ADDIS ABABA UNIVERSITY

INSTITUTE OF BIOTECHNOLOGY

Application and evaluation of LAMP assay for detection of enterohemorrhagic Escherichia coli (O157:H7)

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BY

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DECLARATION

I, the undersigned, declare that this thesis is my original work and has not been presented for a degree in any other University, and that all sources of information used for the thesis have been duly acknowledged.

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This thesis has been submitted for examination with my approval as University advisor.

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ABBREVIATIONS

A/E - Attaching and Effacing

AR system – Acid Resistance system

ARRT-Acute renal replacement therapy

B3 - Backward outer primer

BIP - Backward Inner Primer

BLP - Backward Loop Primer

Bst - Bacillus stearothermophilus

CFU - Colony Forming Unit

CRP - cAMP receptor protein

EHEC - Enterohemorrhagic Escherichia Coli

EPEC - Enteropathogenic Escherichia Coli

EPHI - Ethiopian Public Health Institute

EspA - E. coli secreted protein A

F3 - Forward outer primer

FDA - Food and drug administration

FIP - Forward Inner Primer

FLP - Forward Loop Primer

Gb3 - Globotriaosylceramide-3

HC - Hemorrhagic colitis

HlyA - Hemolysin A

HUS - Hemolytic uremic syndrome

LAMP - Loop Mediated Isothermal Amplification

LEE - Lcus of enterocyte effacement

LP - Long polar

mAb - monoclonal antibody

OMP - outer membrane protein

PD - peritoneal dialysis

Stx - Shiga toxins

TTP - thrombotic thrombocytopenic purpura

WHO - World health organization

ABSTRACT

O157:H7 *E. coli* are one of the enterohemorrhagic *E. coli* strains which cause bloody diarrhea and hemorrhagic colitis as well as hemolytic uremic syndrome in humans. This pathogen has been implicated in the worldwide outbreak of food and water borne diseases. Cost effective, sensitive rapid time detection of this pathogen is beneficial. The objective of the current study was to design and evaluate LAMP diagnostic assay for detection of EHEC O157:H7. In this study, putative fimbria protein coding gene (Z3276) was used to design a loop mediated isothermal amplification (LAMP) assay for the rapid and specific detection of EHEC O157:H7. A total number of 40 pathogenic and non-diarrheic *E. coli* as well as non *E. coli* bacteria were used to evaluate the assay. The LAMP amplified DNA samples were visualized as turbid DNA with naked eye as well as using gel electrophoresis and staining. The assay performed with 100% sensitivity, 97.05 % specificity, as well as 97.5% efficiency. The assay was also 10 times more sensitive than the conventional PCR assay; sensitivity evaluation was done through serial dilution. Additionally, the two assay results showed very high agreement (k = 0.97) on detection of the studied isolates. However, faecal samples spiked with the bacteria (DNA extracted by crude lysate) were not successfully detected by both LAMP assay and PCR method.

Key words: Enterohemorrhagic *E. coli* (O157:H7), Lamp, PCR, Sensitivity, Specificity

1. INTRODUCTION

Escherichia coli (E. coli) is a Gram-negative, rod-shaped, facultative anaerobic bacterium in which most of strains are found as normal flora of gut of animals and humans as non-pathogen (Lim et al., 2010). However, there are many pathogenic *E. coli* strains that can cause infections in intestinal and in a variety of extraintestinal sites such as the urinary tract, meninges, and bloodstream in animals and humans (Donnenberg and Whittam, 2001; Hill et al., 2008). These pathogenic *E. coli* strains are evolved by acquiring virulence factors through plasmids, transposons, bacteriophages, and/or pathogenicity islands (Lim et al., 2010). The pathogenic *E. coli* strains fell in to different categorizes based on serogroups, pathogenicity mechanisms, clinical symptoms, or virulence factors of these bacteria (Kaper et al., 2004). Among them, enteropathogenic *E. coli* (EPEC) that are associated with infantile diarrhea and enterohemorrhagic *E. coli* (EHEC) that are associated with hemorrhagic colitis and the life-threatening sequelae hemolytic uremic syndrome (HUS) in humans lead to serious renal damage and even death are defined as pathogenic *E. coli* (Kaper et al., 2004; Lim et al., 2010). EHEC pathotypes are transmitted through consumption of contaminated food and water as well as can be transferred from person to person and from animal to person. Enterohemorrhagic *Escherichia coli* (EHEC) are enteric pathogenic which colonize the large and small intestines of humans (Thomassin et al., 2012).

EHEC causes diseases by their characteristics of attaching and effacing (A/E) lesions in the gut epithelium (Thomassin et al., 2012). Enteropathogenic *E. coli* cause outbreaks of diarrhea in the developed and developing countries but lesser in developed countries when compared with developing countries. This bacterium has the ability to attach intimately to the host cell membrane, destroy it and produce bloody diarrhea (Wong et al., 2011). Whereas Enterohemorrhagic *E. coli* are characterized by their capacity of producing large quantities of toxins (Shiga toxins) that can damage the intestinal lining and cause bloody diarrhea. There are 200 shiga toxins producing *E. coli* strains but the most common strains are O157 (O157:H7) antigen producing *E. coli* bacteria. Particularly serotype O157:H7 has been implicated worldwide in outbreaks of food and water-borne disease. But since 2000, there are also other non O157 shiga toxin producing *E. coli* strains such as O26, O45, O103, O111, O121, and O145 causing diseases (Wong et al., 2011). Stx1 and Stx2 genes are responsible for prevalence form of toxins that are found in pathogenic strains and acquired from bacteriophage (Donnenberg and Whittam, 2001). In addition to Stx1 and Stx2 genes, EHEC serotype O157:H7 strains also characterized by their eae gene which is responsible for production of intimin, which causes attaching and effacing (A/E) lesions in the intestinal mucosa (Donnenberg and

Whittam, 2001). Z3276 gene is the most mandatory gene that is used for detection of *E. coli* O157:H7 strains. This gene has indicated that gene sequences are highly conserved among EHEC O157:H7 (Qin et al., 2017).

Ethiopia is one the developing countries which faces hygiene problems that could lead to occurrence of diarrhea (Ashenafi Feyisa Beyi et al., 2017). According to WHO report in June 2016, diarrhea outbreak occurred in Addis Ababa when 1,608 new diarrhea cases with 49 deaths were reported; whereas across the country, a total case number of 19,176 with 182 deaths were reported. The total number does not include the cases from Somali Region (WHO, 2016). Tizeta Bekele et al. (2014) also reported the existence of *E. coli* O157:H7 from raw beef, sheep meat and goat meat of human consumed foods in Addis Ababa which leads to food born diarrheic disease. Detection of those Enterohemorrhagic *E. coli* (EHEC O157:H7) of human and animal pathogens are needed for diagnosis purpose.

