Molecular Detection of *Balamuthia mandrillaris* in Soil Samples from Kathmandu Valley

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A dissertation submitted
in partial fulfillment of the requirements for the award of the degree
of Master of Science in Zoology with special paper Parasitology

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Declaration

I hereby declare that the work presented in this dissertation "Molecular detection of *Balamuthia mandrillaris* in soil samples from Kathmandu Valley" been done by myself and has not been submitted elsewhere for any degree award. All information sources have been explicitly acknowledged by referencing the author(s) or institution(s).

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Recommendation

This is to recommend that the dissertation entitled "Molecular detection of *Balamuthia mandrillaris* in soil samples from Kathmandu Valley" has been carried out by Tsunami Thapa Magar for the partial fulfilment of Master's Degree of Science in Zoology with special paper Parasitology. This is her original work and has been carried out under my supervision. To the best of my knowledge, this dissertation work has not been submitted for any other degree in any institution.

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Letter of approval

On the recommendation of supervisor Associate Prof. Dr. Kishor Pandey this dissertation submitted by Tsunami Thapa Magar entitled "Molecular detection of *Balamuthia mandrillaris* in soil samples from Kathmandu Valley" is approved for the examination in partial fulfillment of the requirements for Master's Degree of Science in Zoology with special paper Parasitology.

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Certificate of acceptance

This dissertation work submitted by Tsunami Thapa Magar entitled "Molecular detection of *Balamuthia mandrillaris* in soil samples from Kathmandu Valley" has been accepted as a partial fulfilment for the requirements of Master's Degree of Science in Zoology with special paper Parasitology.

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Abstract

Balamuthia mandrillaris is an emerging free-living amoeba (FLA) that inhabits in a wide range of environments including soil, water, air, dust, contact lens storage case, distribution systems, drinking water treatment, and cooling towers. This pathogen is responsible for the amoebic encephalitis caused known as *Balamuthia* amoebic encephalitis (BAE) with over 90% of cases of this pathogen results in death. The biology and environmental distribution of *B. mandrillaris* are still insufficiently understood, and the environmental isolation of this amoeba is infrequent. The aim of this study is to detect the presence of *B. mandrillaris* in soil samples taken from various sites within the Kathmandu Valley. This study assesses the detection of the pathogen in a variety of soil environments that influences its presence, employing molecular approach. Twenty-five (25) soil samples were collected for the investigation and stored in sterilized zip-lock plastic bags at 4°C. *B. mandrillaris* was identified by isolating DNA from the soil samples, and PCR amplification. The positive samples were separated through electrophoresis and validated the result by sequence analysis and phylogeny. A phylogenetic tree was constructed in MEGA 11 software using the maximum likelihood estimation (MLE) and a substitution Jukes-Cantor model to verify the evolutionary relation and genetic association between predicted *B. mandrillaris* with previously published GenBank sequences. Out of 25 samples, 5 samples were tested positive for *Balamuthia* specific DNA in which two were collected from distinct river banks, one from park, one from nursery and one from goat farm premises soil. The NCBI BLAST analysis of the sequences confirmed 100% homology with the corresponding previously reported *B. mandrillaris* sequence in the GenBank database from Japan. The detection of *B. mandrillaris* from multiple environmental conditions emphasizes the ubiquitous character of this pathogen. Of the 25 samples, *B. mandrillaris* was found in five (20%) soil samples through molecular method and sequencing which demonstrate the significantly higher incidence of *Balamuthia* in environmental habitats. The discovery highlights the importance of public health activities aimed at raising awareness regarding the risks associated with *B. mandrillaris*.

सोध सार

बालामुथिया म्यान्ड्रिलारिस (*Balamuthia mandrillaris*) एक उदयमान स्वतन्त्र जीवित अमिबा (FLA) हो जुन माटो, पानी, हावा, धुलो, कन्टयाक्ट लेन्स स्टोरेज केस, वितरण प्रणाली, पिउने पानी प्रशोधन संयन्त्र, र कूलिङ टावरहरू सहित वातावरणको विस्तृत दायरामा बस्छ । यो रोगजनक अमिबिक इन्सेफलाइटिसको लागि जिम्मेवार छ जसलाई बालामुथिय अमिबिक इन्सेफलाइटिस (बीएई) भनिन्छ जसमा यस संक्रामक एजेन्टको कारणले ९०% भन्दा बढी मामिलामा मृत्यु हुने गरेको छ। बालामुथिया म्यान्ड्रिलारिसको जीवविज्ञान र पर्यावरणीय पहिचान को बारेमा अझै पनि अपर्याप्त रूपमा बुझिएको छ, र यो अमिबाको वातावरणीय पर्यावरणीय पहिचान विरलै हुन्छ। यस अध्ययनको उद्देश्य काठमाडौं उपत्यका भित्रका विभिन्न स्थानबाट लिइएको माटोको नमूनामा बालामुथिया म्यान्ड्रिलारिसको उपस्थिति पत्ता लगाउनु हो। यो अध्ययनले आणविकीय दृष्टिकोण प्रयोग गरी विभिन्न माटोको वातावरणमा बालामुथिया म्यान्ड्रिलारिसको उपस्थितिलाई प्रभाव पार्ने कुराको पत्ता लगाउने, मूल्याङ्कन गर्ने लक्ष्य राखेको छ। अनुसन्धानका लागि २५ वटा माटोको नमूना सङ्कलन गरी ४ डिग्री सेल्सियस तापक्रममा जिप-लक प्लास्टिकको झोलामा भण्डारण गरिएको थियो। बालामुथिया म्यान्ड्रिलारिस माटोको नमूना र डीएनए एक्स्ट्र्याक्सन गरेर पीसीआर प्रवर्धनबाट पहिचान गरिएको थियो । सकारात्मक नमूनाहरू इलेक्ट्रोफोरेसिस मार्फत अलग गरिएको थियो, र अनुक्रम विश्लेषण र फाइलोजेनी द्वारा परिणाम प्रमाणित गरियो । मेगा ११ सफ्टवेयरमा अधिकतम सम्भाव्यता अनुमान (एमएलइ) र प्रतिस्थापन जुक्स क्यान्तोर मोडेलको प्रयोग गरी पूर्वानुमानित गरिएको बालामुथिया म्यान्ड्रिलारिस बीचको विकासवादी सम्बन्ध र आनुवंशिक सम्बन्धलाई पहिले प्रकाशित जीनबैंक अनुक्रमहरू प्रयोग गरी फाइलोजेनेटिक रूख निर्माण गरिएको थियो। पच्चिस वटा नमूनामध्ये पाँच वटा नमुनामा बालामुथिया विशिष्ट डीएनए पोजेटिभ देखिएको थियो जसमा दुई वटा छुट्टाछुट्टै नदी किनारको माटोबाट, एउटा पार्कबाट, एउटा व्यावसायिक नर्सरीबाट र एउटा बाख्रा फार्म परिसरको माटोबाट संकलन गरिएको थियो । अनुक्रमहरूको NCBI BLAST विश्लेषणले जापानबाट जीनबैंक डाटाबेसमा पहिले रिपोर्ट गरिएको बालामुथिया म्यान्ड्रिलारिस अनुक्रमसँग १००% होमोलोगि पुष्टि गर्यो। पच्चिस नमूनाहरू मध्ये, बालामुथिया म्यान्ड्रिलारिस आणविक विधि र अनुक्रम मार्फत पाँच (२०%) माटो नमूनाहरूमा फेला पऱ्यो जसले वातावरणीय बासस्थानहरूमा बालामुथियाको उल्लेखनीय रूपमा उच्च घटनाहरू देखाउँछ। यस खोजले बालामुथिया सँग सम्बन्धित जोखिमहरूको बारेमा जागरूकता बढाउने उद्देश्यले सार्वजनिक स्वास्थ्य गतिविधिहरूको महत्त्वलाई हाइलाइट गर्दछ।

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List of abbreviations

Abbreviated form	Details of abbreviations
BAE	*Balamuthia* Amoebic Encephalitis
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
dNTPs	Deoxynucleotide Triphosphate
FASTA	Fast Adaptive Shrinkage Threshold Algorithm
FLA	Free-Living Amoebae
GAE	Granulomatous Amoebic Encephalitis
GPS	Global Positioning System
MM	Master Mix
NFW	Nuclease-Free Water
NGS	Next Generation Sequencing
NNA	Non-Nutrient Agar
PCR	Polymerase Chain Reaction
qPCR	Quantitative Real time PCR
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
RT	Room Temperature
RT-PCR	Real-Time Polymerase Chain Reaction
TBE	Tris-Borate-EDTA
UV	Ultraviolet

1. Introduction

1.1 Background

Balamuthia mandrillaris is an emerging opportunistic Free-living amoeba (FLA) recognized for its high case fatality rate with over 90% cases results in death (Schuster et al., 2009). *B. mandrillaris* also called as "Brain eating amoeba" (Cope et al., 2019) are amphizoic in nature (Page, 1988) that is they are opportunistic or pathogenic (Javanmard et al., 2017; José Maschio et al., 2015; Pazoki et al., 2019; Soares et al., 2017). It was formerly identified as a leptomyxid amoeba and was discovered in the brain of mandrill baboon (*Papio sphinx*) who died at the San Diego Zoo Animal Park in California from meningoencephalitis (Siddiqui & Khan, 2008; Visvesvara et al., 1990). Later, it was identified as a novel protozoa in 1993 (Visvesvara et al., 1993) while remaining closed to *Acanthamoeba*, based on microscopy, pathogenicity testing, as well as antigenic and genetic analysis (Amaral Zettler et al., 2000; Booton et al., 2003; Visvesvara et al., 1993).

B. mandrillaris causes Granulomatous amoebic encephalitis (GAE) typically known as *Balamuthia* amoebic encephalitis (BAE) often with skin lesions (cutaneous Balamuthiasis) in both immunocompromised (associated with the cases of HIV/AIDS, alcoholism, drug users, cancer, diabetes, liver diseases, malignancies and malnutrition situations) and seemingly immunocompetent individuals (Badirzadeh et al., 2011; Jackson et al., 2014; Javanmard et al., 2017; Lorenzo-Morales et al., 2013; Niyyati et al., 2015; Pazoki et al., 2020; Schuster et al., 2009). The infection is typically associated with the exposure to contaminated soil or water, while gardening, agricultural activity, playing with mud, inhalation of cyst from soil, outdoor recreational pursuits, soil excavation, and contaminated water (Bravo & Seas, 2012; Dunnebacke et al., 2004; Maciver, 2007; Matin et al., 2008; Schuster et al., 2003, 2009; Siddiqui & Khan, 2008). This amoeba enters the human body through an open wound, lower respiratory tract or through oral and nasal passage, then enter the central nervous system via hematogenous dissemination (Cabello-Vílchez et al., 2014; Kiderlen & Laube, 2004; Lorenzo-Morales et al., 2013; Martinez & Visvesvara, 1997; Schuster & Visvesvara, 2004). Furthermore, organ transplantations are possible mode of infection (Basavaraju et al., 2014; Farnon et al., 2016; Gupte et al., 2014). The infection is rare, but due to its complicated and non-specific symptoms, as well as the lack of expertise among healthcare professionals, it is frequently misdiagnosed (Bhosale & Parija, 2021; Cope et al., 2019; Glaser et al., 2008).

Since the discovery, over 200 cases of human infection have been documented worldwide (Parija et al., 2015; Wang et al., 2020; Yohannan & Feldman, 2019). Along with human beings, this amoeba also infect dogs, horses, and other primates (Finnin et al., 2007; Hawkins et al., 2021; Hodge et al., 2011; Kinde et al., 1998; Rideout et al., 1997). The Americas have recorded the highest number of infections, especially in the Southwest of the USA (Arizona, Texas, and California), and Latin America, with cases documented in Mexico, Peru, Venezuela, Argentina, Brazil, and Chile (Lares-jiménez et al., 2014; Lorenzo-Morales et al., 2013; Yousuf et al., 2013). One hundred nine instances of infection have been reported till 2016 in the America (Cope et al., 2019; Yohannan & Feldman, 2019). Children and young men of Hispanic origin have been reported to comprise the majority of cases in the USA (Matin et al., 2008; Schuster et al., 2004). Few cases have been reported in Asia (Bando et al., 2012; Intalapaporn et al., 2004; Prasad et al., 2008), Australia (Doyle et al., 2011; Hill et al., 2011), and Europe in United Kingdom, Portugal, and Czech Republic (Jayasekera et al., 2004; Kodet et al., 1998; Tavares et al., 2006). This amoeba inhabit in diverse environment such as soil, water, dust, contact lens storage case, distribution system, drinking water treatment plant, and cooling towers (Rodríguez-Zaragoza, 1994; Schuster & Visvesvara, 2004). Globally, higher incidence observed in young (under 15 years) and elderly (over 60 years), possibly due to their weaker immune systems (Matin et al., 2008). Surprisingly, there are no reports of cases from Africa except an Ivory Coast investigation found a significant seroprevalence of *B. mandrillaris* in healthy individuals who showed no symptoms of the infection (Kiderlen et al., 2010). The most common habitat is presumed to be soil, where an abundance of nutrients allow *B. mandrillaris* to feed on other amoeba (Visvesvara et al., 2007). The first environmental isolation of *B. mandrillaris* was documented in Northern California from soil samples (Schuster et al., 2003). *B. mandrillaris* amoeba have lately been identified in soils both close and far from a BAE case (Dunnebacke et al., 2004; Schuster et al., 2003). The survival of this pathogen in the environment can be influenced by various factors such as soil type, moisture level, organic matter content, and human activities (Cabello-Vílchez et al., 2014; Khan & Siddiqui, 2015; Matin et al., 2008; Yamanouchi et al., 2018).

