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Molecular Detection of *Listeria monocytogenes* among Sudanese Pregnant Women with Pervious Miscarriage in Khartoum State, 2017

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الآيـة

بسم الله الرحمن الرحيم

قال تعالى:

{لَا يُكَلِّفُ اللَّهُ نَفْسًا إِلَّا وُسْعَهَا لَهَا مَا كَسَبَتْ وَعَلَيْهَا مَا اكْتَسَبَتْ رَبَّنَا لَا تُؤَاخِذْنَا إِنْ نَسِينَا أَوْ أَخْطَأْنَا رَبَّنَا وَلَا تَحْمِلْ عَلَيْنَا إِصْرًا كَمَا حَمَلْتَهُ عَلَى الَّذِينَ مِنْ قَبْلِنَا رَبَّنَا وَلَا تُحَمِّلْنَا مَا لَا طَاقَةَ لَنَا بِهِ وَاعْفُ عَنَّا وَاغْفِرْ لَنَا وَارْحَمْنَا أَنْتَ مَوْلَانَا فَانْصُرْنَا عَلَى الْقَوْمِ الْكَافِرِينَ}

صدق الله العظيم

سورة البقرة الآيه(286)

DEDICATION

This work is dedicated to:

Mom and Dad

Who gave me love

Acknowledgement

First of all, thanks to **ALLAH** who guided me to the true way and gave me the strength, blessings and patience to complete this work.
I thank my supervisor **Prof. Yousif Fadlalla Hamedelnil**.

All thanks and praise to everyone who helped me to accomplish this work

ABSTRACT

Listeria monocytogenes is an emerging food borne pathogen and causative agent of listeriosis. Clinical manifestation of invasive listeriosis is usually severe and includes sepsis and meningoencephalitis.

The objective of the study was to determine the prevalence of *L. monocytogenes* in pregnant women with spontaneous abortions or having a history of spontaneous abortions using PCR.

In this cross sectional study, a total of 50 samples (vaginal and high vaginal swabs) were collected from 50 women with spontaneous abortion hospitalized in Omdurman Maternity Hospital and Alsudi Hospital in Khartoum State. Each sample was immersed in plastic swab tube containing 5 ml of Tris Hcl buffer (PH 8.0) and transported to research laboratory in Sudan University of Science and Technology as soon as possible for the direct DNA extraction and PCR.

L. monocytogenes DNA was detected from 10% samples. 3/50 (6%) and 2/50 (4%) were detected from vaginal and high vaginal swabs respectively. The most affected age group with Listeria infection was 31-36 years old represented 2/19 (10.5%) of aborted women. The most aborted women 3/25 (12%) with *Listeria* infection were had previous abortions within second trimester.

ملخص الأطروحه

اللستريه المستوحدة هي أحد العوامل المسببة للأمراض التي تنتقل عن طريق الغذاء وهي المسببة لداء اللستريات. المظاهر السريرية لداء اللستريات تشمل الإنتان والتهاب السحايا والدماغ.
كان الهدف من الدراسة هو تحديد مدى انتشار حالات اللستريه المستوحدة في النساء المصابات بالإجهاض التلقائي أو لهن تاريخ من الإجهاض التلقائي باستخدام تحليل الكشف الجزيئي.

في هذه الدراسة المقطعية ، تم جمع 50 عينة من مسحات مهبلية ومن أعلى المهبل من 50 امرأة عانين من إجهاض تلقائي في مستشفى أمدرمان للولادة ومستشفى السعودي في ولاية الخرطوم. تم غمر كل عينة في أنبوب مسحة بلاستيكية تحتوي على 5 مل من محلول Tris Hcl (PH 8.0) وتم نقلها إلى المختبر البحثي بجامعة السودان للعلوم والتكنولوجيا في أقرب وقت ممكن لاستخراج الحمض النووي والكشف الجزيئيز

تم اكتشاف الحمض النووي لي اللستريه المستوحدة من 10% من العينات. بنسبة 50/3 (6%) و 50/2 (4%) من مسحات المهبل ومسحات أعلى المهبل على التوالي. كانت الفئة العمرية الأكثر تأثراً بالعدوى الليستريه هي 31-36 سنة ممثلة في 19/2 (10.5٪). النساء الأكثر تعرضًا للإجهاض 25/3 (12٪) كان لهن تاريخ اجهاض سابق خلال الثلث الثاني من الحمل.

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CHAPTER ONE

INTRODUCTION AND OBJECTIVES

1. INTRODUCTION

1.1. Introduction

Listeriosis is a severe food borne disease that rarely occurs in humans and primarily affects the elderly, persons with impaired immunity, pregnant women and unborn or newborn babies. Although uncommon, compared to other foodborne infections, listeriosis is associated with high mortality (Goulet *et al.*, 2011). It is caused by *L. monocytogenes*, a Gram positive, non-spore forming, facultative intracellular and adaptable environmental bacterium. Although most of bacteria do not grow or grow weakly at temperatures below 4°C, *L. monocytogenes* survives in low temperatures. Therefore, *L. monocytogenes* is an important food born pathogen in ready-to-eat foods that have been refrigerated (Salyers and Whitt, 2002; Ramaswamy *et al.*, 2007) .

L. monocytogenes has been found in 10% or more of healthy people, usually in the gut (Eslami *et al.*, 2014). All the 13 serovars of *L. monocytogenes* are reported to cause human listeriosis, but serovars 1/2a, 1/2b and 4b are implicated with most of the cases (Bracegirdle *et al.*, 1994).

Pregnant women are particularly prone to infection. The placenta provides a protective niche for the growth of *L. monocytogenes*, thereby resulting in spontaneous abortions, stillbirth neonatal infection, severe necrotizing hepatitis, placental necrosis and increased risk of post implantation loss (Abram *et al.*, 2003, Bakardjiev *et al.*, 2005). Latent listeriosis in pregnant women leads to habitual abortions, intrauterine deaths and fetal malformations (Winkhaus-Schindl *et al.*, 1966, Romana *et al.*, 1989).

Listeriosis can occur at any time during pregnancy but it is most often recognized in the third trimester (from 28 weeks of pregnancy) (Awofisayo *et al.*, 2015). Pregnancy-related cases of listeriosis are classified into early onset and late onset depending on how long after birth the baby starts to develop symptoms. An early onset case is defined as a newborn with symptoms at birth or within 48 h of birth. This is usually attributed to in-utero infection either through ascending spread from vaginal colonization, or more commonly through transplacental transmission from maternal bacteraemia. Late onset is defined as a newborn that develops symptoms 48 hrs after birth. Infection is thought to occur as the baby passes through the birth canal or as a nosocomial infection from another early onset case (Awofisayo *et al.*, 2015).