Different detection methods of these pathogens have been developed elsewhere; to list some of them selective culture media, enzyme immunoassays and PCR assays have been used. Selective culture media is not sensitive and specific as well as time consume procedure so that difficult to effectively isolate and detect those pathogens and enzyme immunoassay is used by rare laboratories since there is few commercial assay approved by FDA for diagnosis (Mahony et al., 2016). Whereas molecular technique detection through PCR is not widely used because it requires skilled technicians and specialized instruments specially this technique is not affordable in developing countries.

In order to solve the above problem, easy, specific and sensitive as well as chip detection method of entrohemorrhagenic *E. coli* O157:H7 is required. Recently, loop-mediated isothermal amplification (LAMP) has been applied to detect these *E. coli* pathogens in human stools, environment and food (Hill et al., 2008). LAMP assay could be used as a rapid, simple and visual diagnostic technique for the detection of all known intimin variants. Loop mediated isothermal amplification (LAMP) is outstanding DNA gene amplification which uses constant temperature for annealing and denaturation as well as for elongation of new strand. Both amplification and detection of nucleic acid sequences can be completed in single step by incubating the mixture of sample, primers, Bst DNA polymerase at a constant temperature (Nagamine et al., 2001). This method eliminates the need for specialized equipment or expertise (Hill et al., 2008). Compared to other DNA amplification method (PCR), LAMP is very sensitive and specific for diagnosis of diseases (Nagamine et al., 2001). Lamp can amplify a few copies of DNA in to 10^9 in less than an hour (Notomi et al., 2000). Since 2013, Lamp has been established to diagnose several micro-organisms, including bacteria, viruses, parasites and fungi (Xue-han et al., 2013). LAMP is a rapid and simple detection technique that can be used for diagnosis of pathogenic *E. coli*. It is a promising diagnostic tool that could provide the results rapidly, and with high analytic sensitivity and specificity without the use of specialized instrumentation (Xue-han et al., 2013). Lamp needs four to six primers that can specifically target the part of the amplified gene; forward and backward outer primer (F3 &B3), forward and backward inner primer (comprised two binding domains F1c & F2 and B1& B2 respectively) and it operates under constant temperature (60-65 ^{o}C). The result of amplified product can be also visualized with naked eye by addition of SYBR Green I. In addition to this, the LAMP assay may be monitored by measuring turbidity of magnesium pyrophosphate, a by- product of LAMP, measuring fluorescence using a DNA intercalating dye such as SYBR green II and ethidium bromide (Heidarnejhad et al., 2015).

The aim of this work was to apply and evaluate a simple, rapid, specific and sensitive molecular diagnostic assay for enterohemorrhagic *E. coli* (O157: H7) by using the loop mediated isothermal amplification and to evaluate the sensitivity as well as specificity of this assay, compared to PCR, was been also. This assay was mainly concern on the amplification of the important virulence genes (Z3276) of EHEC O157:H7 strains.

2. OBJECTIVE OF THE STUDY

2.1 General objective

The general objective of this study is to apply LAMP diagnostic assay in order to detect Enterohemorrhagic *E. coli* O157:H7.

2.2 Specific objectives

1. To evaluate the specificity and sensitivity of LAMP assay in comparison with PCR
2. To evaluate the efficiency of LAMP diagnostic assay for detection Enterohemorrhagic E. coli O157:H7.

3. LITRATURE REVIEW

3.1 Introduction

E. coli is grouped in gamma enterobacteria class and gram negative bacteria as well as it has the ability to ferment glucose and lactose, but it often doesn't produce H2S. *E. coli* was first coined by a German pediatrician, Theodore Esherich, in 1884 from faces of human neonates (Khan and Steiner, 2002). *E. coli* can be recovered easily from clinical specimens on general or selective media at 37°C under aerobic conditions, e.g. Mac-Conkey, eosin methylene-blue agar. *E. coli* serotypes are specific O-group/H-antigen combinations. There are more than 700 antigenic types (serotypes) that are recognized by the presence of O, H, and K antigens. So far 170 different O antigens and at least 56 H antigens have been recognized (Tu et al., 2006).

Escherichia coli is a commensal of the lower gastrointestinal tract of mammals. However, some pathogenic strains have been evolved through horizontal gene transfer mechanisms. Enterohemorrhagic *E. coli* is one of them.

3.2 Characterization of EHEC

EHEC is characterized by their Shiga toxin, intimin, and enterohemolysin producing of key virulence factors for the pathogenesis (Wang et al., 2014). Shiga toxins inhibit protein synthesis resulting in the death of host cells and are key virulence factors of other Shiga toxin-producing *E. coli* infections. Intiman is an effector protein which is secreted through type III secretion system and facilitates the intimate attaching of EHEC on intestinal epithelium. Enterohemolysin is the causative agent for enterocyte effacing (Wang et al., 2014) and also it affects extra intestinal cells as well as has the ability to affect several cells, such as lymphocytes, granulocytes, erythrocytes, and renal cells causing severe effect (Ashgan et al., 2015).

3.3 Geographic Distribution

EHEC 0157:H7 infections are worldwide in distribution. However, the lineages of this organism are reported to differ between regions, potentially influencing the incidence and severity of human disease. Non-O157 EHEC are also widely distributed. The importance of some EHEC serotypes may vary with the geographic area. EHEC O104:H4 caused a major outbreak in Europe, but it has also been identified on other continents including Africa and Asia (Khan and Steiner, 2002).

3.3.1 Distribution of EHEC in Ethiopia

The distribution of E. Coli O157:H7 in Ethiopia is not well studied. Some of studies for prevalence of EHEC were also carried out based on biochemical test and selective culture media test. However, unpublished data which were studied in different part of Ethiopia reveal the prevalence of pathogenic E.Coli in lambs and children. Because of those studies were carried out through PCR method (molecular level of detection), the results are more accepted than selective media method or/and biochemical method. Rosa Abdissa et al (2017) reported the prevalence of O157:H7 E.Coli in the fecal samples and intestinal mucosa of cattle beef. Isolation, identification and characterization of this study was done using standard microbiological methods. This finding was mainly extrapolated to a larger cattle population of Ethiopia albeit with caution; cattle slaughtered at processing plants. Thus to diminish the contamination of O157:H7 during and after slaughter, good carcass management at the retail shops and at abattoirs are important (Rosa Abdissa et al., 2017). Metasebia Aklilu et al (2013) also reported the high prevalence of pathogenic E.Coli in diarrheic lambs and E.Coli strains which was isolated from diarrheic lambs were categorized in to fifteen different biotypes according on their sugar fermentation.