Over the years, numerous techniques developed to successfully diagnose *B. mandrillaris* infection includes Microscopy, Culture method, Immunodiagnostic and Molecular assays, Neuroimaging techniques, Metagenomics and Mass Spectrometry (Bhosale & Parija, 2021; Kiderlen et al., 2007; Tavares et al., 2006). Few media, such as BM-3 and modified Chang's

special medium, have been described for axenic cultivation of *B. mandrillaris*, but they require multiple components and laborious preparation. Non-nutrient agar (NNA) coated with bacterial cultures has been used for isolation, but it's ineffective in promoting growth; nonetheless, *B. mandrillaris* colonize in other amoeba plates (Niyyati et al., 2009; Schuster & Visvesvara, 2004; Siddiqui et al., 2008). Conventional PCR, real time PCR, and nested PCR have been used to detect the amoeba in both water and soil samples (Ahmad et al., 2011; Qvarnstrom et al., 2006) where nuclear 18S rRNA gene, the RNAse P gene, and mitochondrial 16S rRNA gene specific to *B. mandrillaris* are the common targets (Booton et al., 2003; Kiderlen et al., 2008; Qvarnstrom et al., 2006; Wang et al., 2020; Yagi et al., 2005). Next-generation sequencing (NGS) is an important diagnostic tool to identify the amoeba where raw sequencing data can be linked to the existing *B. mandrillaris* genome through bioinformatics analysis. Numbers of studies have recently detailed the use of NGS to confirm *B. mandrillaris* infection by focusing on the 18S and 16S rRNA genes (Greninger et al., 2015; Wu et al., 2020; Yang et al., 2020).

In Nepal, there is a lack of information regarding *B. mandrillaris*, and no specific research on molecular detection of this amoeba in soil samples from the Kathmandu Valley have been discovered in available studies. However, the global context suggests that *B. mandrillaris* is a rare but perhaps fatal amoeba. The Kathmandu Valley, with its dense human population and unique geographical characteristics may provide conditions for the survival and proliferation of soil-dwelling microorganisms. The discovery of *B. mandrillaris* in soil samples from the Kathmandu Valley can profound public health consequences, as it could indicate the presence of the amoeba in the environment and a risk of infection. Assessing the molecular detection of *B. mandrillaris* in soil samples from the Kathmandu Valley is critical for reducing the risk and expanding our understanding of this pathogen. By elucidating the detection, distribution, and environmental factors influencing the presence of *B. mandrillaris* in soil samples, this research aims to contribute to the broader understanding of this pathogen.

1.1.Statement of problem

B. mandrillaris, prevalent in environment, is a major concern to public health because it can cause serious and often deadly neurological infections. The Kathmandu Valley presents a unique ecological environment with diverse soil types, rapid urbanization and anthropogenic activities increases the risk of environmental contamination with *B. mandrillaris* and raises concern about potential risks of human exposure to the infection.

Despite the increasing recognition of *B. mandrillaris* as a potential pathogen, the detection and distribution of this amoeba in environmental samples remain largely unexplored in Kathmandu Valley which underscore the urgency of research in this area. The molecular identification and characterization of this amoeba from the soil samples in the Valley has not gotten sufficient attention up to the point that hinders accurate assessment of its presence and incidence. The lack of extensive research and surveillance efforts for *B. mandrillaris* poses a significant gap in our ability to assess the public health risk posed by this amoeba and to implement appropriate preventive measures against this emerging pathogen. In the absence of effective management strategies and targeted preventive measures, this amoeba has substantial economic consequences due to the high case fatality rate. With the increasing incidence of GAE cases worldwide, there is an urgent need to investigate the existence, distribution and molecular identification of *B. mandrillaris* from soil samples collected from diverse locations within Kathmandu Valley.

1.3. Objectives

1.3.1. General objective

- To determine the presence, and distribution of *B. mandrillaris* in diverse soil samples from Kathmandu Valley employing molecular identification technique.

1.3.2. Specific objectives

- To detect the presence *B. mandrillaris* in soil sample using molecular method i.e., PCR and sequencing analysis.
- To determine the distribution of *B. mandrillaris* across diverse soil settings, such as soil type, moisture, pH and temperature.

1.4. Research question

- Is *B. mandrillaris* present in soil samples from Kathmandu Valley?
- Does the distribution of *B. mandrillaris* vary depending on soil environment factors?
- Is there any association between the occurrence of *B. mandrillaris* and soil environments?

1.5. Significance of study

B. mandrillaris is an emerging pathogen with potentially devastating consequences for both human and animal health. This study investigates the existence, and distribution of *B. mandrillaris* in diverse soil environments within Kathmandu Valley, Nepal. By focusing on

soil samples, this study aims to uncover the environmental reservoirs of *B. mandrillaris*. Accurate detection of existence of this pathogen from soil sample is fundamental for assessing the true burden of infection and potential risk of exposure to the pathogen. The molecular technique employed for detecting and identifying this amoeba enhances the diagnostic capabilities for *B. mandrillaris* and is critical in assessing the risk of infection to humans and animals. Molecular approaches provide remarkable sensitivity and specificity when compared to traditional methods, which enables the detection of low-abundance pathogens and differentiation from closely related species. This study provides sequencing of *B. mandrillaris* genome, which will improve the sensitivity of sequencing-based efforts for the detection of this amoeba. The genomic data can also be used to guide the development of targeted assays for genotyping and detection. The investigation of *B. mandrillaris* in soil samples can shed light on various factors such as soil pH, temperature, and moisture levels that promote the growth and persistence of pathogen. The findings expand the understanding of *B. mandrillaris* in the Kathmandu Valley, contributing to the monitor and manage the pathogen's spread, particularly in locations where it is considered an emerging threat. The study adds to the scientific understanding of *B. mandrillaris* and provide novel insights into the environmental reservoir of the pathogen contributing to the broader field of public health. Overall, the study has significant implications for national programs and public health initiatives, for the development of effective prevention and control measures, as well as educational campaigns to raise awareness of the pathogen.

1.6. Limitations of study

- The study is restricted to the Kathmandu Valley, and samples are obtained from a limited geographical area inside the Valley, which may not properly represent the diversity of soil environments and geographic regions, where the pathogen could be present.
- Certain areas and soil types may be underestimated or not included in the study, limiting the generalization of the findings.
- The limited number of samples may not capture the true prevalence and diversity of pathogen in the region as well as the limited samples may be insufficient for statistical analysis.
- The scope of the study may not include other potential reservoirs or transmission routes of *B. mandrillaris* beyond soil, such as water.

2. Literature review

B. mandrillaris is the only species within the genus *Balamuthia* that is known to cause GAE (Cabello-Vílchez et al., 2014; Lorenzo-Morales et al., 2013). Worldwide, there have been over 200 cases of *B. mandrillaris* with very few survival even after treatment (Parija et al., 2015; Wang et al., 2020). This amoeba is distributed in all the continents globally but with limited occurrence in environmental sources. Several epidemiological investigations carried out in different countries throughout the world to detect the existence of *B. mandrillaris* in soil and other environmental samples, such as water and dust, have demonstrated the occurrence of this amoeba in several nations, such as Iraq, California, Iran, Guinea-Bissau, Costa Rica, Italy, United Kingdom, Portugal, Mexico, Peru, South Africa, Jamaica, and Pakistan (Otero-ruiz et al., 2022). The biological characteristics and environmental circulation of *B. mandrillaris* are still insufficiently understood, and environmental isolation constitutes a very uncommon event. The sporadic occurrence of this amoeba in environmental condition around the world makes it challenging to evaluate the true prevalence and distribution of *B. mandrillaris*, which presents difficulties in assessing its biology and isolation in the environmental settings (CDC, 2019).

2.1 Molecular detection of *B. mandrillaris*

Molecular detection of *B. mandrillaris* in the soil samples, primarily focus on the use of Polymerase Chain Reaction (PCR) and sequencing. The rarity of this pathogen combined with severity, frequently results in detection too late in environmental settings as well as medical interventions (Krasaelap et al., 2013). PCR is widely used and sensitive method for detection of *Balamuthia* specific DNA (Ahmad et al., 2011; Al-Aboody et al., 2021) even at a low concentrations. To limit the chance of nonspecific amplification and sample cross-reactivity, as well as to detect various strains, more than one target area of the pathogen's genome are amplified simultaneously (Kiderlen et al., 2008). The nuclear 18S rRNA gene, the RNAse P gene, and the mitochondrial 16SrRNA gene specific to *B. mandrillaris* are the common targets of molecular detection technique (Kiderlen et al., 2008; Qvarnstrom et al., 2006; Wang et al., 2020; Yagi et al., 2005). A study described the development of primers that produces a *Balamuthia*-specific PCR product using the mitochondrial small-subunit-rRNA (16S rRNA) gene, by utilizing DNA sequence information from the mitochondrial small-subunit-rRNA enabling for rapid detection of *B. mandrillaris*. The same study additionally investigated the DNA sequences of Nuclear

small-subunit-rRNA (rns) from various *B. mandrillaris* isolates and discovers no sequence variation among them, whereas mitochondrial rns exhibit variation among isolates (Booton et al., 2003). Similar method was employed to detect *B. mandrillaris* DNA in clinical specimen using PCR targeting the mitochondrial 16S rRNA gene in California and successfully detected *Balamuthia* in five clinical samples. The study discovered that *Balamuthia* DNA was detected in PCR mixtures including mitochondrial DNA, with only 1 to 0.2 amoeba per reaction mixture (Yagi et al., 2005). The study broadened the application of PCR as a potential technique for the quick identification of the pathogen infections in vivo and in clinical settings. Real-time PCR assays have been developed for the specific detection of *B. manrillaris* DNA in soil samples.

A multiplex real-time PCR technique for the simultaneous identification of *Acanthamoeba* spp., *B. mandrillaris*, and *Naegleria fowleri* in clinical samples was developed, as described in a study (Qvarnstrom et al., 2006). The assay was intended to target the 18S rRNA gene of each pathogen. The multiplex real-time PCR technique was validated with the DNA taken from each amoeba culture, demonstrating its ability to identify all three pathogens in a single reaction with high specificity and sensitivity. Similarly, a study described a real-time PCR method that targeted the RNase P gene, and showed great specificity and sensitivity in detecting at least two genomes of *B. manrillaris* DNA in varied samples. This assay was compared to a previously published real-time PCR targeting the 18S rRNA gene sequence of *B. mandrillaris*, as well as the duplex assay. This assay did not react with DNA from closely similar *Acanthamoeba* species or other pathogens, ensuring specificity and reliability (Kiderlen et al., 2008). Additionally, quantitative real-time PCR (qPCR) was utilized to detect FLA in soil samples from Turkey. The study used the quantitative real-time PCR test to determine the quantitative concentration of FLA in soil and demonstrated considerably greater incidence (45.71%) of FLA with 20% cases of *B. mandrillaris* (Aykur & Dagci, 2023). Furthermore, nested PCR approach was devised to detect FLA including *B. mandrillaris* in 186 soil and water samples collected from France, Southern California, South Africa, Italy, the United Kingdom, and Portugal. Two sets of primers were utilized in PCR amplification of mitochondrial 16S rDNA (ribosomal DNA), yielding 500bp with *Balamuthia* and the results were confirmed by sequencing of PCR product. Nested PCR was designed to yield 403 and 201 bp products from the outer and inner primer sets, respectively, resulting in 41% (77/186) positive cases of *B. mandrillaris*, in which 24.6% (19/77) positive samples were from soil samples. The study indicated that the advancement

of the nested PCR technique and DNA extraction allowed for a deeper comprehension of environmental distribution and public health risk of *B. mandrillaris* (Ahmad et al., 2011). *B. mandrillaris* is mainly confirmed using NGS together with bioinformatic analysis (Greninger et al., 2015; Qin et al., 2024; Wang et al., 2020).

Research in Northern California to assess the environmental isolation of *B. mandrillaris* documented the first-time isolation of this amoeba from soil associated with a fatal case. The study extracted DNA from the 18 different soil and water samples collected from the surrounding and indoor areas of the child home who died of the amoebic encephalitis, and cultivated the samples in NNA growth media then confirm the presence of *B*. *mandrillaris* by PCR and sequencing of 18S rRNA gene that produced amplimer of 230 bp. *B*. *mandrillaris* positive sample was obtained from the potted plant soil. The study highlighted the significance of molecular techniques in detecting and characterizing *B. mandrillaris* from environment (Schuster et al., 2003). Similar microbiological and molecular techniques have been followed to detect *B*. *mandrillaris* from soil and water samples in various studies conducted in the Americas. Studies carried out in Costa Rica (24 dust and 12 water samples) and Mexico (10 water and 10 soil samples) revealed the presence of *B. mandrillaris* in 1 dust sample from Costa Rica (Retana-moreira et al., 2014) and, 7 water and 1 soil sample from Mexico (Lares-jiménez et al., 2014). The detection was carried out via NNA culture, PCR amplification of the mitochondrial 16S rRNA gene, producing 1075bp, and sequencing, which confirmed 97% similarity with known GenBank sequences indicating accuracy of PCR method. Other studies, such as in Peruvian soils (Cabello-vílchez et al., 2014) and Jamaican soils (Todd et al., 2015) have confirmed the presence of *B. mandrillaris* in soil environments by NNA culture, PCR amplification of 16S rDNA (ribosomal DNA), and sequencing to validate the presence exhibiting 97% to 100% homology.