The incidence of listeriosis in general population is 0.7 in 100000 but the prevalence is 12 in 100000 in pregnant women (which is a 17-fold increase) (Kaur *et al.*, 2007), this is because during pregnancy the immune system is modulated, with the placenta serving as a protective environment for the growth of the bacterium (Bakardjiev *et al.*, 2005).
The fetus suffers more damage than the pregnant women, leading to a clinical syndrome known as granulomatosis infantiseptica (Klatt *et al.*, 1986). *L. monocytogenes* causes meningitis and hydrocephalus in children born of infected mothers (Gogate and Deodhar, 1981). These reports highlight the importance of the pathogen as a cause of spontaneous abortions and infant mortality(Aljicević *et al.*, 2005).

Unlike developed countries, systematic studies done on the association of pathogenic *L. monocytogenes* with spontaneous abortions are lacking, especially in the Sudan.

1.2. Rationale

Listeria monocytogenes is a ubiquitous bacterium found in soil, decaying vegetation and the faeces of animals. Infection of *L. monocytogenes* causes listeriosis among the immunocompromised, pregnant women, the unborn, newborns and the elderly.

Listeriosis is one of the most important food-borne diseases of humans. The disease can induce septicemia, meningitis and encephalitis. *Listeria monocytogenes* has also been associated with gastroenteritis with fever and it cause severe invasive disease in human.

In pregnant women, intrauterine or cervical infections may result in spontaneous abortion or stillbirths, and may be preceded by influenza-like signs, including fever (Bhat *et al.*, 2012).

The incidence of listeriosis in pregnancy is 12 per 100000, compared with a rate of 0.7 per 100000 in the common people (Janakiraman, 2008). Pregnant women have 17-fold increased risk of developing *listeria* bacteraemia, two-third of babies born to such mothers develop clinical listeriosis (Al-dorri, 2016). The source of infection is generally from animals through undercooked meat or chicken (Bortolussi, 1990). About one-third of reported human listeriosis cases happen during pregnancy, which may result in spontaneous abortion in second or third trimester (Lotfollahi *et al.*, 2011). Overall, pregnant women are more susceptible to acquiring listeriosis, and the risk of listeriosis in pregnant women has been reported to be 17 times that of the normal population (Ciesielski *et al.*, 1988,Gellin et al., 1991, Mylonakis *et al.*, 2002).

Pregnancy-associated listeriosis is a challenging issue not only for researchers but also for clinicians because of the asymptomatic or nonspecific clinical symptoms linked with the disease such as mild flu-

like symptoms, fever, muscle ache, backache, headache, nausea or diarrhea (Lamont *et al.*, 2011, Awofisayo *et al.*, 2015).

The unknown reason of recurrent miscarriage is a major and worrying problem if untreated.

1.3. Objectives

1.3.1. General objective

To study the frequency of *Listeria monocytogenes* among Sudanese pregnant women by using PCR.

1.3.2. Specific objectives

1. To identify and determine the *Listeria monocytogenes* in second and third trimester pregnancy by using PCR.

CHAPTER TWO

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1. Historical background

Listeria monocytogenes is a Gram positive rod, facultative intracellular, foodborne pathogen responsible for cases and out-breaks of listeriosis.

Earlier studies reported that *L. monocytogenes* has been isolated from tissue sections of patients in Germany in 1891, from rabbit liver in Sweden in 1911, and from spinal fluid of meningitis patients in 1917 and 1920 (Pratapa, 2017)**.**

Listeria was first described in 1926 by Murray *et al.* who discovered it while investigating an epidemic infection among laboratory rabbits and guinea pigs (Murray *et al.*, 1926). At that time, it was given the name *Bacterium monocytogenes* because infection in the animals was characterized by monocytosis. The following year, Pirie isolated an identical bacterium from the liver of several gerbils in South Africa. and proposed the name *Listerella hepatolytica* for the genus in honor of Lord Lister a prominent surgeon of the time (Pirie, 1927). Despite considerable confusion in the nomenclature of the pathogen until 1940, the official name *Listeria monocytogenes* was adopted in the Sixth Edition of Bergey's Manual of Determinative Bacteriology (Gray and Killinger, 1966), and the word "monocytogenes" means monocyte producing, since it produced a typical monocytosis during an illness in the diseased animal (Pratapa, 2017).

The first cases of human listeriosis were reported by Nyfeldt in 1929 (Nyfeldt, 1929). The increased number of reported cases during the 1980s in several countries, and the evidence of foodborne transmission, turned

listeriosis into a recognized foodborne disease (Ueda *et al.*, 2005, Shetty *et al.*, 2009).

2.2. *Listeria monocytogenes*

2.2.1. Characteristics

L. monocytogenes is a Gram-positive, facultative anaerobic, non spore forming, rod-shaped bacterium (Collins *et al.*, 1991, Sallen *et al.*, 1996). This organism, presents in decaying plant material as a saprophyte (Hain *et al.*, 2006), is widely distributed in the environment but also acts as an intracellular pathogen (Vázquez-Boland *et al.*, 2001). *L. monocytogenes* has been recovered from soil, vegetable matter, silage, sewage and fecal matter of healthy animals and humans (Farber and Peterkin, 1991, McLauchlin *et al.*, 2004). *L. monocytogenes* can grow at temperatures of 0-45 °C, at high salt concentrations (10%) and at pH of 4.4-9 (Grau and Vanderlinde, 1990). *Listeria* species are motile at 10-25 °C (Hain *et al.*, 2006).

2.2.2. Epidemiology

The incidence of listeriosis in pregnancy is 12 per 100,000, compared with a rate of 0.7 per 100,000 in the general population (Kaur *et al.*, 2007). The CDC monitors cases of listerial infection, and estimates that there were about 800 cases in 2007 (Control and Prevention, 2008). Cases were spread evenly throughout the United States. Based on known incidences, approximately 200 of those likely occurred during pregnancy (Temple and Nahata, 2000). The incidence of listeriosis in the newborn is estimated at a rate of 8.6 per 100,000 live births (Control and Prevention, 2008). Listeriosis is most often a food-borne illness, and sporadic cases as well as outbreaks have been linked to contaminated food (Southwick and

Purich, 1996). Outbreaks of listeriosis are more common in the summer. *Listeria* is a resilient organism; it can survive at temperatures ranging from 4°C to 37°C. *Listeria* maintains its motility best at room temperature, where it can multiply rapidly in a short period of time (Temple and Nahata, 2000). The incubation period for *Listeria* has not been well established; according to case reports, the incubation period is from 24 hours to 70 days (Southwick and Purich, 1996). The FDA and the USDA perform routine screening and surveillance for listerial contamination (Janakiraman, 2008). In 2008, there have been recalls of prepared chicken, pork, and seafood products in the United States due to concerns of listerial contamination (Janakiraman, 2008).