3.4 Prevalence and transmission

Raw meat can harbor pathogenic bacteria; animal feces are reservoir of EHECO157. Thus, food contamination with EHEC O157 can occur in farms (Wang et al., 2014). The infectious dose for humans is estimated to be less than 100 organisms, and might be as few as 10. Transmission of *E. coli* serotype O157:H7 is via fecal-oral route, due to improperly washed hands or ingestion of undercooked foods from animal origin harboring the organism specially meat and the meat products, milk and dairy products as well as contaminated water (Ashgan et al., 2015). Additional outbreaks have been linked to lettuce, spinach, various sprouts and other contaminated vegetables, unpasteurized cider, nuts and even pickled vegetables. Contaminated irrigation water is an important source of EHEC O157:H7 on vegetables. This organism can attach to a variety of edible plant material, although it seems to bind more readily to some fruits and vegetables than others. EHEC O157:H7 can be internalized in the tissues of some plants including lettuce, where it may not be susceptible to washing. EHEC is also zoonotic and can be transmitted from animal to human and person to person. Person-to-person transmission of EHEC and EAHEC can contribute to disease spread during outbreaks, via the fecal-oral route. Young children tend to shed these organisms longer than adults. Humans do not appear to be a significant reservoir for EHEC O157:H7. Cattle are a major reservoir of EHEC, but unlike in humans, EHEC colonization in adult ruminants is asymptomatic. EHEC O157:H7 can remain viable for long periods in many food products. It can survive for at least nine months in ground beef stored at -20°C (-4°F). It is relatively tolerant of acidity, and remains infectious for weeks to months in acidic foods such as mayonnaise, sausage, apple cider and cheddar at refrigeration temperatures. Salt might increase its resistance to inactivation in highly acidic foods such as pickles. It also resists drying (Wang et al., 2014, Ashgan et al., 2015).

Cattle transmit EHEC to humans by shedding the pathogen in their faeces. Supper shedding animals excrete large amount of EHEC than other animals. Once shed evacuate into the environment, humans acquire EHEC by consuming contaminated bovine-derived products such as meat, milk, and dairy products or contaminated water, unpasteurized apple drinks, and vegetables. Whereas, direct contact with ruminants at petting zoos or through interactions with infected people within families, daycare centers, and healthcare institutes represent another source of EHEC trans- mission (Nguyen and Vanessa, 2012). Average incubation period of this bacteria ranges from 1 to 9 day (mean 3.1 to 3.9 d) during community outbreaks and from 1 to 14 d (mean 4 to 8 d) (Christina and Bertil, 2005). Carcass contamination occurs through skin-to-carcass or fecal-to-carcass transfer of the pathogen during slaughter process at processing plants; and this is the major risk factor for human infection. Isolation of *E. coli* O157: H7 from the fecal samples and intestinal mucosal swabs indicates carriage of the pathogen by the animal (Rosa Abdissa et al., 2017).

During infection, EHEC must encounter and attach to one or more cell types found in the intestinal mucosa, evade host defenses, and compete for nutrients with other bacterial species (Alfredo et al., 2002). In addition to intimin, intestinal tropism may also involve for adherence of EHEC on the gastro intestine tract. The LP (long polar) fimbriae were synthesized by the lpf operon which contains lpfC and lpfC for proteins and that increased adherence of EHEC (Alfredo et al., 2002).

3.5 EHEC Pathogenesis

The pathogenesis of EHEC has multistep process, involving interactions between several additional bacterial and host factors. It expresses more than one virulence genes which leads to disease in humans (Christina and Bertil, 2005).

EHEC is a pathogenic *E. coli* that cause watery and bloody diarrhea, hemorrhagic colitis (HC), hemolytic-uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP) (Wang et al., 2014). In humans, EHEC O157 is recognized as a major pathogenic agent of these diseases. Escherichia coli O157:H7 is one of the most important foodborne pathogens that cause significant losses among the human population in the past two decades (Ashgan et al., 2015).

3.5.1 Attaching and effacing adherence

The adherence mechanism of EHEC is as similar as EPEC. EHEC colonizes the large bowl and it can able to attach intimately to the surface of intestinal epithelial cells by exploiting the host cell's cytoskeleton, producing a characteristic pathology called the attaching and effacing (A/E) lesion(Bruce et al, 2002). The A/E phenotype is responsible for focal degeneration of the brush border microvilli, intimate bacterial adhesion and host cytoskeletal reorganization, leading to the accumulation of polymerized actin (Bruce et al, 2002). This renders to produce pedestal like structure for adherence and colonization of bacteria on the intestinal lineage, remain there to cause infection. During infection, EHEC releases a shiga toxin that damages intestinal tissues and, in some cases, causes renal failure, neurological disease, thrombocytopenia and hemolytic anemia (Bruce et al, 2002;Nguyen and Vanessa, 2012). The infectious dose of EHEC is very low, between 1 and 100 CFU, which is a much lower dose than for most other intestine pathogens (Christina and Bertil, 2005). For gram negative *E. coli*, their 41 genes responsible for the virulence of A/E pathogens that are located within a distinct chromosomal region, termed a pathogenicity island. This region, called the locus of enterocyte effacement (LEE), is a 36 kb segment of the EPEC chromosome. One factor of LEE intimate adherence promotes by result of intimine coding gene called eaeA, 94-kDa outer membrane protein (OMP) called intimine. This gene is not virulence factor rather it uses for anchor the bacterium on the human gut. The mutation of this gene can result to loss its E/A ability (Christina and Bertil, 2005).

An important bacterial structure which enhances for adherence is called fimbriae, thread-like structures that reach out from the bacterial surface. Their three types of fimbriae are responsible for adherence. To list them type I fimbriae that makes intimate adherence and A/E lesion formation, P fimbriae that recognizes a disaccharide of uroepithelial cells and human erythrocytes, S fimbriae; mediates adherence to endothelial cells, urinary tract epithelial cell and brain endothelial cells (Christina and Bertil, 2005). In order to inject the virulence factor of this bacterium into the host, it uses type III secretion system (Bruce et al, 2002).

