الوبر الصخري المختبرة (عدد = 16) ، استبعدت أربع عينات من الدراسة من بينها الطحال (عدد = 2) والدم (عدد = 1) والأنسجة الأنفية (عدد = 1). لذلك ، طبقت التحليل على اثني عشر عينة فقط، اكتشفت الليشمانيا في أربع عينات من الطحال (عدد = 3) والأذن (عدد = 1). تم التعرف على ثلاثة منها على أنها اللِّيشْمانِيَّةُ المَدارِيَّة *L. tropica* بينما حددت العينة الرابعة على انها من جنس الليشمانيا فقط . جمعت هذه العينات من من عنبتا - طولكرم وحيفا حيث تنتشر حالات مرض الليشمانيات الجلدي البشري الناجم عن اللِّيشْمانِيَّةُ المَدارِيَّة *L. tropica*. علاوة على ذلك وعلى المستوى البكتيري ، اكتشفت المُتَفَطِّرَةُ السُّلِّيَّةُ (MTC) في ثلاثة عينات من الوبر الصخري من حيفا وفقوعة جنين والعمارة بالقرب من بئر السبع. من ناحية أخرى ، كشف عن مسببات الأمراض البشرية مثل النيسرية السحائية في عينتين بالإضافة إلى نوع البُورْديتيلَة الذي اكتشفت في عينة واحدة فقط. هناك نوعان من الفيروسات في ثلاث عينات من الوبر الصخري ، والفيروسان السائدان هما فيروس الهربس وفيروس بروكابيا كابينسيس(*Procavia capensis gammaherpesvirus*) .

في الختام ، أظهرت النتائج التي توصلنا إليها أنه يمكن للوبر الصخري نقل مسببات أمراض خطيرة و الضارة للإنسان لأن الوبر الصخري له عمر طويل وعادات سربية ومشاركة المسكن مع عدد من ناقلات الامراض. أكدت نتائجنا وجود الحمض النووي الخاض باللِّيشْمانِيَّةُ المَدارِيَّة *L. tropica* في الوبر الصخري في فلسطين ودعمت دورها المحتمل كخازن لليشمانية الجلدية البشرية. يجب تطبيق تحليل الجينوم المتقدم على أساس تجميع تسلسل الحمض النووي من الكروموسومات بأكمله. وبالتالي ، يمكن الحصول على الجينوم الكامل لمسببات الأمراض البشرية مثل المكورات السحائية النيسرية والفيروسات التجريبية البشرية التي تم اكتشافها في عينات الوبر الصخري ، وتحليلها ومقارنتها مع تسلسلات عالمية أخرى. يجب أن تركز الجهود المبذولة لمنع داء الليشمانيات البشري على كسر دورة العدوى التي تشمل مكافحة ناقلات ذبابة الرمل والخازن الحيواني.