2.2.3. Taxonomy

The genus *Listeria* consists of a group of low Cytosin/guanin content bacteria in the phylum of Firmicutes and is closely related to the genera *Bacillus*, *Clostridium*, *Enterococcus*, *Streptococcus* and *Staphylococcus* (Hain *et al.*, 2006). Members of the genus *Listeria* are *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. grayiand* the recently described *L. marthii*, *L. rocourtiae*, *L. fleischmanii* sp. nov., *L. wihenstephanensis* sp. nov., *L. floridensis* sp. nov., *L. aquatic* sp. nov., *L. cornellensis* sp. nov., *L. riparia* sp. nov., *L. grandensis* sp. nov., *L. booriae* sp. nov. and *L. newyorkenesis* sp. nov. (Cabanes *et al.*, 2011, Bertsch *et al.*, 2013, den Bakker *et al.*, 2013, Halter *et al.*, 2013, den Bakker *et al.*, 2014, Weller *et al.*, 2015). Only *L. monocytogenes* and *L. ivanovii* are pathogenic (Zhang, 2015). *L. monocytogenes* is pathogenic to humans while *L. invanovii* is mainly an animal pathogen but has been reported to cause gastroenteritis in humans (Guillet *et al.*, 2010).

Phylogenetic analysis of rRNA genes and other genes indicate that *L. monocytogenes* and *L. innocua* form one group while *L. welshimeri*, *L. seeligeri* and *L. ivanovii* form another group (Zhang, 2015). Within the latter group, *L. seeligeri* and *L. ivanovii* are more closely related to each other than to *L. welshimeri* (Zhang, 2015). *L. grayi* is distantly related to *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. ivanovii* (Schmid *et al.*, 2005).

Listeria species may have evolved from a pathogenic ancestor. Comparative genomic analysis of the pathogenic species (*L. monocytogenes*) versus non-pathogenic species (*L. welshimeri* and *L.seeligeri*) revealed genomic reduction resulting from deletions within *L. welshimeri* and *L. seeligeri* genomes (Zhang, 2015). For instance, the chromosomal locus that consists of six virulence factors key to the intracellular life cycle is not found within non-pathogenic *Listeria* species except *L. seeligeri*. Gene insertion within the chromosomal locus of *L. seeligeri* disrupted gene function within the chromosomal locus (Hain *et al*., 2006).

2.2.4. Sub typing

There are many sub typing methods for *L. monocytogenes* isolates that provide varied degrees of discrimination. Sub typing is necessary to identify relationships between isolates. In addition, sub typing is useful for identifying the source of infection and tracking the infection in outbreak and sporadic cases (Graves *et al.*, 2007).

Serotyping was the first method developed for sub typing *L. monocytogenes* isolates. Serotyping is based on the use of high quality antisera specific for somatic (O) and flagellar (H) antigens of *Listeria*. Strains of *Listeria* species are divided into serotypes according to which

O antigens and H antigens are detected by high quality antisera (Zhang, 2015). There are at least 12 serotypes: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e and 7 (Liu, 2006). Only three serotypes, 1/2a, 1/2b and 4b, cause 95% of human infections (Graves *et al.*, 2007). Serotyping has poor discriminating power compared to other sub typing methods (Zhang, 2015).

Molecular sub typing methods have been developed for *L. monocytogenes* and is used to place *L. monocytogenes* strains isolated from human outbreaks into epidemic clone (EC) groups EC1, EC1a, and EC2 (Vázquez-Boland *et al.*, 2001, Brehm-Stecher and Johnson, 2007). Strains belonging to EC1 and EC1a are from earlier documented human listeriosis outbreaks in the late 1970s and early 1980s associated with coleslaw and Mexican-style cheese, respectively (Gasanov *et al.*, 2005). EC2 strains of *L. monocytogenes* strains were first recognized in a multistate hot-dog associated outbreak in 1998-1999 and are associated with the majority of recent listeriosis outbreaks in the USA (Fluit *et al.*, 1993, Brehm-Stecher and Johnson, 2007, Liu, 2008, Zhang, 2015).

Ribotyping is a type of Southern hybridization analysis (Zhang, 2015). *Listeria* genome DNA is digested and probed with 16S and 23S rRNA specific probes (Graves *et al.*, 2007). This method detects possible restriction fragment length polymorphism associated with the ribosomal operon (Zhang, 2015). Ribotyping is commonly used for phylogenetic studies and long-term epidemiology studies (Graves *et al.*, 2007).

2.2.5. Pathogenesis

L. monocytogenes is a food borne pathogen. After ingestion of contaminated food, the primary entrance point is the gastrointestinal tract. *L. monocytogenes* predominantly invades and translocates through the

small intestine at the apical tips of intestinal villi (Pentecost *et al.*, 2006), villus epithelial folds and junctions between goblet cells (Nikitas *et al.*, 2011). After cellular internalization, *L. monocytogenes* releases from the phagocytic vacuole, multiples and spreads between cells (Vázquez-Boland *et al.*, 2001).

L. monocytogenes primarily colonizes the liver and the spleen via blood or lymph (Zhang, 2015). Although initial tissue colonization is rapid, the incubation period between ingestion of contaminated food to symptoms of invasive listeriosis is 20-30 days (Vázquez-Boland *et al.*, 2001). The majority of *L. monocytogenes* cells are eliminated by resident macrophages in the liver and spleen with the help of cells from innate and adaptive immunity (Vázquez-Boland *et al.*, 2001). In individuals with weak or compromised immune systems, uncontrolled *L. monocytogenes* proliferation occurs in the liver resulting in colonization of secondary target organs such as the brain and placenta (Vázquez-Boland *et al.*, 2001). Severely immunocompromised hosts also suffer from septicemia (figure 2.1) (Vázquez-Boland *et al.*, 2001).