3.5.2 Virulence of Shiga toxin

The most important virulence characteristic of EHEC, once established in the gut, is its ability to produce shiga toxin. This toxin is prototype toxin with by Shigella dysenteriae serotype 1(Christina and Bertil, 2005). EHEC may produce one or more toxin as named as Stx1 and Stx2. Shiga toxin is comprised of A and B two major subunits. A subunit shows an RNA N-glycosidase activity against the 28S rRNA which leads to apoptosis by inhibits of protein synthesis. The B subunit forms a pentamer that binds to globotriaosylceramide-3 (Gb3), and this specificity determines where Shiga toxin mediates its pathophysiology. EHEC colonizes large intestine of humans and produces Shiga toxin released by binds to endothelial cells expressing Gb3 allowing absorption into the bloodstream and dissemination of the toxin to other organs (Nguyen and Vanessa, 2012). In the host cell, shiga toxin catalytically cleaves a single adenine residue from the 28S rRNA component of the eukaryotic ribosomal 60S subunit and it leads to inhibition of protein synthesis and cell death. Hence, shiga toxin production results in acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia, all typical characteristic of HUS which typically affects children (Christina and Bertil, 2005, Nguyen and Vanessa, 2012). Christina and Bertil (2005) provided the following conclusion on the pathogenesis of shiga toxin, the toxin : 1) possess the mechanism to cross the intestinal epithelial barrier; 2) induce a neutrophil-rich inflammatory infiltrate in the gut; and 3) in the bloodstream take a lift on neutrophils which mediate the transport to tissues with high-affinity receptors.

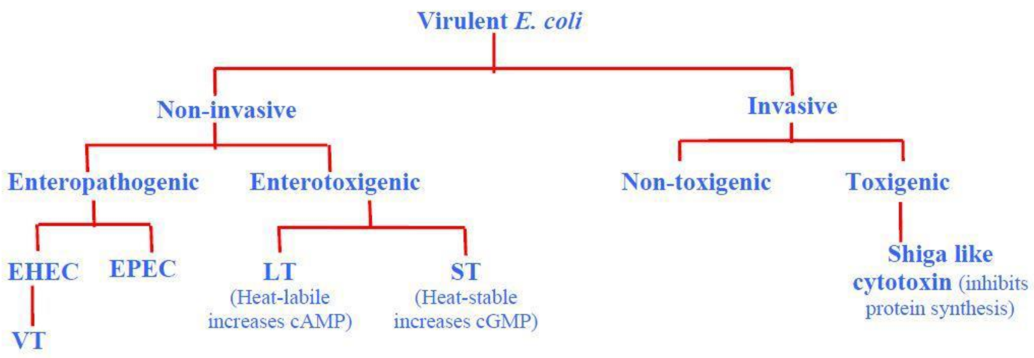


Figure1. Groups of pathogenic *E. coli* bacteria, Source :(Tu, 2006).

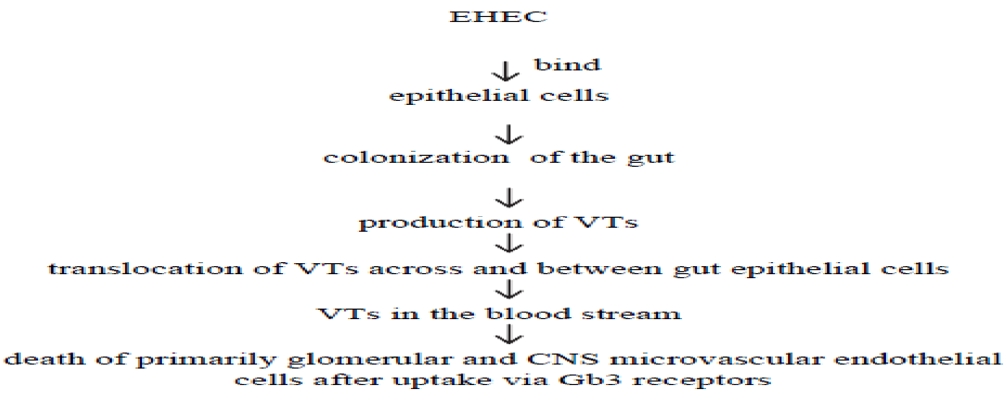


Figure2. Virulence mechanisms of *E. coli*: Source (Tu, 2006).

3.5.3 The plasmid pO157

EHEC had gained their pathogenicity from other pathogenic bacteria through Pathogenesis Island. EHEC carries large plasmids about 75 -100 kb in size, which indicates EHEC pathogenicity. pO157 codes EHEC-haemolysin named

EHEC-hlyCABD(E-hlyA) which associated with defective haemolysin secretion. This haemolysin secretion leads for pathogenesis of EHEC disease is possibly that haemoglobin released by the action of the haemolysin provides a source of iron (Christina and Bertil, 2005). pO157 encodes HEC katP catalase-peroxidase, in addition to chromosomal catalase or hydrogen per oxidase. Hydrogen peroxidase is haem binding enzyme, which used to convert hydrogen peroxidase for electron acceptor whereas catalase is used as defense mechanism against oxygen stress. The other protein which is encoded by pO157 is extracellular serine protease espP, functional tests showed that the espP gene codes for a protease capable of cleaving pepsin A and human coagulation factor V (Christina and Bertil, 2005).

3.5.4 Mechanisms OF EHEC survival in the host

3.5.4.1 Acid resistance

After ingestion of EHEC by animals, EHEC breaches the acidic barrier stomach. In order to reach the RA for colonization, EHEC has an intricate acid resistance (AR) to survive the acidic environment stomach. It has three mechanisms; the AR system 1 (glucose-repressed or oxidative), AR system 2 (glutamate-dependent), and AR system 3 (arginine-dependent) (Nguyen, and Vanessa, 2012). Of the three AR systems, the glutamate- dependent AR system provides the highest level of acid protection Activation of the glucose-repressed AR system depends on the cAMP receptor protein (CRP), and the stress response alternative sigma factor RpoS of global regulators(4). Glutamine and arginine dependent AR system depend on glutamate decarboxylases GadA and GadB enzyme and arginine decarboxylase AdiA which convert glutamine and arginine to γ-amino butyric acid(GABA) and agmatine respectively (Nguyen, and Vanessa, 2012).

3.6 Clinical significance

Vehicles of infection, suspected or confirmed, have been identified for most outbreaks. During the 1980s most outbreaks of *E. coli* O157:H7 were associated with inadequately cooked hamburgers and unpasteurized milk. Some later outbreaks have been traced to other dairy products such as cheese and yogurt. Increasingly, contaminated water has been reported as a source of human infection. This includes drinking water sources contaminated with animal feces and also contaminated lake and pool water used for swimming and playing (Reza et al., 2016).

A large outbreak in Japan, affecting more than 12,000 persons, was associated with contaminated radish sprouts (Stratakos et al., 2016) and other outbreaks have been associated with contaminated fruit juices, melon, and salad greens (Rosa Abdissa et al., 2017). More recently a number of outbreaks have occurred among children visiting farms and petting zoos where they come into direct contact with animals carrying *E. coli* O157:H7 and their environment (Dhama et al., 2014).