In the Asian countries several studies have assessed the presence of *B. mandrillaris* in environmental settings. Studies from Northern Iran (Niyyati et al., 2009) and North-Western Iran (Niyyati et al., 2015) have investigated the occurrence of *B. mandrillaris* in dust samples and soil samples respectively. The study employed culture method in NNA plate, PCR amplification of 16S rRNA in North Iran and 16S rDNA in North-Western Iran, and confirmed the presence by sequencing which showed sequence homology between 93-96% (northern Iran) and 99-100% (north-western Iran). The findings in Northern Iran showed a previously unidentified environmental reservoir for the pathogen, as well as an

urgent need for improved techniques to extract the amoeba from its environment in North-Western Iran. Another investigation in Northern Iran, carried out in mineral and hot spring water samples (66), found two (3.03%) *B. mandrillaris* by PCR amplification of 16S rRNA and revealed sequence homology between 96-99% GenBank sequences (Latifi et al., 2016). An investigation assessed the prevalence of rotavirus and FLA in 20 drinking water filtration plants and residential drinking water samples in Karachi, Pakistan. The pathogen was confirmed by NNA culture technique, PCR amplification producing a 171 bp amplicon, and sequencing to confirm the pathogen. Thirteen (65%) of the samples from the filtration plant tested positive for *Acanthamoeba* spp., and 1 (5%) tested positive for *B. mandrillaris* with no cases of *B. mandrillaris* in drinking water sources (Yousuf et al., 2017). A study employed PCR amplification of the mitochondrial rRNA gene that generated 1075 bp PCR product, to identify *B. mandrillaris* in 75 soil samples in the west Asian nation of Iraq. Sequencing verified that five water and four soil samples were positive for *B. mandrillaris* (Al-Aboody et al., 2021). A related survey conducted in Japan on 13 soil samples successfully verified that 1 of the samples had PCR amplification products specific to *Balamuthia*. These *Balamuthia* strains had 99% homology to previously reported *B. mandrillaris*.

In Europe, a child's clinical specimen infected with amoebic encephalitis in California was compared to two distant soils samples from Great Britain. Antimicrobial sensitivity, immunological response, growth circumstances, and overall morphology were all compared in the study. The presence of *Balamuthia* DNA was confirmed by traditional culture method using NNA plate, PCR amplification and 16s rRNA gene sequencing in a soil sample that yielded an amplimer of 1075 bp which was comparable to that of the two prior isolates from California, SAm and RP5 (Dunnebacke et al., 2004). The nested PCR approach was implemented to detect *B. mandrillaris* in environmental samples from Italy, the United Kingdom, and Portugal in a combine study with Southern California and South Africa. The study suggested the discovery of an environmental DNA extraction method and nested PCR can address environmental distribution and may contribute in the development of better isolation procedures (Ahmad et al., 2011).

Several studies have been done to discover the presence of *B. mandrillaris* in African nations' environmental sources. In a study conducted in Guinea-Bissau, Western Africa, the prevalence of potentially pathogenic FLA, including *B. mandrillaris*, was assessed in 22 well water samples. Of these, 15 (68.2%) water samples tested positive for FLA. A

combination of NNA culture, DNA extraction, PCR amplification, and gene sequencing was used to identify the amoeba strain. The 16S rDNA gene was amplified and sequenced to identify *B. mandrillaris*. The results showed a single sample producing a 1075 bp product, indicating a low frequency of the pathogen prevalence. The results indicated the need of better culture procedures for isolating and cultivating the amoeba in axenic environments (Baquero et al., 2014). An additional investigation focused on the existence of *B. mandrillaris* and other FLA in South Africa's hospital water distribution system. Temperature, pH, residual chlorine, and total dissolved solids were measured at the collection site. The study analyzed 71 water and biofilm samples using microscopy and molecular techniques, confirming the presence of FLA in 63 (88.7%) of them and *B. mandrillaris* in 12 (19.4%). The study emphasized the need of development of enhanced culture techniques and detecting methods (Muchesa et al., 2015).

2.2 Distribution of *B. mandrillaris* in various environmental conditions

The particular distribution, niche, or preferred food source of *B. mandrillaris* in the environmental sources remains unknown despite minimal success. This might be due to a variety of aspects, such as the fact that *B. mandrillaris* amoeba are less common in the environment than other FLA, that they are slow-growing and difficult to isolate, or a combination of the above (Bhosale & Parija, 2021; Cope et al., 2019; Matin et al., 2008). Regardless, many research carried out worldwide have documented its existence in a variety of environmental settings and situations (Figure 1).

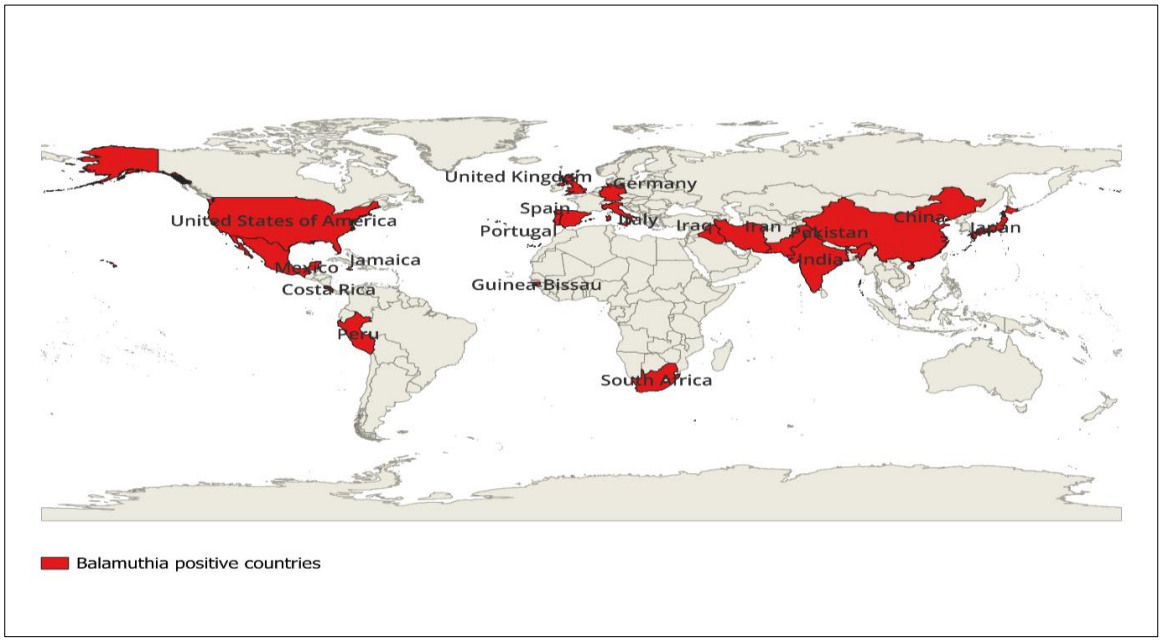

Figure 1. Map showing *B. mandrillaris* detected countries globally

The first environmental isolation of *B. mandrillaris* was related to fatal case of amoebic encephalitis from the flowerpot soil sample taken from the surrounding of deceased person (Schuster et al., 2003). The Central American country, Costa Rica revealed the amoeba in dust sample (Retana-moreira et al., 2014). Mexico identified eight new strains of *B. mandrillaris* in samples of nursery soil and artificial lagoon water, broadening the range of its ubiquity (Lares-jiménez et al., 2014). Research conducted in various parts of the Americas, such as Peru, found *B. mandrillaris* in soil samples from rose gardens, deserts, and dried forests (Cabello-vílchez et al., 2014), and Jamaica identified the amoeba in a soil sample from a mineral spring where local residents and visitors use the therapeutic mud (Todd et al., 2015).

Asian continent have documented the existence of *B. mandrillaris* in a variety of environments such as in Tehran, Iran dust samples from large buildings in urban settings without prior involvement of infection (Niyyati et al., 2009), East Azerbaijan, North-Western Iran in soil samples from parks, university gardens, and a school that receives frequent visitors (Niyyati et al., 2015), water samples from hot springs in Mazandaran province, Northern Iran (a hotel spa with a pH of 4.2 and a temperature of 42°C, and a bridge spa with a pH of 3.5 and a temperature of 32°C) (Latifi et al., 2016), Pakistan in a water filtration plant, indicating post-treatment as a source of infection (Yousuf et al., 2017), Iraq in soil samples with bird and lizard wastes, as well as water samples from river, tank, stagnant water, and air conditioning water (Al-Aboody et al., 2021), and Japan in soil samples from farms and paddy fields in a heavy snow region, indicating a wider habitat range (Yamanouchi et al., 2018). A recent study in Japan attempted the soil analysis to determine the growth conditions of *B. mandrillaris* as previous attempts of extracting *B. mandrillaris* from soil in Japan using the culture method with BM3 medium and COS-7 cells did not succeed. The results revealed *B. mandrillaris* positive soils were alkaline, with high electrical conductivity and nutrient-rich loam and clay loam features, which may contribute to the high frequency of this amoeba causing GAE among people working in agriculture-related occupations (Yamanouchi et al., 2024).

In Great Britain, *B. mandrillaris* was detected in soil samples from unrelated outdoor potted plants (Dunnebacke et al., 2004). Similarly other nations in European continent have documented the environmental identification of *B. mandrillaris* from water, water with sediments and soil samples such as Italy, United Kingdom, and Portugal (Ahmad et al., 2011). In African continent a study conducted in West Africa, Guinea Bissau *B.*

mandrillaris was reported in a water sample which is often flooded by high tides during rainy season and high salinity (Baquero et al., 2014). Likewise, *B. mandrillaris* was found in water and swab samples from hospital showerheads and taps in Johannesburg, South Africa (Muchesa et al., 2015). The temperature range of the water was 19.0 °C to 27.0 °C (mean = 23.1 °C), with a pH range of 7.5 to 8.0 (mean = 7.9), total dissolved solids of 110 mg/L to 187 mg/L (mean = 109 mg/L), and residual chlorine of 0.04 mg/L to 0.17 mg/L (mean = 0.08 mg/L). The study suggested that the comparatively lower temperatures of the water samples may have contributed to the high prevalence outcome in their investigation. Though the limited occurrence of *B. mandrillaris*, isolation from environmental sources worldwide, indicate its presence in diverse geographic regions (Khan & Siddiqui, 2015).

The molecular detection techniques for various FLA have advanced substantially, yet there is still a lack of research regarding the environmental isolation of pathogenic *B. mandrillaris* in an urban area like the Kathmandu Valley. However, this amoeba has been identified as a rare but potentially dangerous pathogen, the existing research are primarily focused on clinical cases. As a result, there is a lack of comprehensive data on the geographic distribution and potential factors related with *B. mandrillaris* in the soil environment, which restricts the effective public health interventions and control approaches. There has been a lack of investigation into the existence, distribution, and prevalence of this amoeba in soil samples from around the world. Thus, through investigating the molecular identification of *B. mandrillaris* in soil samples from the Kathmandu Valley, this study intends to gain an exhaustive knowledge of the pathogen's presence and prevalence.

3. Materials and methods

3.1 Study area

Nepal, a country with a vast geographical diversity, is divided into three major regions: terai, hilly, and Himalayan. The country covers 147,516 square kilometers, stretching 885 kilometers from east to west. It is situated between longitudes 80° and 88°E and latitudes 26° and 31°N.

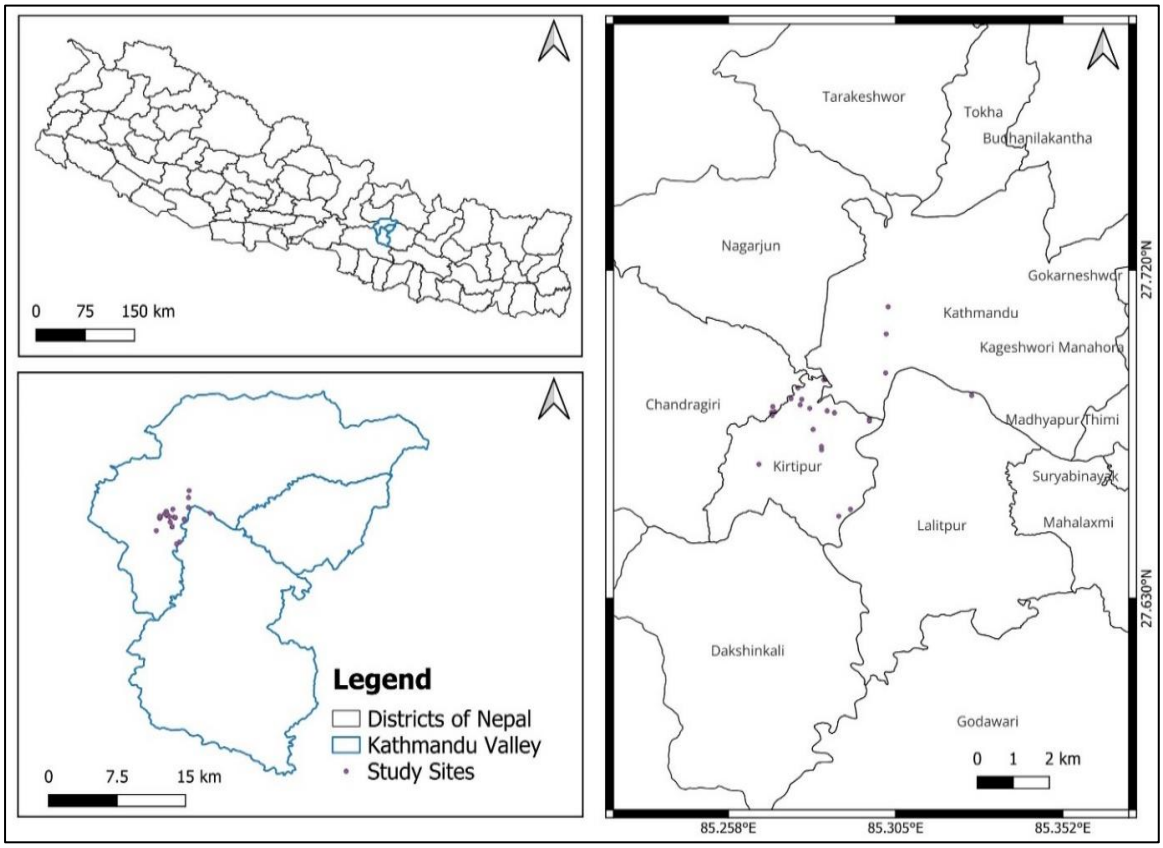

Figure 2. Map showing study area, the Kathmandu Valley

Kathmandu Valley is a bowl-shaped Valley in central Nepal that covers an area of 570 square kilometers (220 square meters). This Valley is located in Nepal's Himalayan mountains between latitudes 27°32'13" and 27°49'10" N and longitudes 85°11'31" and 85°31'38" E. The Valley has an average elevation of 1,200 meters above sea level and measures approximately 25 kilometers long and 15 kilometers wide. The Valley comprises of three districts Kathmandu, Lalitpur, and Bhaktapur. The average summer temperature in the Kathmandu Valley ranges from 20°C and 35°C, while the average winter temperature is between 2°C and 12°C. The Kathmandu Valley is densely populated urban area with a variety of land uses such as residential, commercial, open water, agricultural area, forestry,

recreation, and transportation, which may enhance the likelihood of pathogen contamination in the environment.