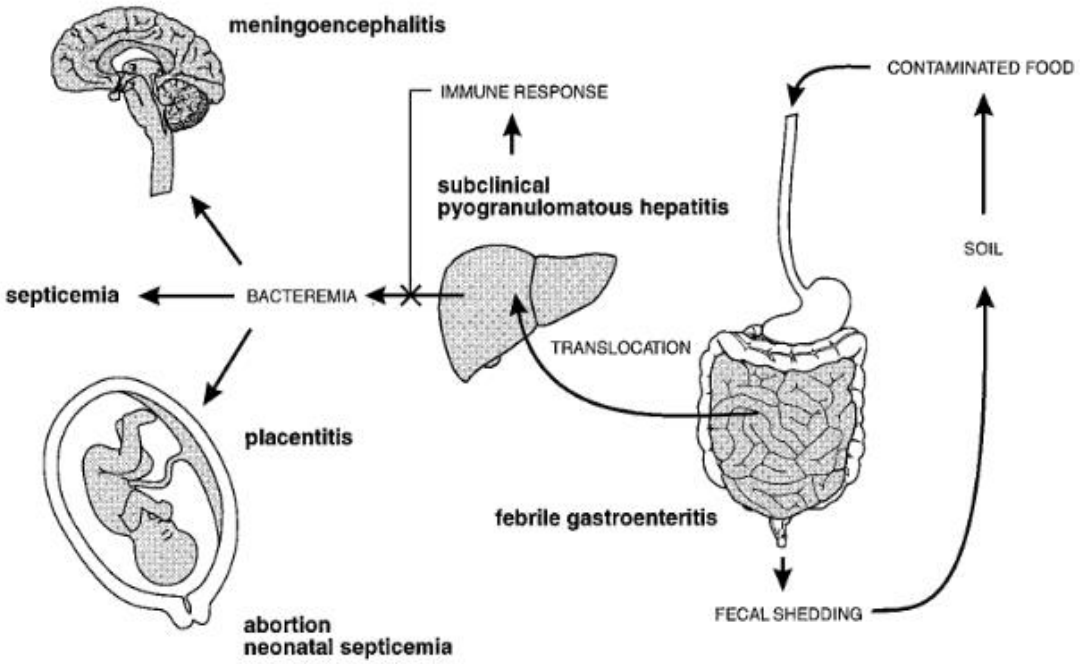

Fig 2.1 Schematic representation of the pathophysiology of *Listeria* infection (Vázquez-Boland et al., 2001).

2.2.6. Listeriosis Manifestation

There are two forms of listeriosis: non-invasive and invasive.
Non-invasive listeriosis can develop in any population when foods highly contaminated with the bacteria (>103 colony forming units (CFUs)/g) are consumed (Zhang, 2015). The average incubation time in otherwise healthy individuals is 24 hours and manifests as febrile gastroenteritis (Ooi and Lorber, 2005).

Invasive listeriosis can be life-threatening. Although invasive listeriosis is rare with respect to other food borne illnesses, it accounts for 3.8% of food borne disease hospitalization and 27.6% of food borne disease deaths (Mead *et al.*, 1999). It occurs in the elderly, pregnant women and individuals with weak or compromised immune systems. Invasive

listeriosis frequently occurs in non-pregnant adults with at least one underlying illness such as heart disease, corticosteroid therapy, cancer, renal disease, diabetes and HIV infection (Schuchat *et al.*, 1992). Non-pregnant patients who acquire invasive listeriosis commonly suffer from meningitis and bacteremia (Vázquez-Boland *et al.*, 2001).

Most cases of listeriosis during pregnancy occur in healthy women. The infected mother experiences non specific flu-like symptoms while the fetus develops systemic infection resulting in miscarriage, stillborn or premature birth of an infant with septicemia and meningitis (Painter and Slutsker, 2007, Ryser and Marth, 2007).

Although antibiotic treatment of the mother, with early detection, can cure the infant of listeriosis, non specific symptoms of the disease makes diagnosis difficult (Ryser and Marth, 2007, Painter and Slutsker, 2007). The flu-like symptoms experienced by pregnant women are associated with the bacteremic phase of the infection and is the optimal time for blood tests (Painter and Slutsker, 2007, Ryser and Marth, 2007). Hence, all febrile episodes during pregnancy should be assessed with blood cultures (Painter and Slutsker, 2007, Ryser and Marth, 2007).

Neonatal infection is serious and often fatal. In early onset neonatal listeriosis, the fetus acquires the infection *in utero* through the placenta from the bloodstream of the mother (Painter and Slutsker, 2007, Ryser and Marth, 2007). Illness occurs at birth or shortly after, within the first week of life. Between 45-70 % of neonatal listeriosis is early onset (Painter and Slutsker, 2007, Ryser and Marth, 2007). Symptoms include respiratory distress, fever and neurological abnormalities. Less commonly, abscesses in multiple internal organs can develop (Painter and Slutsker, 2007, Ryser and Marth, 2007).

Late onset neonatal listeriosis occurs at least one week after birth (Painter and Slutsker, 2007, Ryser and Marth, 2007). Infants are often born from pregnancies without complications. Infants that have late onset neonatal listeriosis more frequently suffer from meningitis (Painter and Slutsker, 2007, Ryser and Marth, 2007). Unlike the early onset disease, the transmission of bacteria in late onset neonatal listeriosis is less clear (Zhang, 2015). The transmission of late onset neonatal listerosis can be transplacental, acquired during passing through the birth canal or contact with an external source (Ryser and Marth, 2007, Painter and Slutsker, 2007).

2.3. *Listeria* Diagnostics

2.3.1. Conventional identification methods

For the identification of *Listeria* to the species level, typical colonies from the selective/ differentiation agar plates are subjected to a battery of tests like Gram-staining reaction, catalase, motility, haemolysis and carbohydrate use (OIE, 2014).

To observe tumbling motility, a hanging drop preparation made from tryptone soya yeast extract broth incubated at room temperature for 8–24 hours is used. For semisolid motility agar, stab inoculation (about 1 cm) is followed by incubation at 20–28°C for 24 hrs. *Listeria* swarm through the medium, which becomes cloudy. At about 0.5 cm below the surface of the agar, a characteristic layer of increased growth is observed, like an umbrella. This occurs because of the better development of *Listeria* under aerobic conditions as opposed to strictly anaerobic conditions (OIE, 2014).

For haemolysing activity, inoculation should be done by piercing the horse and sheep blood containing agar plates with 18-24 hrs young

culture from TSB broth and incubation at 37°C for 24 hrs. *L. ivanovii* exhibits a wide zone of hemolysis and that of *L. monocytogenes* is narrow, frequently not extending much beyond the edge of colonies. In this case, removal of the colonies could help interpretation. Rare strains of *L. monocytogenes* are not hemolytic (OIE, 2014).

The Christie–Atkins–Munch–Peterson (CAMP) test is a very useful tool to help identify the species of *Listeria*. It is used in the ISO and some AOAC protocols and it is considered to be optionalin the FDA and USDA-FSIS methods. It consists of streaking a ß-haemolytic *Staphylococcus aureus* and *Rhodococcus equi* in single straight lines in parallel, on a sheep blood agar plate or a double-layered agar plate with a very thin blood agar overlay. The streaks should have enough separation to allow test or control *Listeria* strains to be streaked perpendicularly, in between the two indicator organisms, without quite touching them (separated by 1–2 mm). After incubation for 24–48 hours at 35–37°C, a positive reaction consists of an enhanced zone of β-haemolysis, at the intersection of the test/control and indicator strains.
Listeria monocytogenes is positive with the *S. aureus* streak and negative with *R. equi*, whereas the test with *L. ivanovii* gives the reverse reactions (Quinn *et al.*, 2000).