Humans can be infected asymptomatically with EHEC, or they may develop watery diarrhea, hemorrhagic colitis and/ or hemolytic uremic syndrome. Most symptomatic cases begin with diarrhea. Some resolve without treatment, but others progress to hemorrhagic colitis within a few days. Hemorrhagic colitis is characterized by diarrhea with profuse, visible blood, accompanied by abdominal tenderness, and in many cases, by severe abdominal cramps. Nausea, vomiting and dehydration may also be seen. Some patients have a low–grade fever; however, fever often resolves by the time hemorrhagic colitis appears, and can be absent. Many cases of hemorrhagic colitis are self–limiting and resolve in approximately a week. Complications in severe cases may include intestinal necrosis, perforation or the development of colonic strictures.

Hemolytic uremic syndrome occurs in a minority of patients with hemorrhagic colitis. This syndrome is most common in children, the elderly and those who are immunocompromised. It usually develops about a week after the diarrhea begins, when the patient is improving, but there are occasional cases without prodromal diarrhea. HUS is characterized by acute kidney injury, hemolytic anemia and thrombocytopenia. The relative importance of these signs varies. Some patients with HUS have hemolytic anemia and/or thrombocytopenia with little or no renal disease, while others have significant kidney disease but no thrombocytopenia and/or minimal hemolysis. Extrarenal signs can range from lethargy, irritability and seizures to paresis, stroke, cerebral edema or coma; respiratory syndromes (e.g., pleural effusion, fluid overload, adult respiratory distress syndrome); elevation of pancreatic enzymes or pancreatitis; and uncommon complications such as rhabdomyolysis, bacteremia, deep abscesses or myocardial involvement. The form of HUS usually seen in adults, particularly the elderly, is sometimes called thrombotic thrombocytopenic purpura (TTP). In TTP, there is typically less kidney damage than in children, but neurological signs are more common. Deaths occur most often in patients with serious extrarenal disease. Long-term renal complications of varying severity can be seen in some patients, although many or most children recover from HUS without permanent damage. There may also be residual extrarenal problems such as transient or permanent insulin-dependent diabetes mellitus, pancreatic insufficiency, or neurological defects such as poor fine-motor coordination (Paul and Karl, 2012). In rare cases, EHEC including EHEC OH157:H7 have caused urinary tract infections, with or without diarrhea and/or HUS.

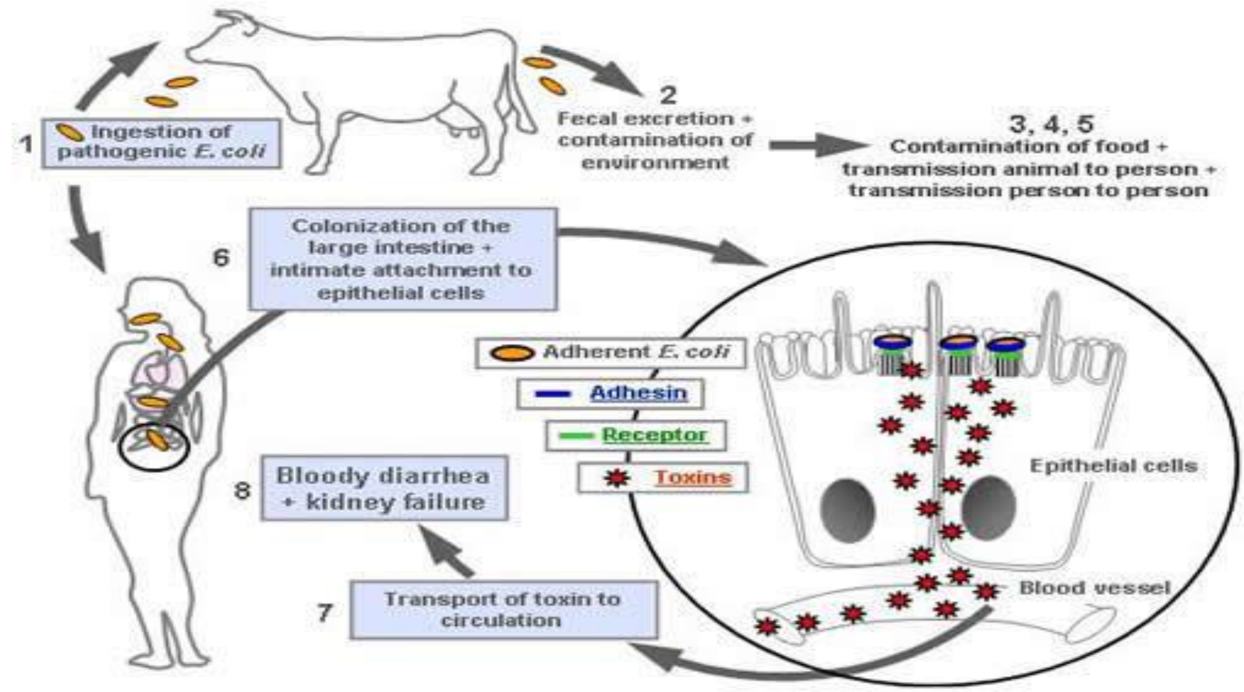


Figure 3: How zoonotic shiga toxin-producing Escherichia coli (STEC) cause bloody diarrhea and hemolytic uremic syndrome in humans ([www.ecl-lab.ca) (down](http://www.ecl-lab.ca)load date Dec 23, 2018).

EHEC clinical cases caused can occur sporadically. For EHEC O157:H7, the estimated annual incidence ranges 0.5 up to 50 cases per 100,000 populations in various countries. During the warmer months in temperate climates, EHEC O157:H7 infections occur due to shading factors of animal (Paul and Karl, 2012). But other EHEC do not necessarily follow the same seasonal pattern, and might peak at other times. In clinical cases, the mortality rate varies with the syndrome. Hemorrhagic colitis alone is usually self–limiting, although deaths can occur. EHEC-associated HUS/ TTP is estimated to be fatal in 1-10% of children and up to 50% of the elderly.

3.7 Treatment of HUS

HUS comprises acute renal failure and its consequential perturbation of fluid and electrolyte balance, hemolysis, risk of stroke as well as it will result production of toxin, and complement complex formation. To address this, multidrug approach is used. This includes implementing of antiplatelet and thrombolytic agents and thrombin inhibitors, selective use of antimicrobials, probiotics, toxin neutralizer and antibodies against key pathogenetic pathway elements to interrupt pathological processes are necessary to eliminate diseases. Acute renal replacement therapy (ARRT); for example, peritoneal dialysis (PD) or hemodialysis is also another approach to treat this disease (Paul and Karl, 2012). Antibiotics are usually used to avoid or treat the toxic which are released from the dead bacteria cell. Monitoring of hemoglobin, hematocrit and platelet count is also essential to control hematological issues (Paul and Karl, 2012).