3.2. Research design

The study design of this study entails the selection of study area within the Kathmandu Valley and collection of soil samples from the study locations. Data on various soil characteristics were noted. The samples were transported to the laboratory and stored at 4°C. The sample were then analyzed through DNA extraction, PCR amplification, DNA sequencing and phylogenetic analysis. The obtained results were analyzed and interpreted (Figure 3).

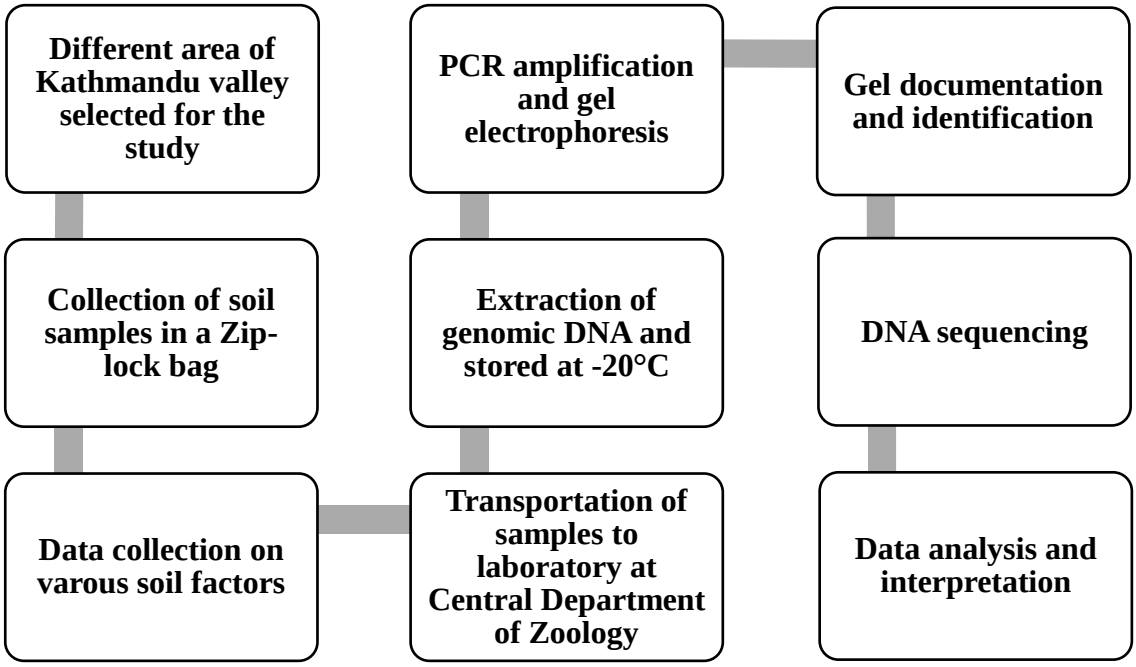

Figure 3. Flow diagram showing framework of study

3.3. Sampling site selection

A comprehensive literature review was carried out initially to learn about previous sites where studies were carried out and *B. mandrillaris* was discovered. Twenty-five sampling sites from 25 different locations of the Kathmandu Valley were selected through purposive sampling methos and multiple sampling points within a study site were also selected randomly to minimize the biasness and ensure the representative sampling. Various soil characteristics such as soil type, moisture level, pH, temperature, light condition and GPS (Global positioning system) coordinates were carefully considered. The soil samples were collected from diverse environment such as agricultural land, commercial nursery,

vegetable garden and farms, animal husbandry area, grazing area, park, school playground, dumping site, animal farm premises, river banks flower garden, paddy field, and kitchen garden (Table 1).

Table 1: Different study area in the Kathmandu Valley

Sample no.	Study area
1	Goat farm premises, Kirtipur
2	Goat farm premises, Kalanki
3	Commercial nursery, TU road, Balkhu
4	Commercial nursery, TU road, Balkhu
5	Commercial vegetable farm, Kirtipur
6	Commercial vegetable farm, Kirtipur
7	Naga vegetable farm, Kirtipur
8	Cow farm premises, Kirtipur
9	Balkhu river, Kirtipur
10	Grazing area, Kirtipur
11	Bagmati river bank, Kirtipur
12	Park, Dallu, Kathmandu
13	Bishnumati river bank, Kalimati
14	Park, Lalitpur
15	Teku dovan, Kathmandu
16	Animal farm premises, Kirtipur
17	Cow farm premises, Kirtipur
18	Kitchen garden, Kirtipur
19	Kitchen garden, Kirtipur
20	Rose garden, Tribhuvan university, Kirtipur
21	Coronation garden, Tribhuvan university, Kirtipur
22	Paddy field, Bhajangal, Kirtipur
23	Vegetable farm, Panga, Kirtipur
24	School playground, Kirtipur
25	Dumping site premises, Kirtipur

3.4 Field sampling, transportation, and preservation

The sample collection for the study was conducted between December 2023 to February 2024. The flow diagram of the sample collection, transportation, and preservation is shown in the (Figure 4). First, clean sampling equipment like auger, spade, sampling zip-lock bag, scoops, gloves, and plastic bags were assembled and disinfected using 70% alcohol. In the

sampling field multiple sampling points within a site were selected. The zip-lock bag used to keep the sample was labelled properly with unique identifier including sampling site location, date, and time. Next, removed all the debris like leaves, twigs, grasses, plastic garbage, pebbles, and stones from the periphery and a sterile spade or auger was used to dig in the ground at a depth of about 10 cm. In a note book, details such as the date, location, pH, temperature, moisture content, light and location coordinates were noted. With the help of a scoop or auger about 100 gram of soil sample was collected in total from all the sampling points and mixed thoroughly in a clean newspaper then put in a labelled zip lock bag. To minimize the risk of exposure and contamination, protective masks and gloves were worn during field procedure. The samples were brought to the laboratory at Central Department of Zoology in Kirtipur, Kathmandu within two hours of collection and stored in refrigerator at 4°C for the further laboratory analysis.

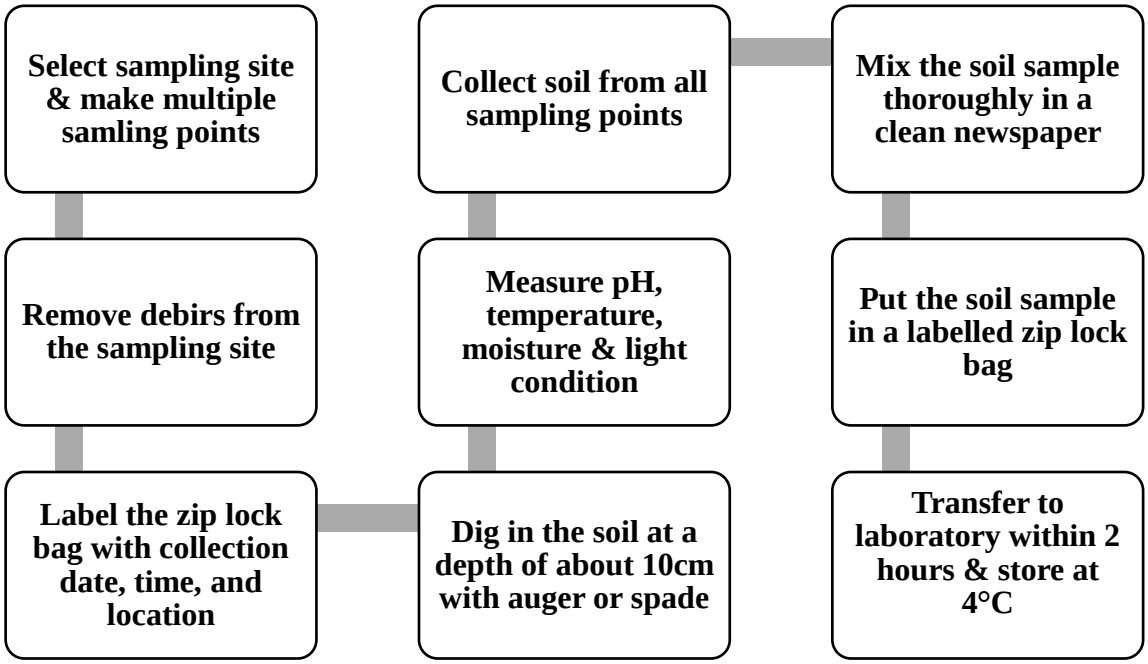

Figure 4. Flow diagram showing sample collection and preservation method

3.5 DNA extraction from soil

The DNeasy PowerMax Soil Kit (Cat. no. 12988) from QIAGEN was used to extract DNA from the soil samples following their protocol (QIAGEN, 2018). In a PowerMax Bead Tube, 15 ml of PowerBead solution was added. 10g of soil sample was weighted and added to the PowerMax Bead Tube containing PowerBead solution. The tube was vortexed vigorously for one minute. Next, 1.5ml of solution C1 was added to the PowerMax Bead Tube and vortexed vigorously for 30 seconds. The PowerMax Bead Tube was then placed

in a water bath set at 65°C and shake at the interval of 2 minutes for 30 minutes. The tube was centrifuged at 2500 x g rpm for 3 minutes at room temperature (RT) (25°). The supernatant was transferred to a labelled clean collection tube avoiding pellet and added 5ml of solution C2. The tube was inverted twice to mix and incubated at 2-8°C in ice for 10 minutes. After incubation, it was centrifuged at 2500 x g rpm for 4 minutes at RT and the supernatant was transferred to a labelled clean collection tube avoiding pellet. Next, 4ml of solution C3 was added and inverted twice to mix then incubated at 2-8°C for 10 minutes in ice.

The C4 solution was shake to mix and pipetted out 30ml to the supernatant then inverted twice to mix. The supernatant was then filled in an MB Maxi Spin Column tubes and centrifuged tubes at 2500 x g for 2 minutes at RT. Discarded the flow-through and the tube was cleaned by gently tapping inverted tube in tissue. Second volume of supernatant was added to the same MB Maxi Spin Column and centrifuge again at 2500 x g for 2 minutes at RT. The process was repeated until entire volume had been processed which took up to 4 total spins. After all the volume of supernatant was processed, 10ml of solution C5 was pipetted out and centrifuged at 2500 x g for 3 minutes at RT then, discarded the flow-through. The tube was again centrifuged 2500 x g for 5 minutes at RT. The MB Maxi Spin Column was carefully placed in a new Collection Tube, avoiding the splashing solution C5 onto the column. Then, 5ml of sterile solution C6 was added to the center of MB Maxi Spin Column membrane and centrifuged at 2500 x g for 3 minutes at RT. The MB Maxi Spin Column was discarded and the DNA was ready for concentration and PCR amplification. The isolated DNA was stored at -20°C.

3.6 Concentration of eluted DNA

The final volume of isolated DNA was 5 ml. DNA concentration is mainly performed for the quantification of DNA, to assess sample quality, and ensure DNA purity (Gupta, 2019). The extracted DNA was taken out from freezer and kept outside until the solution reached to RT. In the laboratory 5M NaCl was prepared, and 70% and 100% ethanol were cooled in a refrigerator. Then, 0.2ml (200µl) of 5M NaCl was added with a help of pipette in tube containing DNA and inverted 3-5 times to mix. Afterward, 10.4 ml of 100% cold ethanol was added to the tube and inverted 3-5 times to mix. The tube was centrifuged at 2500 x g for 5 minutes at RT and decanted all the liquid. By this point, the DNA pellet was visible at the tube's side or bottom walls. The pellet was washed with 5ml of 70% ethanol. To remove the remaining liquid without disturbing the pellet, gently invert the tube and tapped

it on a piece of clean tissue paper. The remaining liquid was air dried and the precipitated DNA was resuspended in 100µl of sterile 10mM Tris i.e., C6 solution. Finally, the concentrated DNA was stored at -20°C to prevent degradation (QIAGEN, 2018).

3.7 Polymerase Chain Reaction (PCR)

Conventional PCR amplification of all the concentrated DNA was performed with *Balamuthia* specific primer provided by Dr. Arima from Japan. PCR amplification was carried out using the primer set, 5' Balspec 16S and Balspec 16Sr 610 (Itoh et al., 2015) which amplifies 230bp portion of the mitochondrial 16s ribosomal DNA (rDNA) gene from putative *B. mandrillaris* (Table 2). The PCR products were electrophoresed on a 2% agarose gel, and stained with Ethidium Bromide (EtBr) (Yamanouchi et al., 2018).