2.3.2. Molecular tests

Identification of *Listeria* spp. and *L. monocytogenes* using molecular methods is becoming increasingly popular because these techniques are extremely accurate, sensitive and specific.

2.3.2.1. Polymerase chain reaction (PCR)

PCR has had an immense impact on all molecular applications since its introduction. PCR is a technique whereby segments of DNA are amplified using a Heat stable DNA polymerase and two primers (short DNA sequences specific to a particular gene) and the amplified fragments are then detected, usually using agarose gel electrophoresis (Gasanov *et al.*, 2005). In contrast to DNA hybridization, where comparatively large amounts of target DNA or RNA are required to perform the test, PCR amplifies large amounts of DNA from minute amounts of target DNA. PCR is now established as a reliable and reproducible technique for identification of *Listeria* spp. and more importantly for the differentiation of *L. monocytogenes* from other *Listeria* species using primers targeting genes of virulence factors or RNA sub unit genes (Gasanov *et al.*, 2005).

CHAPTER THREE

MATERIALS AND METHODDS

3. MATERIAL AND METHOD

3.1. Study design

Cross sectional study.

3.2. Study area

The samples were collected from selected government hospitals in Khartoum State (Omdurman Maternity Hospital and Alsudi Hospital). The genotypic identification of the clinical samples was carried out in Medical Microbiology Research Laboratory of Sudan University of Science and Technology.

3.3. Study duration

The study was carried out form March 2017 to February 2018.

3.4. Study population

The samples were collected from women with spontaneous miscarriage or having a history of recurrent miscarriage, with different ages and different trimesters, who attended to the selected hospital during the study period.

3.5. Sample size

Fifty clinical specimens (25 vaginal swabs and 25 HVS) were included in this study.

3.6. Data collection

A structured questionnaire was used to collect the data. The questionnaire contains questions on respondent's socio-demographic characteristics, obstetrical history and other Bio data (appendix I).

3.7. Samples collection and preservation

The vaginal swab was taken by trained and qualified doctor or sister, the high vaginal swab was taken by trained and qualified doctor or sister with a speculum, by inserting the speculum 3–4 cm into the vagina and rotating the swab with a circular motion, leaving it in the vagina for approximately five seconds. Then the swab was inserted into plastic tube containing 5 ml of Tris Hcl buffer (PH 8.0). Pellet from these samples were obtained by centrifugation and then re suspended in 2 ml Tris Hcl buffer and stored in falcon tube at -20 °C until used (Jacobs *et al.*, 1995).

3.8. Genotypic analysis of bacterial isolates

3.8.1. DNA Extraction

DNA was extracted by thermal lysis (boiling method) (de-Paris *et al.*, 2011), and other modified method (Mashouf *et al.*, 2014).

3.8.2. Polymerase chain reaction (PCR)

Polymerase chain reaction was carried out using thermo cycler (TECHNE TC-312, UK) (Appendix-III). Specific primer was used for detection of *L. monocytogenes* by conventional PCR.

3.8.2.1. Primers

The primer 5’-TATGTCGGGCAAGCGTTC-3’ and 5’-GCGCTTGCGTGGTAATTC-3’ was used, with product size 281bp.

3.8.2.1.1. Preparation of primers

3.8.2.1.1.1. Stock primer

Centrifugation of primer vial was done firstly then 230µl of sterile DW was added into each primer vial.

3.8.2.1.1.2. Working primer

From each stock primer 10µl was dissolved in 90µl of distilled water and stored at -20˚C.

3.8.2.2. Master Mix

Master Mix kits (iNtRON's Maxime PCR PreMix, Korea) containing all reagents for PCR except water, template and primers was used. Storage of the master mix was carried out at -20˚C.

3.8.2.2.1. Preparation of reaction mixture

Table 3.1: Preparation of PCR reaction mixture

Reagents	Volume (µl)
WFI	18
Forward primer	1
Reverse primer	1
Template	5

3.8.2.3. Amplification conditions of PCR

The amplification was done by using 0.2 PCR eppendorf tubes that subjected to thermo cycler. The amplification conditions listed in table 3.2

Table 3.2. PCR amplification conditions which recommended by iNtRON's Maxime PCR PreMix, Korea manufacture

Phase	PCR conditions	Number of cycles
Initial denaturation	94º C for 2 mines	1 cycle
Denaturation	94º C for 30 sec	35 cycle
Annealing	48º C for 30 sec	
Extension	72º C for 40 sec	
Final extentin	72º C for 5 mines	1 cycle

3.8.3. Gel electrophoresis and visualization under UV light

3.8.3.1. Preparation of 10X TBE

One Liter of 10x stock solution prepared by dissolving 48.4g Tries base and 55g of boric acid in 40ml of 0.5 M EDTA

3.8.3.2. Preparation of 1X TBE

Ten ml of 10X TEB was diluted by addition of 90ml of de ionized water.

3.8.3.3. Preparation of ethidium bromide

One gram of ethidium bromide was added to 100ml of DW, 1% (10mg/ml) of the solution transferred to dark bottle and stored at room temperature.

3.8.3.4. Preparation of agarose gel

Agarose gel (2%) was prepared by melting 1gm agarose in 50 ml of 1X TBE Buffer using a microwave oven for 1 minute. The melted agarose was allowed to cool to about 50ºC then 2 µl of ethidium bromide was mixed. Agarose gel was poured into gel tray, comb was placed and any air bubbles were removed. After solidification of the gel, the comb was removed and 50ml of 1X TBE buffer was poured into the gel tank to barely submerge the gel.

3.8.3.5. Visualization of the DNA products

The gel casting tray was put into the electrophoresis, tank flooded with 1x TBE buffer just to cover the gel surface, 6µl of PCR products from each sample was added to wells of electrophoreses, 5µl of 100-bp DNA ladder (iNtRON, Korea), was added to the well in each run. The gel electrophoresis apparatus was connected to power supply (100 V, 500 mA, UK). The electrophoresis was carried out at 75Volts for 30 minutes and the gel tray was removed from the electrophoresis apparatus and the buffer was discarded. Then the gel was visualized for DNA bands by U.V transilluminater and photographed (Uvitec –UK) (Jalali *et al.*, 2015).

3.9. Data analysis

Data was analyzed by using Statistical Package for Social Science Program (SPSS) version (16.0) for frequency and percentage.

CHAPTER FOUR

RESULT

4. Result

In this study, 50 clinical samples were collected during 2017. Clinical specimens obtained from patients with spontaneous miscarriage hospitalized in Omdurman Maternity Hospital and Alsudi Hospital, including: vaginal and high vaginal swabs.