Targeting the ligand like Synsorb PK mimics of the receptor for Stx, globotriaosylceramide (Gb3), binding to Stx in the gastrointestinal tract with the intention of preventing the spread of toxin to extra intestinal sites have been proposed as a new approach. Antibodies are used for therapeutic purpose through neutralize the toxin. Monoclonal antibodies targeting the A subunit epitopes of Stx1 have been shown as neutralize agents. For instance, orally administered mAb such as Rotavirus and Gastrogard-R, are applied for gastro intestinal infection (Islam and Stimson, 1990). Other binding agents also used to treat the disease. Most of these agents bind to toxin directly and inhibit the binding to its receptor present on the target cells.

Attenuated and recombinant vaccine producing of EHEC also indicates another novel approach to eradicate this disease. Several vaccine strategies have been shown in animal model (Gu et al. 2011). Recombinant virulence proteins such as Stx, intimin and E. coli secreted protein A (EspA) or peptides or fusion proteins of A and B subunits of Stx2 and Stx1 such as Stx2Am-Stx1B or a virulent ghost cells of EHEC O157:H7 are used as a strategy to design vaccines. On the other hand live attenuated bacteria like salmonella as a carrier for vaccine proteins against mucosal pathogens including EHEC as well as protein-based vaccines and DNA vaccines are also used as new way of strategies (Paul and Karl, 2012). Moreover, Fluid and electrolyte imbalance can be treated with Intravenous fluids, acute renal failure. Acute can be treated by renal replacement therapy, apheresis and antihypertensives and the last further effects of toxin can be prevented by antibiotics (Paul and Karl, 2012). Vaccines against EHEC O157:H7 may reduce shedding, and have received full or conditional approval in some countries including the U.S. and Canada, but are not in wide use.

3.8 Approaches to cease pathogen transmission

The best way to diminish the transmission of this pathogen is keeping the hygiene. This pathogen is transmitted through ingestion of contaminated foods and water. There is no substitute for the proper handling of meat and other food products to prevent contamination with pathogenic bacteria. Similarly, proper water treatment should also limit the transmission of these organisms (Bruce et al., 2002). Proposed interventions include the application of disinfectants (e.g., chlorhexidine), various antimicrobials or bacteriophages to the terminal rectum; the use of probiotics that would preferentially colonize the gastrointestinal tract; dietary manipulations; reductions in animal density in feedlots to decrease transmission. Management practices to decrease EHEC in the environment include the storage of effluents on a cement floor for 3 months or longer before discharge, and the collection of all liquids in a trap to minimize leaching of liquid manure into groundwater. Composting manure before use as a fertilizer may reduce transmission from this source; however, the survival of the organism varies with the size and composition of the compost heap, the temperature attained, and other biological processes (aerobic and anaerobic digestion), heat drying, and/or chemical treatments have been proposed to sanitize farm effluents before discharge into the environment (Bruce et al., 2002).

3.8.1 Disinfection

E. coli can be killed by numerous disinfectants including 1% sodium hypochlorite, 70% ethanol, phenolic or iodine–based disinfectants, glutaraldehyde and formaldehyde. This organism can also be inactivated by moist heat (121°C [250°F] for at least 15 min) or dry heat (160–170°C [320-338°F] for at least 1 hour). Foods such as ground beef can be made safe by cooking them to a minimum temperature of 160°F/ 71°C. Ionizing radiation or chemical treatment with various substances, such as sodium hypochlorite or acetic acid, may reduce or eliminate bacteria on produce.

Bacteria in biofilms are more difficult to destroy, and longer treatment times are usually necessary. Heat, steam and other physical means combined with disinfectants can be more effective than disinfectants alone.

3.9 EHEC diagnosis methods

Different methods of detecting O157: H7 have been developed throughout the world (i.e selective culture, immunological method, molecular method PCR and LAMP). The selective methods used to detect EHEC O157:H7, but do not identify EHEC O157: H– or non-O157 EHEC, which are biochemically similar to other *E. coli* and do ferment sorbitol. These techniques are labor-intensive and not widely available, non-O157 EHEC is generally detected by their verotoxin-production, and sent to a reference laboratory for further identification. Moreover, conventional selective culture methods based on biochemical features are tiresome and typically require up to 72 h; therefore, the development of rapid techniques for detection based on immunological and genetic targets has become the focus of attention (Reza et al., 2016).

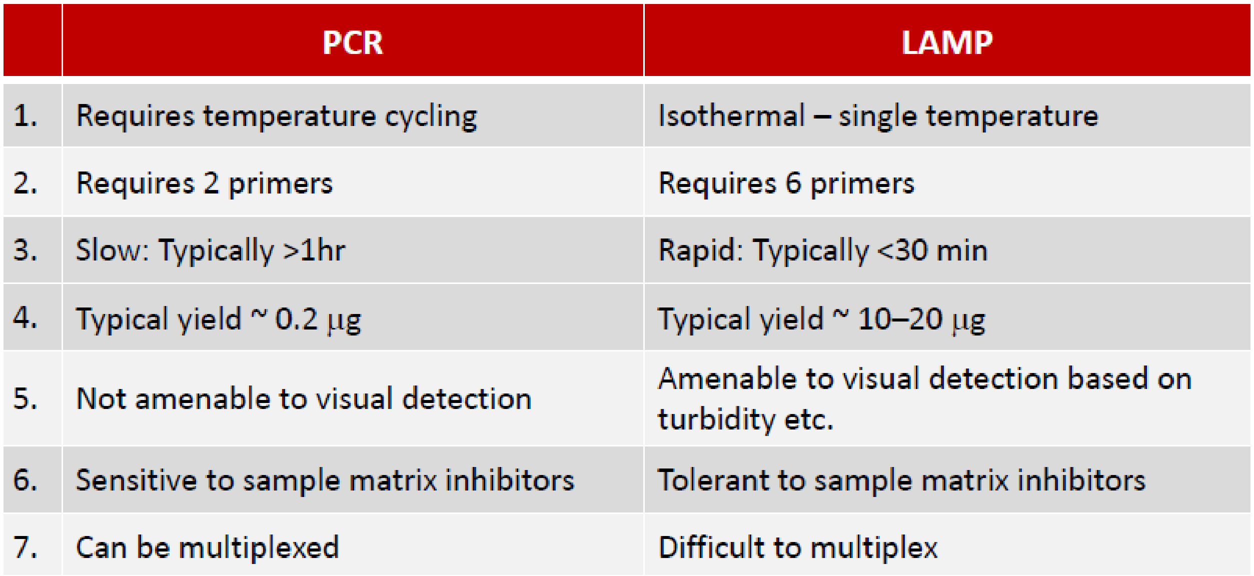
Rapid immunological tests that detect O and H antigens or various genes associated with EHEC are used with human clinical samples or food samples, but some kits validated for this purposes may lack sensitivity when testing fecal samples from animals. The techniques based on the detection of O157 somatic and H7 flagellar antigens also are inadequate due to their lack of specificity (Paton and Paton, 1998). Enzyme immunoassay, a rapid detection method, has shown a high requirement for the population of pathogens. Additionally, less number of antibodies are developed for the purpose of detection. Such methods have failed to differentiate between O157:H7 and other less-virulent (Reza et al., 2016). EHEC and several Shiga toxins may not be detected at all.