Table 2: Primers used in PCR amplification

Name of primers	Sequence of primer	Size
5' Balspec 16S	5'-CGCATGTATGAAAGAAGACCA-3'	230bp
Balspec 16Sr 610	5'-CCCCTTTTTAACTCTAGTCATATGT-3'	

3.7.1. Preparation of Master mix (MM) and PCR mixture

The master mix (MM) typically consists of components required for PCR such as Taq DNA polymerase enzyme, buffer solution, nucleotides (dNTPs), primer set targeting specific regions of putative pathogen, and Nuclease-free water (NFW). All the chemicals were carefully calculated and mixed in right quantities to achieve the optimal amplification efficiency and specificity (Table 3). The MM was prepared using Premix Taq (Ex Taq version 2.0) which was a premade mixture. The Premix Taq included an optimized buffer, PCR enzyme, dNTPs mixture, gel loading dye (green), and a density reagent in a 2× premix format (Yamanouchi et al., 2018).

Table 3: Reagents and volume of Master mix (MM) used to make 50µl of PCR mixture

Component	Volume (µl) per reaction
	50µl reaction
Taq (Ex Taq version 2.0)	25µl
Primer (Forward)	1µl
Primer (Reverse)	1µl
DNA template	20µl
Nuclease-free water	3 µl

To make PCR mixture in total volume 50 µl

A 27µl of MM for each single sample included 25µl Premix Taq, 1µl of forward primer, and 1µl of reverse primer (Table 3). This volume of mixture was multiplied by the number of DNA samples using for PCR, plus an additional one amount for probable losses during pipetting. All the reagents were taken out from the freezer and placed in an ice filled clean container while they were not in use. A PCR tube was labeled where the reagents were pipetted out precisely and tapped gently to mix. Finally, the MM was ready to use. While running PCR, 20µl of template DNA and remaining portion of nuclease-free water were added in 27µl of MM to make 50µl of PCR mixture. For a positive control (*Balamuthia* positive sample), 1µl of DNA was added to 27µl of MM with the remaining of the nuclease-free water. In the negative control, 27µl of MM and the remaining volume of water were added to make a 50µl PCR mixture excluding DNA.

3.7.2. Thermal cycler condition of PCR amplification

The PCR cycles were set up in a thermal cycler (MyGene™ I Series Pelter Thermal Cycle) and run the machine. The thermal cyclic condition applied during the course of PCR was 39 cycles of initiation at 95°C for 5 minutes, thermal denaturation at 95°C for 30s, annealing at 50°C for 1 minute, extension at 72°C for 2 minutes, and final extension at 72°C for 15 minutes (Table 4). Overall, the PCR amplification took about 3 hours and 12 minutes to complete (Yamanouchi et al., 2018).

Table 4: Thermal cycler conditions used for PCR

Stages	Program	Temperature (°C)	Time	Cycle
Stage I	Initiation	95°C	5 minutes	1 cycle
Stage II	Denaturation	95°C	30 seconds	39 cycles
Stage III	Annealing	50°C	1 minute	
Stage IV	Extension	72°C	2 minutes	
Stage V	Final extension	72°C	15 minutes	1 cycle
Stage VI	Holding	10°C	∞	Infinity

3.7.3. Gel electrophoresis

Following PCR, the amplified DNA products were separated, and bands were confirmed by electrophoresis on a 2% agarose gel. For the preparation of agarose gel, 0.8 gram of agarose powder was mixed with 40 ml of 1× TBE (Tris-borate-EDTA) buffer into an

Erlenmeyer flask. The mixture was heated in an oven without boiling until the agarose dissolved completely in the buffer then allowed to cool to room temperature. Next, 4µl Ethidium Bromide (EtBr) was added carefully to the mixture and shaken vigorously. The agarose gel was left to cool on the benchtop for a while until it was comfortable to touch with hand. A mold was created by taping the open sides of a casting tray. After that, an appropriate comb was inserted into the casting tray to create the wells, and gel was poured into it. The agarose gel was allowed to set at room temperature and the comb was removed upon the complete setting of gel. The gel was placed in the electrophoresis tank filled with the same buffer used to prepare the gel (Yamanouchi et al., 2018).

3.7.4. Loading of sample and running the gel

The loading dye, DNA ladder, DNA sample, and PCR-amplified samples were taken in a box containing ice. In the first well 6µl of DNA ladder of size 1500 bp was loaded with micropipette. In a piece of parafilm, 10µl DNA sample was mixed with 2µl of loading dye and loaded in the well subsequent to the ladder. After loading all the DNA samples, same process was repeated to load the positive and negative control. While performing electrophoresis with various samples, the positive and negative controls were put in the wells in varied ways. The leads of the gel tank were closed and connected to the power source once the loading was finished. The desired voltage and time were set on the program and turn on the power supply. The gel was run at 120 volts for 30 minutes. To make sure everything was functioning, the gel box and power supply were checked timely (Yamanouchi et al., 2018).

3.7.5. Gel documentation of separated DNA fragments

When the electrophoresis was completed, the power supply was turned off, and the gel tank's cover was removed carefully without disturbing the gel and the tank. The gel from the tank was removed, and excess buffer was drained off the gel surface by slightly tilting the gel. The gel was carefully laid down on the transilluminator surface of the gel documentation system and was exposed to UV light. The result was observed using UVITEC software (version UVI. After use, the gel and the running buffer were properly disposed (Yamanouchi et al., 2018).

3.8 Sequencing of PCR positive sample and building phylogenetic tree

The sequencing was performed in National Academy of Science and Technology (NAST), Lalitpur with the help of Dr. Ram Chandra Poudel. In the completion of PCR and gel

electrophoresis, the positive samples were further confirmed and validated through sequencing and phylogenetic analysis. The sequencing was performed using the same forward primer as previously used in PCR amplification. The raw DNA sequence data were assembled, aligned, and conducted the evolutionary analysis.

3.8.1 Sequence editing

The raw sequence data obtained from NAST were trimmed at their end and beginning to remove the low-quality reads and kept the region of high-quality bases using the BioEdit sequence alignment editor software (BioEdit version 7.2.5) and assembled into a FASTA file.

3.8.2 BLAST (Basic Local Alignment Search Tool) analysis

The edited sequences were compared to the known sequences present in the NCBI GenBank to determine species origin by performing BLAST. The NCBI BLAST webpage was accessed and selected the nucleotides BLAST. The previously edited sequence in FASTA file was entered and optimize was selected for highly similar sequences in program selection menu. On the submission of all necessary information BLAST button was clicked to submit the search of closely related sequences. BLAST create an alignment between the query sequence and closely related sequences and score the best possible alignment between the query sequence and database sequence as Max score. Total score provides the overall measure of the similarity between the query and database sequences. BLAST calculate the statistical significance value for each alignment as expected value (E value). The E value represents the probability of finding a match between a query sequence and a database sequence only by chance.

The BLAST generated a list of B. *mandrillaris* sequences deposited previously on GenBank that were similar to the query sequences. The genome showing highest percentage of similarity were selected for multiple sequence alignment randomly and downloaded in FASTA (aligned sequence) format (Zhang et al., 2000). The (Table 5) shows the *B. mandrillaris* sequences selected for analysis. Sequence alignments between GenBank and this study sequences were preformed using ClustalW tool in MEGA 11 software (MEGA version 11.0.13).

Table 5: List of *B. mandrillaris* sequence data used in analysis

Species	Country	Year	Accession number
Balamuthia mandrillaris	Japan	2018	LC349294
Balamuthia mandrillaris	Japan	2023	LC348995
Balamuthia mandrillaris	Peru	2023	OR528011
Balamuthia mandrillaris	Iran	2016	KR908790
Balamuthia mandrillaris	USA	2015	KT030673
Balamuthia mandrillaris	USA	2023	NC_027736
Balamuthia mandrillaris	Peru	2022	OP941128
Balamuthia mandrillaris	Hongkong	2022	OM994889
Balamuthia mandrillaris	Japan	2022	LC706484
Balamuthia mandrillaris	Peru	2021	MN176994
Balamuthia mandrillaris	India	2013	KF246746
Balamuthia mandrillaris	USA	2022	JX524851
Balamuthia mandrillaris	Spain	2014	KJ000399
Balamuthia mandrillaris	Germany	2012	HF558614
Balamuthia mandrillaris	Japan	2013	AB795712
Balamuthia mandrillaris	Malaysia	2017	KX257198
Balamuthia mandrillaris	Russia	2018	MK100243
Balamuthia mandrillaris	India	2018	MF563608
Balamuthia mandrillaris	Malaysia	2017	KX257197
Balamuthia mandrillaris	USA	2015	KT185628
Balamuthia mandrillaris	Korea	2008	EU572722
Balamuthia mandrillaris	Spain	2014	KJ439568

3.8.3 Phylogenetic tree construction

Phylogenetic tree was constructed in MEGA 11 using Neighbor-Joining method and substitution Jukes-Cantor model to confirm the genetic association and evolutionary relationship of *B. mandrillaris*. Bootstrapping with 1000 replications was used to verify the tree topology. The selection of 1000 bootstrap replicates was based on the principle that a greater number of replicates provides a more accurate estimate of the support for a given clade or branches (Holmes, 2003). Genetic divergence was estimated using pairwise distance method and Jukes-Cantor model to understand the genetic distance between species.

4. Results

3.1 Distribution of *B. mandrillaris* in various soil environments

Out of twenty-five soil samples collected from different soil environment, five of the soil samples tested positive for *B. mandrillaris* which were identified by *Balamuthia*-specific PCR amplification and DNA sequencing. The results showed that 20% of the pathogen was detected in samples of soil numbers 1, 3, 11, 13, and 14 which is significantly a higher incidence of this pathogen. The first positive sample (1) was collected from the premises of a local goat farm in Kirtipur, where the surrounding area was filthy with animal manure and waste materials. The sample 3 was collected from the soil of a commercial nursery in Kathmandu where the soil was used in flowerpots. Two positive samples, 11 and 13, were taken accordingly from the Bagmati river bank soil in Kirtipur and the Bishnumati river bank in Kathmandu. The river banks were severely polluted from excessive garbage and waste disposal on the periphery by human individuals. One of the positive samples, 14, was collected at a park in Lalitpur which exhibits an abundance of human activity (Figure 5).

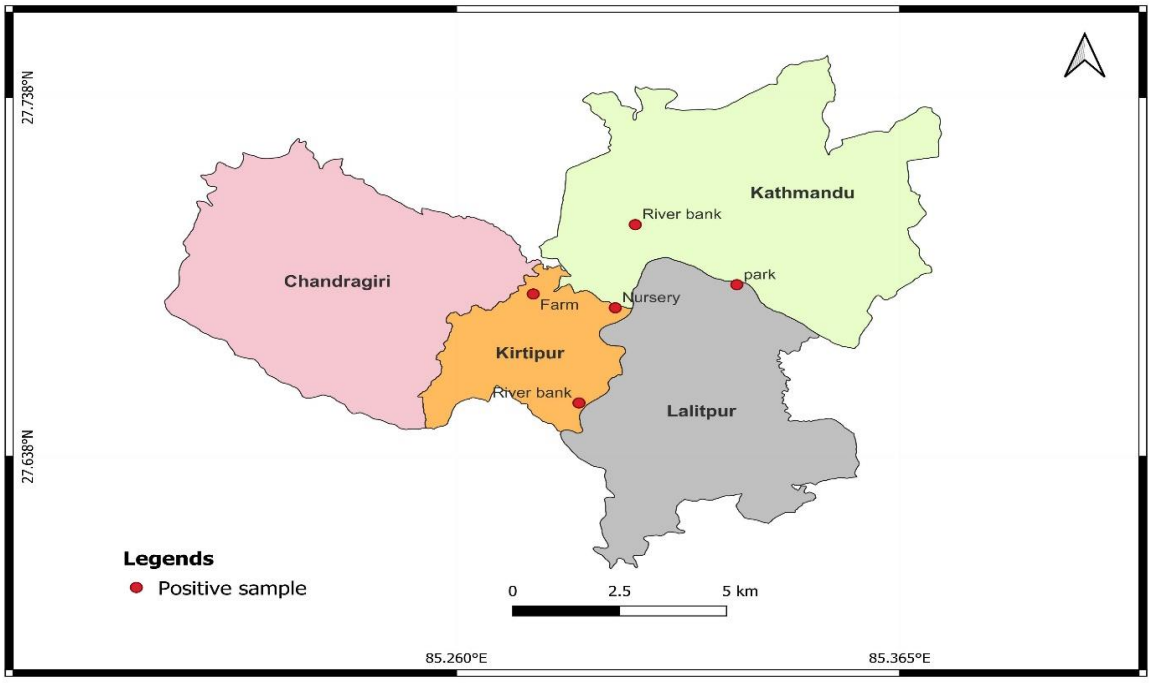

Figure 5. Map showing *B. mandrillaris* positive sites

The soil parameters such as pH, temperature (in °C), moisture, and light condition of the samples collected from the Kathmandu Valley are presented in (Table 6). The mean pH of the soil sample is 6.92 with a median value of 7 & and standard deviation of 0.3. Soil temperature ranged between 13°C and 23°C. The mean temperature is calculated to be

17.12°C, with a median of 17°C, and standard deviation of 2.58°C. Major soil samples were dry indicating low level of moisture while only a few samples are wet. These statistics provide insight into the environmental condition of soil samples (Table 6).