4.1. The percentage of positive samples

The genotypic detection of 50 samples revealed 5 (10%) samples to be positive for *L. monocytogenes* showed in Fig (4.1). The five isolates from clinical samples were recovered from three vaginal swab samples (6%) and two from high vaginal swab samples (4%).

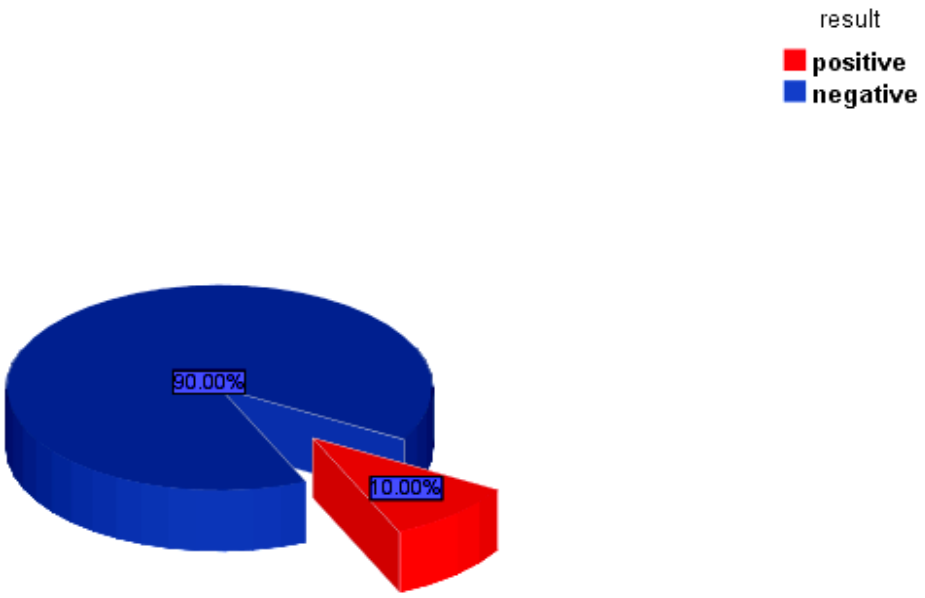

Fig (4.1) The percentage of positive samples

The majority of positive samples (2 out of 5) were found in the age range 31-36 years (table 4.1)

4.2. Table (4.1): Relationship between age and frequency of *L. monocytogenes*

Age	Listeria Spp.		Total
	Positive	Negative	
19-24 yrs	1 16.7%	5 83.3%	6 100.0%
25-30 yrs	1 5.9%	16 94.1%	17 100.0%
31-36 yrs	2 10.5%	17 89.5%	19 100.0%
37-42 yrs	1 12.5%	7 87.5%	8 100.0%
total	5 10%	45 90%	50 100.0%

Three out of five (60%) of the positive cases were recorded in the second trimester of the gestation period (table 4.2).

4.3. Table (4.2): Relationship between gestational age and presence of *L. monocytogenes*

Trimester	Result		Total
	Positive	Negative	
2nd trimester	3 12%	22 88%	25 100%
3rd trimester	2 8%	23 92%	25 100%
total	5 10%	45 90%	50 100%

The majority of positive cases of *L. monocytogenes* 4 (80%) were recovered from women having single abortion during their marriage period (table 4.3).

4.4. Table (4.3): Relationship between the number of abortion of pregnant women and the infection with *L. monocytogenes*

No. of abortion	Result		Total
	Positive	Negative	
One abortion	4 14.3%	24 85.7%	28 100%
Two abortion	0 0%	11 100%	11 100%
≥ 3 abortion	1 9.1%	10 90.9%	11 100%
total	5 10%	45 90%	50 100%

Most of the positive samples infected with *L. monocytogenes* 3 (60%) were obtained from the vaginal swab (table 4.4).

4.5. Table (4.4): Relationship between the location of the sample and the frequency of *L. monocytogenes*

Type of sample	Result		Toyal
	Positive	Negative	
Vaginal swab	3 12%	22 88%	25 100%
HVS	2 8%	23 92%	25 100%
total	5 10%	45 90%	50 100%

The PCR allowed amplification of specific gene of *L. monocytogenes*, the product size is 281bp. Among 50 samples, *L. monocytogenes* was found in 5 samples (Fig 4.6).