Most of the methods currently in use to identify whether EHEC are present in a facal specimen or food, now involve DNA-based techniques. Molecular methods, such as PCR and real-time PCR assay, have been used during the past decade to detect food-borne pathogens (such as *E. coli* O157:H7) by amplifying a number of relevant genes. Nonetheless, the need for trained staff, operating space and sophisticated instrument to maintain discrepancy, post amplification process like electrophoresis, takes more time(3-4 h) to know the result, requires extracted DNA from sample and sensitive for contaminants (PCR inhibitors) has impeded its usefulness (Paton and Paton, 1998, Dhama et al., 2014). Hence, there has been an increasing demand for simple and cost-effective molecular tests. Loop-mediated Isothermal Amplification (LAMP), a novel nucleic acid amplification method that relies on an auto-cycling strand displacement DNA synthesis, is performed by Bst DNA polymerase (Reza et al., 2016).

The advent in the molecular biology has led to development of various types of molecular detection methods, which includes different type of PCR detection methods such as multiplex PCR, real time PCR, reverse transcription PCR, quantitative PCR, immunocapture PCR, PCR-ELISA and so on… (Dhama et al., 2014). To ravage the odd faced by PCR, there are several isothermal amplification of nucleic acid amplification methods have been invented; to list some of them: NASBA(nucleic acid sequence based amplification), 3SR(self-sustained sequence replication) and SDA (strand displacement amplification) along with rolling cycle amplification (RCA), signal mediated amplification of RNA technology (SMART), isothermal multiple displacement amplification (IMBA), helicase dependent amplification (HAD), single primer isothermal amplification (SPIA) and circle helicase dependent amplification (cHDA) (Gill and Cohami, 2008; Lizardi et al., 1998).

All of them have their own drawback. For instance, 3SR, NASBA and SDA bypass from PCR by abolishing heat denaturation. The first two methods use a set of transcription and reverse transcription reactions to amplify the specific genomic material whereas SDA uses asset of restriction enzymes digestion and strand displacement DNA amplification with modified nucleotide as substrates. On the other hand, besides their advantage, they have short comes (Notomi et al., 2000). Among the above isothermal amplification techniques, Lamp is unique and easy. According to world health organization (WHO), the criteria of ideal diagnosis comprise those things: specificity (detect solely the targeted pathogen), sensitivity (detect the pathogen if the microorganism is found in small amount), considerably of low cost, rapidity (technique requires minimum time for detect or result), simplicity, adoptability of any sort of environment variation and easy availability of instrument. Perhaps, PCR fulfills some of WHO requirements for ideal diagnosis such as sensitivity and specificity. In case of Loop mediated isothermal amplification technique, satisfies other qualities which are stated by WHO (Dhama et al., 2014).

Table 1: the discrepancy between PCR and LAMP of molecular diagnosis methods (Source: Dhama et al., 2014).



	PCR	LAMP
1.	Requires temperature cycling	Isothermal – single temperature
2.	Requires 2 primers	Requires 6 primers
3.	Slow: Typically >1hr	Rapid: Typically <30 min
4.	Typical yield ~ 0.2 µg	Typical yield ~ 10–20 µg
5.	Not amenable to visual detection	Amenable to visual detection based on turbidity etc.
6.	Sensitive to sample matrix inhibitors	Tolerant to sample matrix inhibitors
7.	Can be multiplexed	Difficult to multiplex

3.10 Loop mediated isothermal amplification

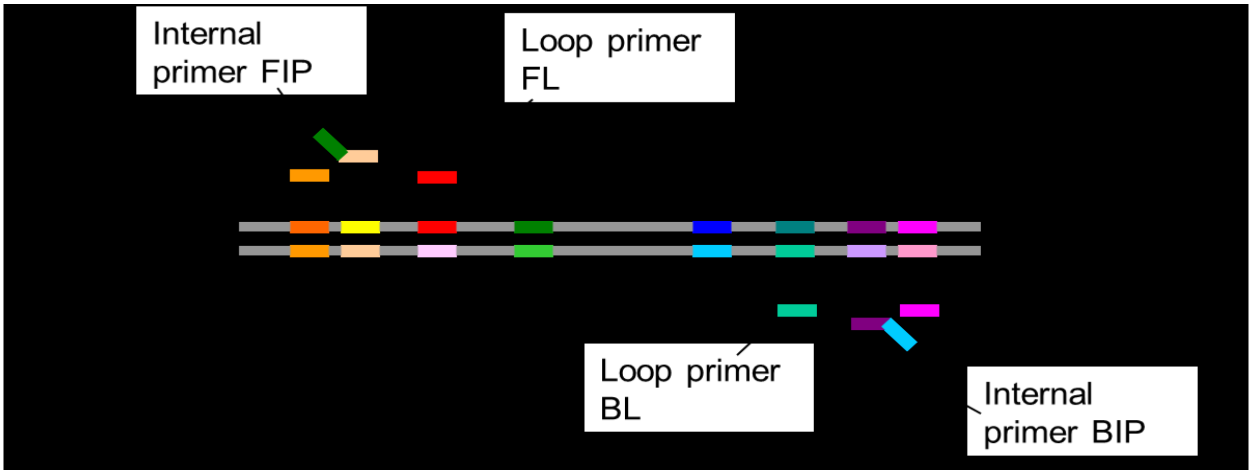
Lamp technique was developed by Notomi and coworker during 2000 GC. AS the name indicates, lamp uses isothermal or constant temperature, so that it diminishes the use of different temperatures required for denaturation, annealing and extension of DNA amplification of PCR (thermal cycler). On the other hand loop means, the final products are mixture of stem loop DNA with various stem lengths and cauliflower structure with multiple loops due to hybridization between alternately inverted repeats in the same strand (Notomi, 2000). The basic principle of this technique is autocycling strand displacement DNA synthesis using specific polymerase enzyme with high strand displacement activity like Bst polymerase which is isolated from *Bacillus stearothermophilus* (Bst) and a set of six primers which includes two loop primers specially designed to bind to unique sites on the target sequence were used. The added advantage of LAMP assay is the overall reaction completes within 30 min and an hour. During this assay, a large amount of white magnesium pyrophosphate precipitate will generate for positive results (Kundapur and Nema, 2016). LAMP amplifies a greatest amount of DNA more than 10^9 DNA copies from negligible amount of DNA within an hour.