Table 6: Characteristics of soil samples collected from study area

Sample code.	pH	Temperature (°C)	Moisture (Normal, wet and dry)	Light (Normal and low)	Results
TS1	7	15°C	Wet	Low	Positive
TS2	7	20°C	Wet	Low	Negative
TS3	7	17°C	Normal	Low	Positive
TS4	5	23°C	Wet	Normal	Negative
TS5	7	17°C	Dry	Low	Negative
TS6	7	17°C	Dry	Low	Negative
TS7	7	16°C	Dry	Low	Negative
TS8	7	18°C	Dry	Low	Negative
TS9	7	17°C	Dry	Low	Negative
TS10	7	18°C	Dry	Low	Negative
TS11	7	17°C	Wet	Low	Positive
TS12	7	13°C	Dry	Low	Negative
TS13	7	14°C	Wet	Normal	Positive
TS14	7	18°C	Dry	Normal	Positive
TS15	7	18°C	Normal	Low	Negative
TS16	7	21°C	Dry	Low	Negative
TS17	7	15°C	Wet	Normal	Negative
TS18	7	18°C	Dry	Low	Negative
TS19	7	17°C	Dry	Low	Negative
TS20	7	18°C	Dry	Low	Negative
TS21	7	18°C	Dry	Low	Negative
TS22	7	14°C	Wet	Low	Negative
TS23	7	18°C	Dry	Low	Negative
TS24	7	14°C	Dry	Low	Negative
TS25	7	17°C	Dry	Low	Negative

The sample taken from the goat farm's surroundings had a pH of 7, was comparatively wet, had a temperature of 15°C, and was exposed to normal levels of light. The pH of the soil sample that was taken from a commercial nursery was 7 and it was 15°C. The soil was normally moist soil with low exposure to light. The nursery had loam soil that was darker in color and rich in organic matter. Positive soil sample taken from the Bagmati River premises had a pH of 7 and a temperature of 17°C, respectively. It was a wet type of soil with low light exposure. Wet soil with a high moisture content, normal light exposure, pH of 7, and temperature of 14°C were present in the positive sample from the Bishnumati

River premises. The soil sample from park had pH and temperature of 7 and 18°C respectively while the soil was comparatively dry and had normal light exposure. The positive samples were gathered from distinct regions of the Kathmandu Valley, including Kirtipur, Kathmandu, and Lalitpur. The results indicate that pathogens were detected at relatively moderate temperatures, between 14°C and 18°C. Additionally, no significant pH variation was seen amongst the positive soil samples, all the positive samples had neutral pH range.

3.2 Molecular detection, sequencing, and phylogenetic analysis of *B. mandrillaris*

The PCR results were electrophoresed on an agarose gel, and when exposed to UV light, the positive PCR products revealed a white band approximately at 230 bp (Figures 6 and 7). The band size was compared to a 1500 bp DNA ladder which showed a bright band at 500 bp. The negative samples did not exhibit any band. All five samples (samples 11, 13, 14, 1, and 5) which were detected positive for *B. mandrillaris* through PCR amplification and gel electrophoresis were validated and confirmed further by DNA sequencing and phylogenetic analysis.

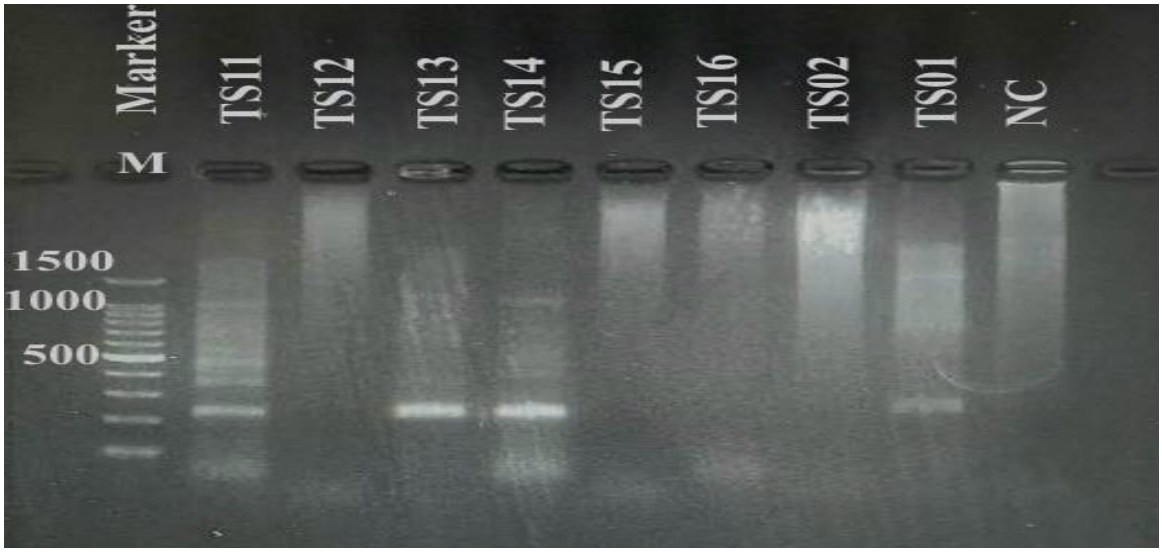

Figure 6. Agarose gel electrophoresis image of PCR product under UV illumination

Balamuthia specific PCR amplification shows white bands on positive samples approximately at 230bp. Lane 1 M: Marker of 1500 bp, lane (2-9): soil samples 11, 12, 13, 14, 15, 16, 02, and 01, lane (10) NC: Negative control.

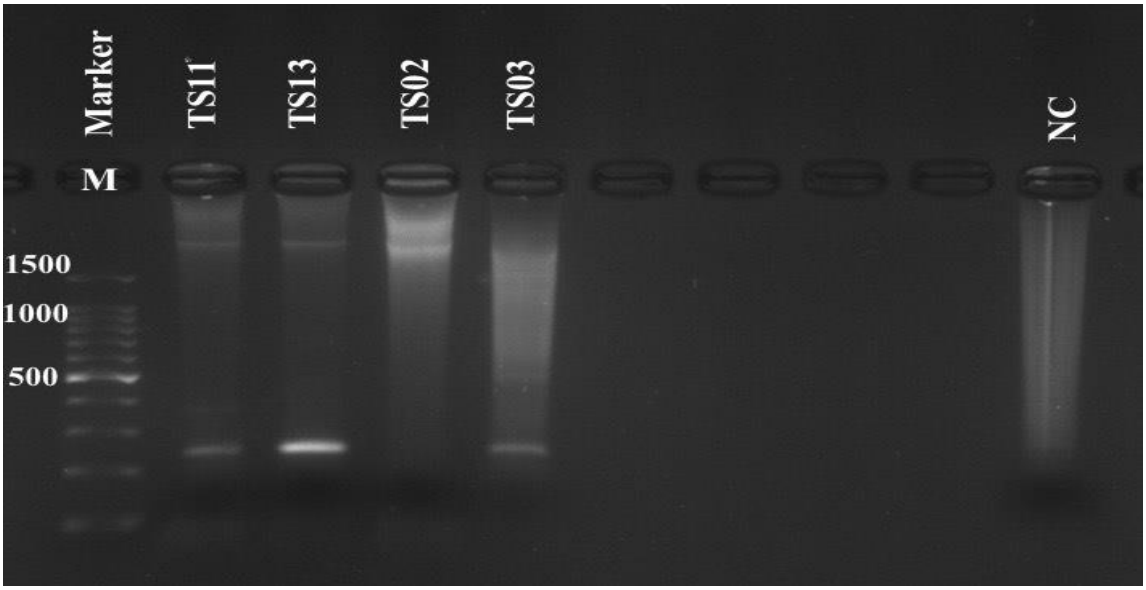

Figure 7. Agarose gel electrophoresis image of PCR product under UV illumination

Balamuthia specific PCR amplification shows white bands on positive samples approximately at 230bp. Lane 1 M: Marker of 1500 bp, lane (2-5): soil samples 11, 13, 02, and 03, lane (10) NC: Negative control

Sequencing of PCR product from the mitochondrial 16S rDNA like gene confirmed the identity of all the five isolates as being representatives of *B. mandrillaris*. The raw chromatograph of sequence (Figure 7) was edited, and multiple sequence alignment was performed in the NCBI BLAST. The query length of five edited samples was 103bp (CDZTU BM 1), 98bp (CDZTU BM 3), 106bp ((CDZTU BM 11), 71bp (CDZTU BM 13), and 98bp (CDZTU BM 14).

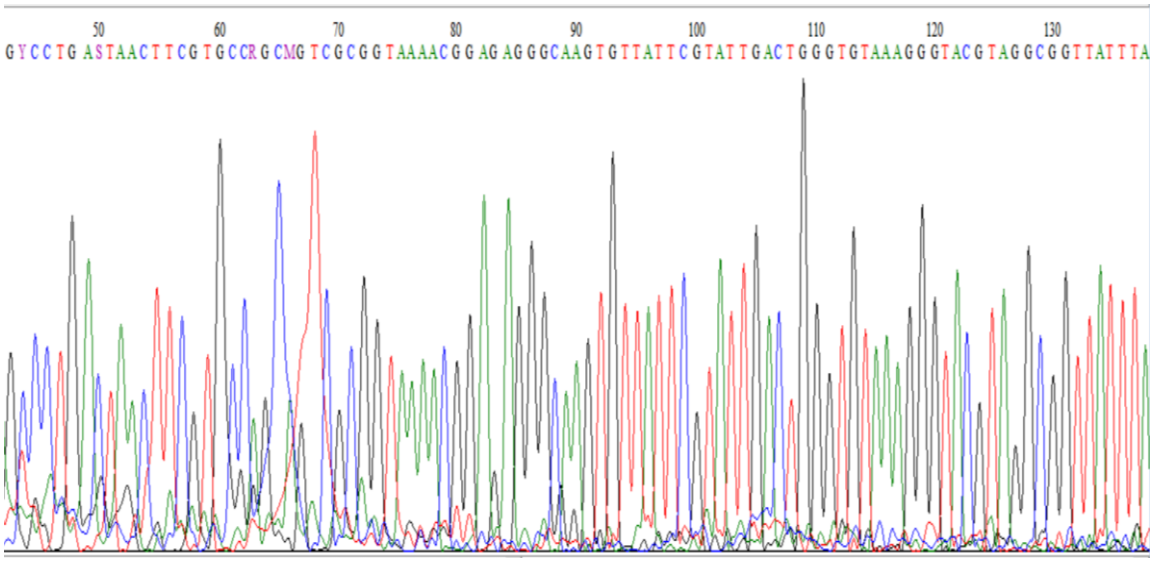

Figure 8. Chromatograph of the raw unedited sequence

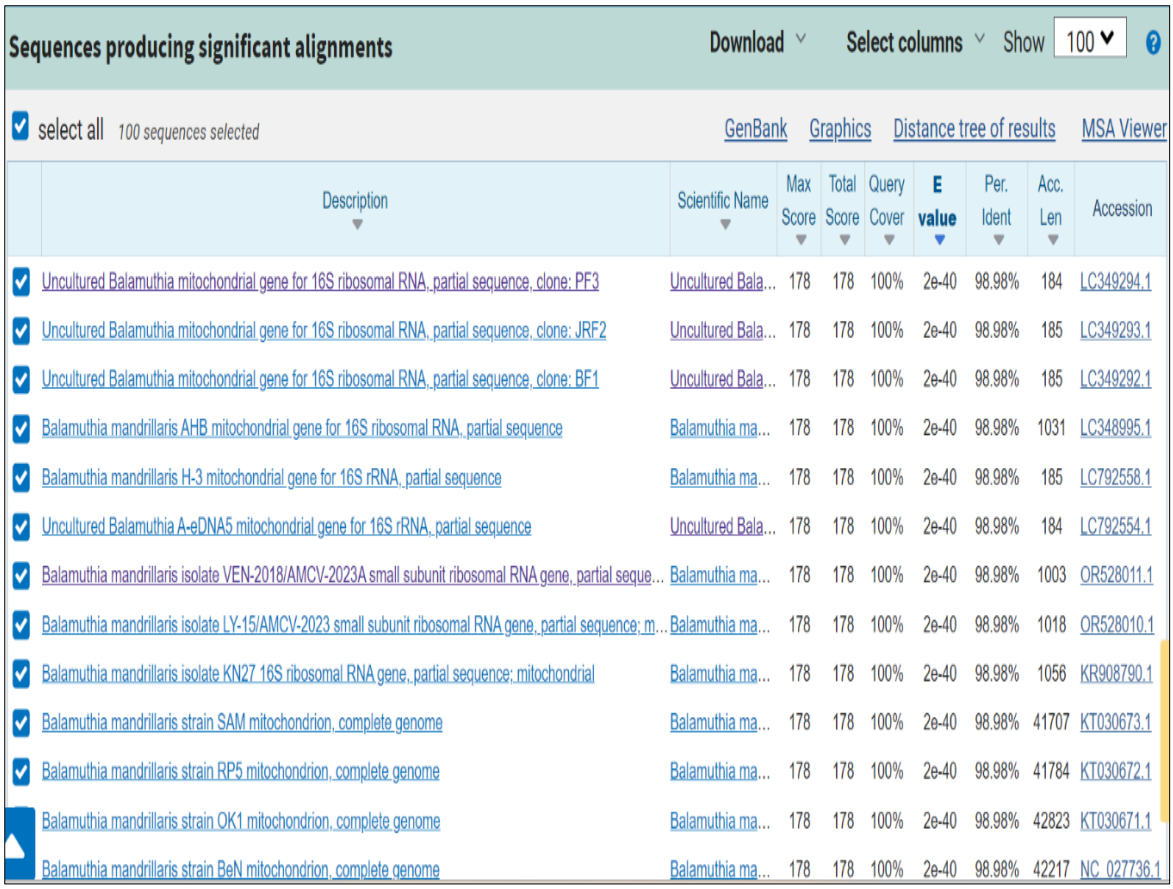

Figure 9. BLAST search result showing aligned sequences

The NCBI BLAST analysis of the query sequences confirmed 100% homology with the corresponding previously reported *B. mandrillaris* sequence from Japan in the GenBank database (accession numbers LC349294). The maximum score and total score obtained for the alignment in BLAST were: 191 (sample TS11), 150 (sample TS13), 193 (sample TS14), 132 (sample TS1), and 178 (sample TS3). The Query cover percentage was 100% for all the five samples. The expected value (E value) was 3e-44 (sample TS11), 3e-32 (sample TS13), 8e-45 (sample TS14), 9e-27 (sample TS1), and 2e-40 (sample TS3). The percent identify value was 100% for all five samples. The BLAST evaluation of sequences in this research demonstrated that the five positive samples had a close association with the *B. mandrillaris* isolates from Japan (Figure. 9). The results of BLAST analysis confirmed the putative *Balamuthia* DNA sequences to be *B. mandrillaris*.