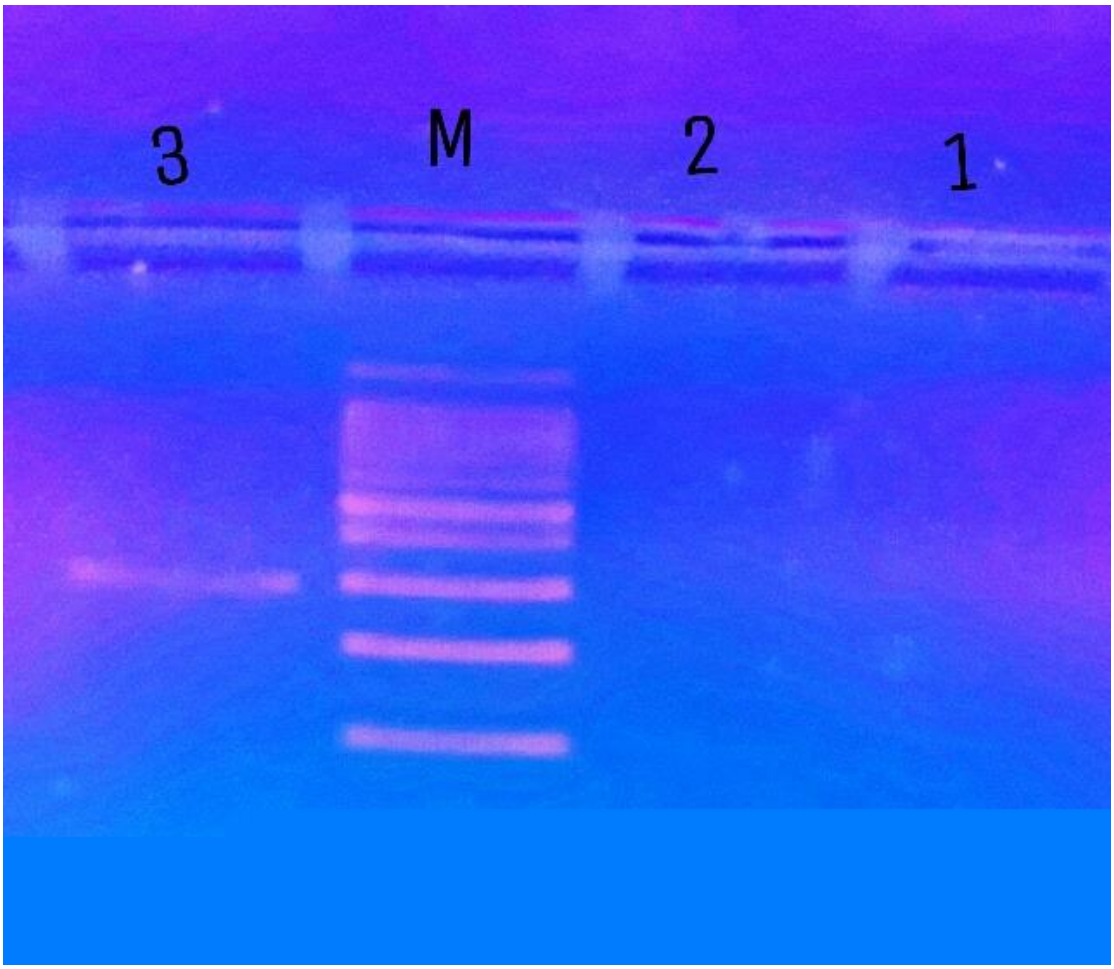

Fig (4.6): Agarose gel electrophoresis of PCR amplification products using specific gene to *L. monocytogenes* isolated from clinical human samples.

Lane M: 100bp ladder as molecular DNA marker, Lane 1: Control negative, Lane 2: Negative sample, Lane 3: Positive sample.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5. Discussion

5.1. Discussion

Reports of listeriosis from humans in Sudan are uncertain, either because of failure to identify the isolate, its rarity, improper isolation techniques, low incidence rate or lack of awareness.

In the present study, the genotypic detection of 50 samples collected from 50 pregnant women with history of spontaneous abortions revealed 5(10%) samples were identified as *L. monocytogenes*, 3(6%) and 2(4%) for vaginal and high vaginal swab, respectively.

A different frequency of *L. monocytogenes* has been reported from several countries. The prevalence of *L. monocytogenes* in this study (10%) was higher than the earlier reports on the isolation of *L. monocytogenes* from three out of 100 (3%) (Bhujwala *et al.*, 1973), nine out of 670 (1.3%) (Bhujwala and Hingorani, 1975), two out of 633 (0.3%) (Dhanashree *et al.*, 2003a), four out of 305 (1.3%) (Kaur *et al.*, 2007), one out of 958 (0.1%) (Stepanović *et al.*, 2007), five from 300 (1.7%) (Soni *et al.*, 2013), seven out of 481 (1.5%) (Sushanta *et al.*, 2015), 18 out of 2200 (0.81%) (Soni *et al.*, 2015), and two out of 295 (0.68%) (Ernest *et al.*, 2015).

The variation reported among the studies can be due to differences in the population under study include culture, race, nutrition, ecological region and also laboratorial diagnosis methods and also difference in sites of samples collection.

Also, the result of this study is in agreement with the earlier from ten out of 100 (10%) (Stephen *et al.*, 1978), twenty-tow out of 428 (5.1%) (Kargar and Ghasemi, 2009), nine out of 100 (9%) (Lotfollahi *et al.*,

2011), fourteen out of 200 (7%) (Shindang *et al.*, 2013), fourteen out of 170 (8.2%) (Kalani *et al.*, 2015), 13 out of 94 (13.8%) (Al-dorri, 2016) and seven out of 100 (7%) (Shayan *et al.* unpublished). These reports highlight the importance of the pathogen as a causative agent for spontaneous human abortions in this ecozone of the world.

25 out of 120 (21%) (Haghroosta *et al.*, 2015) have a higher occurrence compared with a present study.

However, *L. monocytogenes* was recovered from meat and ready to eat food according to studies carried out in Sudan, isolated 204 out of 500 (40%) from Fresh Raw Dressed Broiler Chicken (Alsheikh *et al.*, 2012), and isolated 3% of *L. monocytogenes* from Ready to Eat Vended Food of Meat Origin (Mohammed, 2004), this was indicted to infection of pregnant women with *Liseria* from consumption of contaminated food. In other study in carried out in Sudan, isolated the *Listeria* from blood in case of Puerperal Sepsis (Ahmed and Alsammani, 2013).

The present study shows the most effective age (31- 36 years) this agreed with (Shindang et al., 2013) and (Al-dorri, 2016). This may be attributed to increase marriage and sexual activity with pregnancy at this age group who increased consumption of milk, milk products, fruits and vegetables infected with *Listeria* spp.

The majority of positive cases of *L. monocytogenes* 4 (80%) were recovered from women having single abortion during their marriage period, this agreed with (Eslami *et al.*, 2014).

Most of the positive samples infected with *L. monocytogenes* 3 (60%) were obtained from the vaginal swab, this agreed with (Shaker and Hassanien, 2015).

There is a lack of data for low-income countries and developing countries, the studies only find data from high income and middle-income regions, and said certain assumptions had to be made to produce global estimations. This assumption could not be checked against observed data and so may greatly affect the results(Al-dorri, 2016).

5.2. Conclusion

From this study we can conclude that, *L. monocytogenes* has on association with the spontaneous miscarriage in Sudanese pregnant women.

Also we found different percentage of prevalence between the vaginal and high vaginal swab samples, and the most cases of listeriosis found in second trimester gestational age of pregnant women at sampling.

5.3. Recommendations

1. Large sample size or longitudinal studies is recommended to get conclusive result.
2. Also recommended to collect another samples like blood, placental bit and urine to get more accurate result.
3. Further studies to establish the relationship between vaginal listeriosis and neonatal out come.
4. Further studies are needed to identify all virulence genes of *L. monocytogenes* which responsible for listeriosis, and to determine the physiopathology of this infections to find possible prevention measures.

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APPENDICES

Appendix I Questionnaire

Sudan University of Science and Technology

College of Graduate studies

Molecular Detection of *Listeria monocytogenes* Among Sudanese Pregnant Women with Previous Miscarriage in Khartoum State, 2017

By: Toga Abd ALaziz Awad Mahmmoud

Supervised by: Prof. Yousif Fadlalla Hamedelnill

Name ……

Age ……

Trimester …….

Number of abortion …….

Result

……

……

Signature ……. Date ……

Appendix-II: Preparation of reagents

1. Tris EDTA (PH 8.0)

1 M Tris-HCl PH 8.0
0.5 M EBTA PH 8.0
DW

2. Tris HCl (PH 8.0)

6.0 M Tris HCl
Tris base
0.5 M NaOH

Appendix-III

Fig (1) Microwave

Fig (2) Sensitive balance

Fig (3) Gel electrophoresis and power supply device

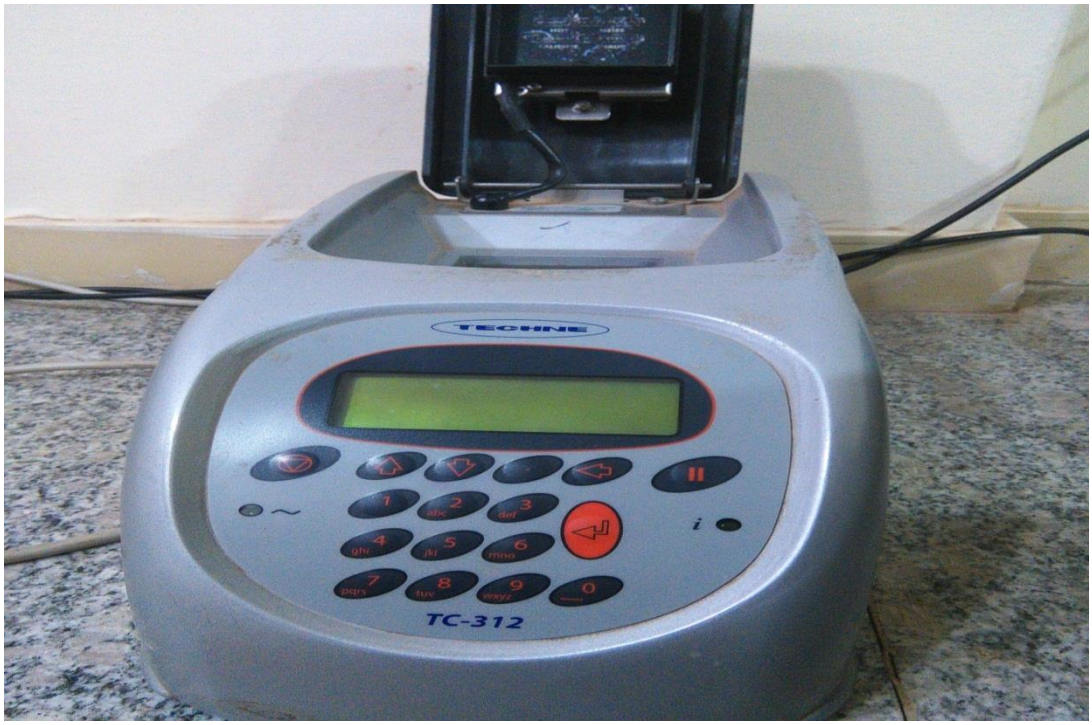

Fig (4) Thermocycler device

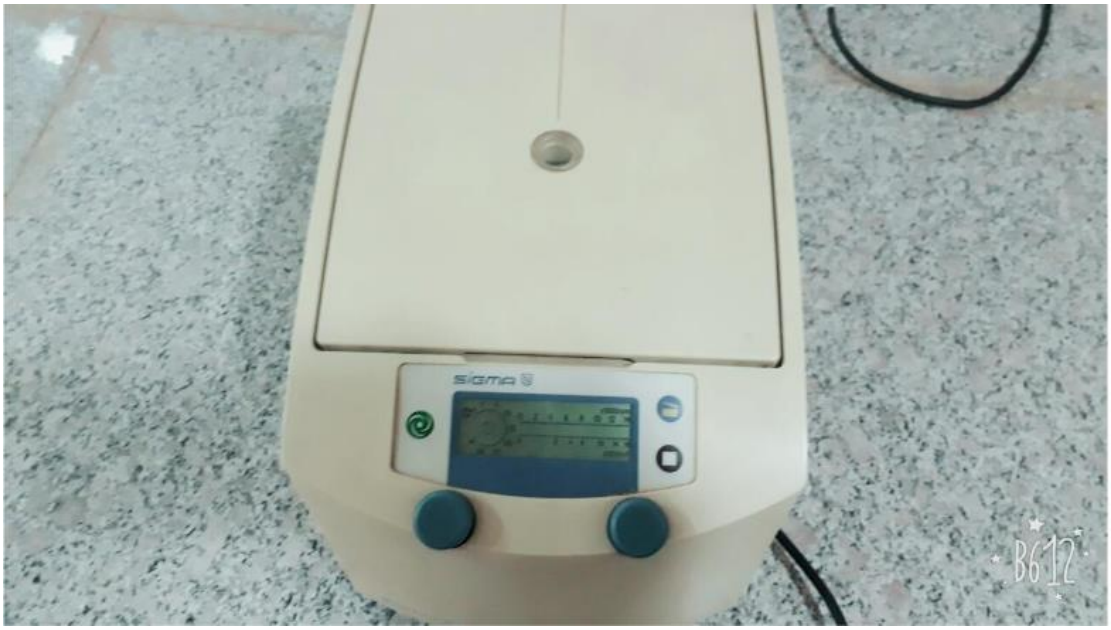

Fig (5) Microcenterfuge device

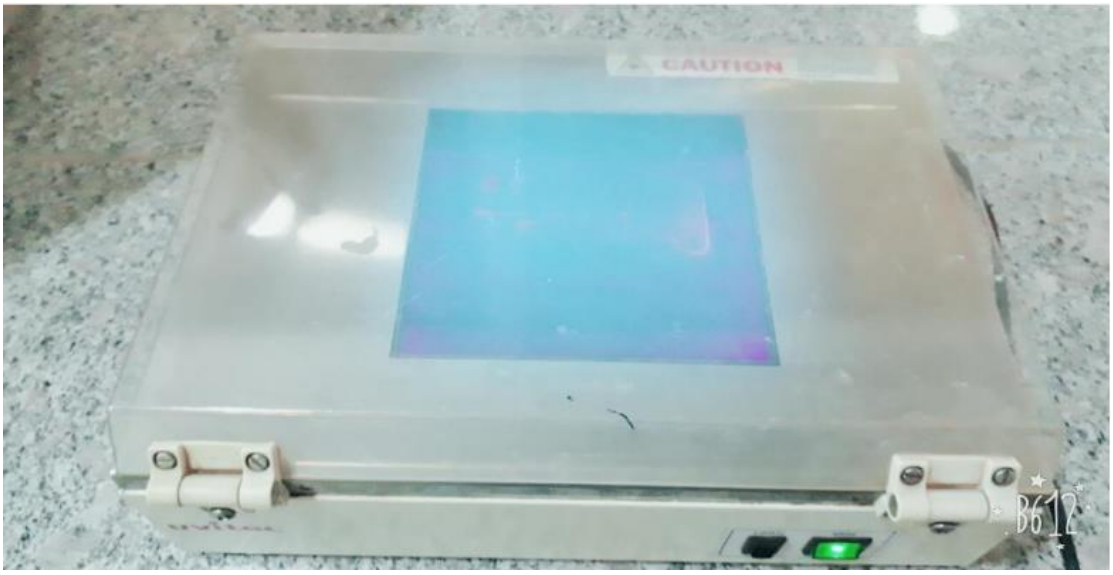

Fig (6) UV Light transilluminater device