Table 7: Genetic distance of *B. mandrillaris* based on 16S rDNA sequences (230bp)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1. 440k 230 F A04 01		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.00
2. 437k 230 F A04 01	-0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.00
3. 438k 230 F B04 04	-0.00	-0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.00
4. 439k 230 F	-0.00	-0.00	-0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.00
5. 441K 230 F	-0.00	-0.00	-0.00	-0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.00
6. Balamuthia LC349294.1 Japan 2018	-0.00	-0.00	-0.00	-0.00	-0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.00
7. Balamuthia LC348995.1 Japan 2023	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.00
8. Balamuthia OR528011.1 Peru 2023	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.00
9. Balamuthia KR908790.1 Iran 2016	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.00
10. Balamuthia KT030673.1 USA 2015	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.00
11. Balamuthia NC 027736.1 USA 2023	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.00
12. Balamuthia OP941128.1 Peru 2022	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.00
13. Balamuthia OM994889.1 Hong Kong 2022	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00		0.00	0.00	0.00	0.00	0.00	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.00
14. Balamuthia LC706484.1 Japan 2022	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00		0.00	0.00	0.00	0.00	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.00
15. Balamuthia MN176994.1 Peru 2021	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00		0.00	0.00	0.00	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.00
16. Balamuthia KF246746.1 India 2013	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00		0.00	0.00	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.00
17. Balamuthia JX524851.1 USA 2022	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00		0.00	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.00
18. Balamuthia KJ000399.1 Spain 2014	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00		0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.00
19. Balamuthia HF558614.1 Germany 2012	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05		0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.03
20. Acanthamoeba AB795712.1 Japan 2013	0.10	0.10	0.10	0.09	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10		0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.04
21. Acanthamoeba KX257198.1 Malaysia 2017	0.12	0.12	0.12	0.10	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.02		0.00	0.00	0.02	0.00	0.00	0.02	0.04
22. Acanthamoeba AB795710.1 Japan 2013	0.12	0.12	0.12	0.10	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.02	-0.00		0.00	0.02	0.00	0.00	0.02	0.04
23. Acanthamoeba MK100243.1 Russia 2018	0.12	0.12	0.12	0.10	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.02	-0.00	-0.00		0.02	0.00	0.00	0.02	0.04
24. Acanthamoeba MF563608.1 India 2018	0.12	0.12	0.12	0.10	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.02	0.03	0.03	0.03		0.02	0.02	0.02	0.04
25. Acanthamoeba KX257197.1 Malaysia 2017	0.12	0.12	0.12	0.10	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.02	-0.00	-0.00	-0.00	0.03		0.00	0.02	0.04
26. Acanthamoeba KT185628.1 USA 2015	0.12	0.12	0.12	0.10	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.02	-0.00	-0.00	-0.00	0.03	-0.00		0.02	0.04
27. Acanthamoeba EU572722.1 Korea 2008	0.12	0.12	0.12	0.10	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.02	0.03	0.03	0.03	0.02	0.03	0.03		0.04
28. Balamuthia KJ439568.1 Spain 2014	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	0.05	0.10	0.12	0.12	0.12	0.12	0.12	0.12	0.12	

The genetic divergence was estimated using the pairwise distance method using MEGA 11 software (Table 7). The genetic divergence between all the five isolates of *B. mandrillaris* from this study with the GenBank *B. mandrillaris* from Germany (accession number HF558614) was calculated to be 5% which suggests moderate level of divergence between these two isolates. The genetic divergence between the *B. mandrillaris* from this study and *Acanthamoeba* was calculated as 1% from Japan (accession number AB795712) which indicate low genetic distance. The genetic distance between four isolates of *B. mandrillaris* that are (CDZTU BM 1), (CDZTU BM 3), (CDZTU BM 11), and (CDZTU BM 14) and *Acanthamoeba* was calculated as 12% from Malaysia (accession no. KX257198), Japan (AB79571), Russia (MK100243), India (MF563608), USA (KT185628), Korea (EU572722) and Spain (KJ439568) respectively while the isolate (CDZTU BM 13) had 10% difference. This result indicates the high genetic differentiation between the species *Balamuthia* and *Acanthamoeba* which is believed to be closely related to *B. mandrillaris* (Amaral Zettler et al., 2000). Absence of genetic variation between the isolated is shown in the table as -0.00 with 0% difference.

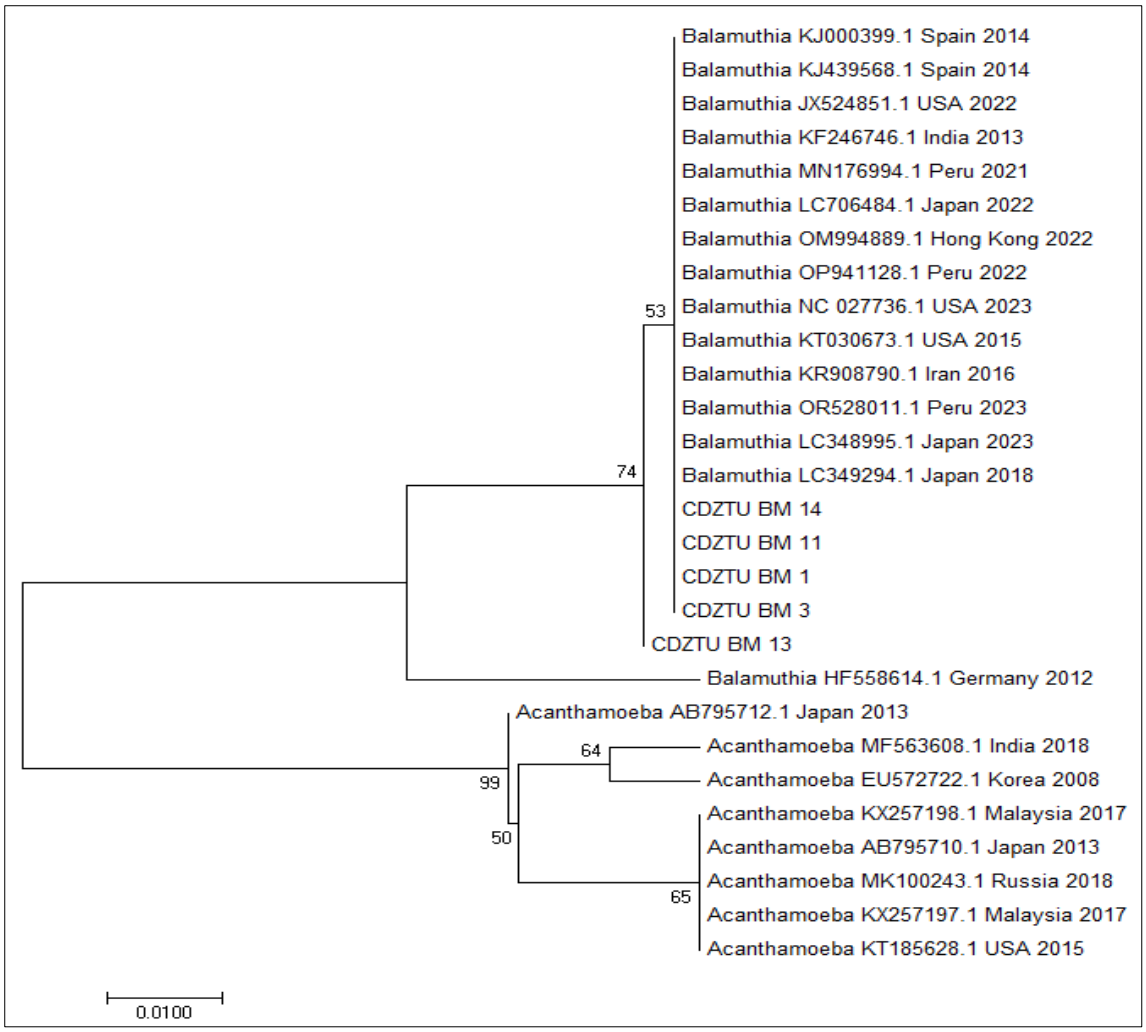

Figure 10. Phylogenetic tree

The result of phylogenetic analysis using MEGA 11 software is shown in the Figure 3. Phylogenetic similarities between the isolates of *B. mandrillaris* found in this study and other isolates of the same species deposited in GenBank for the mitochondrial small subunit rRNA gene sequence. The *B. mandrillaris* isolated in this study are closely related to the uncultured *B. mandrillaris* from Japan. Every sequence in the tree has the isolate name, accession number, and country name labeled on it. The phylogenetic tree denoted the positive samples as (CDZTU BM 1), (CDZTU BM 3), (CDZTU BM 11), (CDZTU BM 13), and (CDZTU BM 14). Phylogenetic evaluation of five *Balamuthia* 16S rDNA nucleotide sequences, including top partial sequence hits in NCBI ranked by BLASTn E-score. Bootstrap values (given as percentages of 500 replications) are displayed at the node of the branch in the phylogenetic tree (Figure 10) and represent the nucleotide substitution per site (number of changes per 100 nucleotide sites). The bootstrap values of 74 and 53 in the branch nodes showed a statistical assurance regarding the relationship grouping represented by the branch. This value implies that, of the 1000 bootstrap replicates created,

almost 74% and 53% of those replicates included the branch or clade supported by a bootstrap value of 74 and 53 respectively. These value of bootstrapping indicate moderate support to evolutionary relationships. The nucleotide substitution rate 0.1 is represented by the bar at the top of the tree.

5. Discussion

B. mandrillaris is an opportunistic FLA and has been associated with cutaneous lesions and *Balamuthia* amoebic encephalitis. Previous studies have revealed the presence of *B. mandrillaris* in the soil. The biology and environmental distribution of *B. mandrillaris* remain poorly investigated, and environmental pathogen isolation is a rare event. Several studies on environmental cases of this pathogen have been confirmed to date in various countries. These include one case confirmed from soil samples of flowerpots in the United States (Schuster et al., 2003), one from Great Britain (Dunnebacke et al., 2004), two studies from Iran, one from city dust sample and one from soil sample (Niyyati et al., 2009, 2015), one from well water in Guinea-Bissau (Baquero et al., 2014), one from dust and water sample in Costa Rica (Retana-moreira et al., 2014), one from water in Mexico ((Lares-jiménez et al., 2014), one from Peruvian soil (Cabello-Vílchez et al., 2014), one from water in South Africa (Muchesa et al., 2015), one from the therapeutic mud bath in Jamaica (Todd et al., 2015), one from hot spring water in Iran (Latifi et al., 2016), one from water in Pakistan (Yousuf et al., 2017), one from soil in Japan (Yamanouchi et al., 2018), and one from Iraq (Al-Aboody et al., 2021). To our knowledge, this is the first report of molecular investigation of *B. mandrillaris* in soil samples in Nepal.

In this study, molecular methods such as PCR amplification and DNA sequencing, and bioinformatic and phylogenetic analysis were employed to analyze 25 soil samples collected from 25 distinct study areas within Kathmandu Valley. *B. mandrillaris* is detected in overall 20% of the soil samples. Out of 25 soil samples included in the study, five soil samples (samples no. 1, 3, 11, 13, and 14) are identified as positive for *B. mandrillaris* samples which were collected from goat farm premises, commercial nursery soil, Bagmati river bank, Bishnumati river bank, and a park respectively highlighting the potential risk associated with these environments. Sequencing of the mitochondrial 16S rDNA gene of *B. mandrillaris* confirmed the positive samples. The NCBI BLAST analysis of the sequences confirmed 100% homology with the corresponding previously reported *B. mandrillaris* sequence in the GenBank database from Japan (Yamanouchi et al., 2018). A closer relative of *B. mandrillaris*, *Acanthamoeba*, is actually more distant than previously thought, based on a comparison of the mitochondrial 16S rRNA gene (Detering et al., 2015). The phylogenetic analysis showed the *B. mandrillaris* isolated in this study is closely related to the *B. mandrillaris* from Japan with the accession numbers LC349294 and LC348995.

The detection of five *B. mandrillaris* specific positive DNA in this study is in accordance with the previous study done in north-western Iran which also confirmed the occurrence of *B. mandrillaris* in five soil samples (Niyyati et al., 2015). The fact that this pathogen has been isolated from soil, water, and dust, suggesting its adaptability to a wide range of environmental niches, could have contributed to the pathogen's considerably greater occurrence in the environmental sample in the study area. Moreover, this amoeba forms cysts that are extremely resilient to chemical and physical changes, enabling them to survive in the environment for a long time (Cope et al., 2019). Similar higher incidences of *B. mandrillaris* in various environmental settings have been reported in several regions such as Southern California (16/17, 94%) (Ahmad et al., 2011), and Peru (4/21, 19%) (Cabello-vílchez et al., 2014). The positive samples were taken from distinct regions of the Kathmandu Valley including Kirtipur, Kathmandu, and Lalitpur. The identification of *B. mandrillaris* from multiple environmental conditions and locations emphasizes the ubiquitous characteristics of this pathogen, which was also described by (Page, 1988). Conversely, modest levels of isolation of this amoeba have been observed in various region of the world, such as Jamaica (1/72, 1.38%) (Todd et al., 2015), Costa Rica (1/36, 2.7%) (Retana-moreira et al., 2014), Northern Iran (5/55, 3.03%) (Latifi et al., 2016), Guinea-Bissau (1/22, 4.5%) (Baquero et al., 2014), Iraq (5/75, 5.3%), and Japan (1/13, 7.69%) (Yamanouchi et al., 2018). The positive samples were collected from the soil having a temperature range from 14°C to 18°C with the pH of 7 same for all the positive samples which represent moderate temperature range and neutral pH level. Comparing the findings of this study to those of several other studies reveals that *B. mandrillaris* can be isolated from a variety of environmental conditions. It has also been found to occur in hot springs with high temperatures between 32°C and 42°C and an acidic pH between 3.5 and 4.2; conversely (Latifi et al., 2016), it has also been isolated from a very cold region in northern Japan with heavy snowfall (Yamanouchi et al., 2018). Another study of *B. mandrillaris* in Guinea-Bissau found it in highly acidic water with a pH of 5.5 and highly saline water (Baquero et al., 2014). In South Africa, *B. mandrillaris* has been isolated from alkaline pH 7.5-8.0 and warmer temperature ranging from 19°C to 27°C (Muchesa et al., 2015). *Balamuthia*'s resistance to harsh conditions, as demonstrated by prior studies that revealed amoeba viability was unaffected by high or low temperatures, acidic or alkaline pH, or both (Salazar-Ardiles et al., 2022).

B. mandrillaris DNA has been found in a local goat farm premises soil. This could be attributed to the possibility that animal wastes have contaminated the soil, adding organic matters that can support *B. mandrillaris* growth and survival (Schuster et al., 2003; Visvesvara et al., 2007). The findings of this investigation, which indicated the presence of *B. mandrillaris* in the soil sample used in flowerpots from commercial nursery settings, align with a study carried out in Mexico where the positive result was also obtained from commercial nursery soil and demonstrated the persistence of this pathogen in various geographical regions. In addition, the observation of *B. mandrillaris* DNA in park soil samples in this research is consistent with the findings from Iran, where positive results were observed in the soil samples taken from parks and gardens, suggesting that recreational activities may expose human beings to the pathogen (Niyyati et al., 2015). Two of the *B. mandrillaris* samples found positive in the soil from Bagmati and Bishnumati river banks in this study is supported by the fact that soil rich in organic additives including chicken manure, earthworm casting, waste disposal, and agricultural practices provide an ideal environment for the growth of bacteria and other microorganisms that *B. mandrillaris* feed on (Dunnebacke et al., 2004; Matin et al., 2008; Schuster et al., 2003). The fact that these rivers travel through the Valley nearer to the human population emphasizes even more the risk of coming into contact with contaminated soil. The presence of *B. mandrillaris* in the river bank highlighted the potential risk associated with these environments. Moreover, the molecular detection of *B. mandrillaris* using PCR represents a sensitive and specific method for identifying the presence of this pathogen in environmental samples (Siddiqui et al., 2016).

Overall, the current work reports the contamination of animal farm, commercial nursery, and river bank soil in Kathmandu Valley caused by *B. mandrillaris*, which is also the first time this pathogen has been isolated from this location. The result of this study showed a significantly high prevalence (20%) of *B. mandrillaris* in the Kathmandu Valley highlighting its presence in diverse environmental settings. The identification of *B. mandrillaris* in this region raises concern about the risk of human exposure and infection. The discovery of *B. mandrillaris* in soil from diverse areas highlights the importance of public health activities aimed at raising awareness regarding the risks associated with *B. mandrillaris*. Additionally, education campaigns, hygiene practices, environmental management strategies, are crucial for minimizing the risk of *B. mandrillaris*.

6. Conclusions and Recommendations

6.1 Conclusions

In the present study, *B. mandrillaris* was isolated in five (20%) soil samples which indicates high prevalence of this pathogen, and the need for urgent preventative and control measures. The positive soil samples collected from diverse environments, including animal farm, commercial nurseries, river banks, and parks, underscore the potential environmental reservoir and ubiquity of this pathogen within urban settings. The pathogens were detected at relatively moderate temperatures (14°C to 18°C) and no significant pH variation was seen among the positive soil samples. *B. mandrillaris* detection in human-frequented places underlines the pathogen's potential for exposure and dissemination. The sequences of *Balamuthia* strains in this study shared 100% homology with the GenBank *B. mandrillaris* sequences from Japan. The homology search results, genetic divergence analysis, and phylogenetic analysis verified the similarity between the *B. mandrillaris* strains in this study and the Japanese strain. Overall, this study contributes to the growing body of knowledge on *B. mandrillaris* providing valuable insight into its environmental existence and highlighting the importance of continued research.

6.2 Recommendations

Based on the findings of the study on molecular detection of *B. mandrillaris* in soil samples via PCR and the conclusions, the following recommendations have been proposed:

- Further research in other locations and diverse soil environments throughout Nepal to understand the presence and geographical distribution of *B. mandrillaris.*
- Employ microscopy and culture-based techniques to ensure the presence of B. *mandrillaris* in the soil samples.
- Implement regular monitoring and surveillance programs focusing on high-risk areas to track the presence and reduce the risk of exposure to *B. mandrillaris*.

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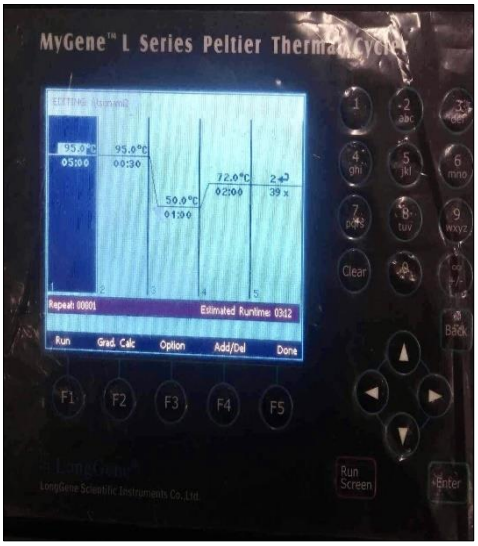
Appendices

Appendix 1. Photographs

Photograph 1: Soil sample collection using auger

Photograph 2: Soil sample collection using spade

Photograph 3: DNA extraction process



Photograph 4: PCR process in thermal cycler

Appendix 2. Preparation of TBE buffer

The 10x stock solution and 1x working solution of TBE buffer in 100ml were prepared following the procedure from Cold Spring Harbor Protocol (Cold Spring Harb Protoc, 2010)

To prepare stock solution of 10× TBE buffer (100ml)

Reagents	**Quantity (For 100ml)**
Tris base	12.11 g
Boric acid	6.18 g
EDTA (disodium salt)	0.74 g
Nuclease-free water	80.97l

- Using an electronic balance weigh out the required amount of Tris base, boric acid and EDTA powder to make 100ml of 10× TBE buffer in a clean aluminum foil.
- In a conical flask, add Tris base and boric acid, then add nuclease-free water and stir with a clean glass stirrer carefully without spilling the mixture.
- Add EDTA in the same conical flask and again stir to make a clear solution which will take some time to dissolve.
- Store the buffer solution at room temperature.

To prepare working solution of 1× TBE buffer (100ml)

For agarose gel electrophoresis, 1:10 dilution of concentrated stock can be used.

- Measure 10 ml of 10× TBE stock solution and pour the buffer into a clean conical flask.
- Add 90ml of nuclease-free water in the same conical flask and mix the solution by gently shaking.
- Store the buffer solution at room temperature.

Appendix 3. Detailed protocol of DNA extraction

Important points before starting

- Shake to mix solution C4 before use.
- If solution C1 has precipitated, heat at 60°C until precipitate dissolves.
- Please wear gloves at all times

Procedure

1. Add 15ml of PowerBead Solution to a well labelled PowerMax Bead Tube.
2. Add up to 10g of soil sample to the PowerMax Bead Tube containing PowerBead Solution. Vortex vigorously for 1 minute.

   Note: After the sample has been loaded into the PowerMax Bead Tube, the tube contains buffer that will (a) help disperse the soil particles, (b) begin to dissolve humic acids and (c) protect nucleic acids from degradation. Vortexing mixes the components in the PowerMax Bead Tube and begins to disperse the sample in the solution.
3. Add 1.2 ml of C1 Solution to the PowerMax Bead Tube and vortex vigorously for 30 seconds.

   Note: Solution C1 contain SDS and other disruption agents required for complete cell lysis. In addition, SDS is an anionic detergent that breaks down fatty acids and lipids associated with the cell membrane of several organisms.
4. Place the PowerMax Bead on a vortex adaptor and vortex for 10 minutes at the highest speed. Alternatively, place the tube in a shaking water bath set at 65°C and shake at maximum speed for 30 minutes.

   Note: Vortexing is critical for complete homogenization and cell lysis. Cells are lysed by a combination of chemical agents from step 1-2 and mechanical shaking introduced at this step. By randomly shaking the beads in the presence of disruption agents, collision of the beads with microbial cells will cause the cells to break open. Use of the vortex adapter will maximize homogenization, which can lead to higher DNA yields. Avoid using tape, which can become loose and result in reduced homogenization efficiency, inconsistent results and reduces yields.
5. Centrifuge at 2500× g for 3 minutes at room temperature.
6. Transfer supernatant to a labelled clean Collection Tube (provided).

   Note: The supernatant may still contain some soil particles and color. The presence of carry-over soil or a dark color in the mixture is expected for many soil types at this step. Subsequent steps in the protocol will remove both carry-over soil and coloration.

7. Add 5ml of Solution C2. Invert twice to mix. Incubate at 2-8°C for 10 minutes.
   Note: Solution C2 is patented IRT. It contains reagent that can precipitate non-DNA organic and inorganic material, including humic substances, cell debris and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.
8. Centrifuge at 2500× g for 4 minutes at room temperature.
9. Avoiding the pellet, transfer the supernatant to a clean Collection Tube (provided).
   Note: The pellet at this point contains non-DNA organic and inorganic material including humic acid, cell debris and proteins. For best DNA yields and quality, avoid transferring any of the pellet.
10. Add 4ml of Solution C3 and invert twice to mix. Incubate at 2-8°C for 10 minutes.
    Note: Solution C3 has IRT and is a second reagent to precipitate additional non-DNA organic and inorganic material including humic acid, cell debris and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.
11. Centrifuge the tubes at 2500× g for 4 minutes at room temperature.
12. Avoiding the pellet, transfer supernatant to a clean Collection Tube (provided).
    Note: The pellet contains additional non-DNA organic and inorganic material including humic acid, cell debris and proteins. For the best DNA yield and quality, avoid transferring any of the pellet.
13. Shake to mix Solution C4. Add 30 ml of Solution C4 to supernatant and invert twice.
    Note: Solution C4 is a high-concentration salt solution. Since DNA binds tightly to silica at high salt concentrations, this will adjust the DNA solution salt concentrations to allow binding of DNA, but non-DNA organic and inorganic material that may still be present at low levels, to the MB Spin Columns.
14. Fill and MB Maxi Spin Column with the solution from step 13.
15. Centrifuge at 2500× g for 2 minutes at room temperature. Discard the flow-through and add a second volume of supernatant to the same MB Maxi Spin Column and centrifuge again at 2500× g for 2 minutes at room temperature. Discard the flow-through. Repeat until entire volume has been processed. This will take up to 4 total spins.
    Note: DNA is selectively bound to silica membrane in the MB Maxi Spin Column device in the high salt solution. Contaminants pass through the filter membrane, leaving only DNA bound to the membrane.

16. Add 10ml of Solution C5. Centrifuge at 2500× g for 3 minutes at room temperature. Discard the flow through.

    Note: Solution C5 is an ethanol-based wash solution used to further clean the DNA that is bound to the silica filter membrane in the MB Maxi Spin Column. This wash solution removes residual salt, humic acid, and other contaminants while allowing the DNA to stay bound to the silica membrane.

17. Centrifuge at 2500× g for 5 minutes at room temperature.

    Note: The second spin removes residual Solution C5 (ethanol wash solution). It is critical to remove all traces of wash solution because the ethanol in Solution C5 can interfere with many downstream DNA applications such as PCR, restriction digests and gel electrophoresis.

18. Carefully place the MB Maxi Spin Column in a new Collection Tube (provided). Avoid splashing Solution C5 onto the column.

19. Add 5ml of Sterile C6 to the center of MB Maxi Spin Column membrane and centrifuge at 2500× g for 3 minutes at room temperature.

    Note: Placing the Solution C6 (sterile elution buffer) in the center of the small white membrane will make sure the entire membrane is wet. This will result in a more efficient and complete release of the DNA from the silica MB Maxi Spin Column membrane. As Solution C6 passes through the silica membrane, DNA that was bound in the presence of high salt is selectively released by Solution C6 (10mM Tris), which lacks salt. Alternatively, sterile DNA-free PCR-grade water (cat. no. 17000-10) can be used.

20. Discard the MB Maxi Spin Column. The DNA is now ready for downstream applications.

    Note: DNA is recommended to store at -20°C to -80°C as Solution C6 does not contain EDTA.