3.10.1 Sensitivity and specificity of LAMP

The specificity of lamp for target sequence is very high compared to other nucleic acid amplification methods. This is achieved by the primers which can recognize six to eight sequences of the target gene at the beginning of amplification and later on four regions or sequences of the target gene (Notomi, etal. 2000). This renders to amplify the target gene which is unique to that pathogen in order to detect and discrepancy from other pathogens. Lamp is very sensitive and able to detect a small amount of DNA or sample. LAMP can detect 78 pg DNA and whereas PCR detects 7.8 ng DNA per test tube (Ranjabar et al., 2016). This indicated that it is 100 times more sensitive than PCR for the detection of EHEC. This implies that if there is a little amount of DNA in a sample, it easily amplifies DNA in to billions of copies. Additionally, lamp is less prone than PCR to the presence of irrelevant DNA and PCR inhibitors; so that DNA can be easily extracted through boiling method.

3.10.2 Principles of primer design

Unlike to that of PCR, lamp has six to eight primers. The performance of lamp mainly relies on crafting of primer which is specific to the target gene. Lamp primers comprise asset of inner primers, two outer primers and two loop primers. The inner primers are known as forward inner primer(FIP) which contains F1C and F2 hybridize with F2C and backward inner primer(BIP) hybridize with B2C and it contains B1C and B2. Outer primers are known as forward outer primer (F3) and backward outer primer (B3) and those comprise F3 and B3 which are complementary to F3C and B3C accordingly (Nagamine et al., 2002). Incorporation of two more primers i.e. loop primers increase the specificity as well as reduce the reaction time (Nagamine et al., 2002).



FIP(Forward Inner Primer)	BIP(Backward Inner Primer)
F3(Forward Outer Primer)	B3(Backward Outer Primer)
FL(Forward Loop Primer)	BL(Backward Loop Primer)

Figure 4. Lamp primer location on the gene. Source: (Nagamine et al., 2002)

3.10.3 Lamp amplified product detection

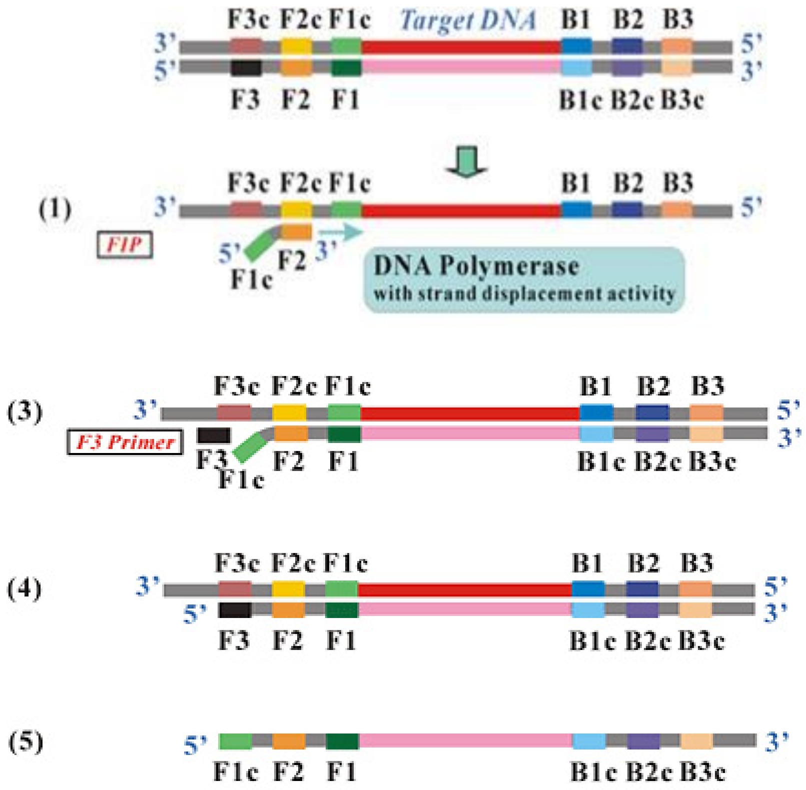
The novel LAMP assay is able to detect and distinguish between generic *E. coli* and VTEC when present on beef or in bovine faeces at levels of >10^2– 10^3 CFU per g, as demonstrated by the results of testing of artificially spiked samples (Stratakos et al., 2016).

Amplified LAMP reaction mixtures contained magnesium pyrophosphate (as a by-product), a white turbidity, which could be observed by the naked eye, emerged in the positive tubes. There is no turbidity in the negative tubes. Naked-eye detection is performed by SYBR Green II or calcien that is added to the reaction mixture, and the color change will observed (Reza et al., 2016).

3.10.4 Lamp reaction mechanism

Lamp reaction is carried out by auto-cycling strand displacement DNA synthesis which is done at 60-68 0c for 30-60 min with the aid of bst Polymerase that is used for strand displacement and polymerase activity, specific primers, dNTPs and Template DNA. The reaction mechanism has three stages; starting material producing stage, cycling amplification stage and elongation and recycling stage (Notomi et al. 2000). In the initial stage, FIP (inner primer) hybridizes with F2 region which is found in the target gene. Thereof, it initiates complementary new strand synthesis. F3 (outer primer) which is short length base and less concentration than inner primers. This render to diminish the hybridization of outer primer (F3) with the target region of the gene before FIP hybridizes. F3 slowly hybridizes with F3c on the target DNA strand and initiates strand displacement DNA synthesis, releases FIP linked complementary strand, which forms looped structure at one end. This single strand used as a template for BIP -initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis leading to the production of a dumb-bell form DNA, which is quickly converted to a stem –loop DNA. Then this stem loop structure is used as a starting material for the next of reaction, LAMP cycling.

The second stage starts by hybridization of FIP with the loop in the stem-loop DNA. Then FIP primes strand displacement and DNA synthesis, generate an intermediate one gapped stem loop DNA with an additional inverted copy of the target sequence in the stem. BIP sequence forms Loop at the opposite end. Subsequent self-primed strand displacement DNA synthesis yields one complementary structure of the original stem-loop DNA and one gap repaired stem-loop DNA with a stem elongated to twice as long and a loop at the opposite end. Both of them are used as template for the next BIP-primed strand displacement in the subsequent cycles, the elongation and recycling step. Mixtures of stem loop DNA with numerous stem length and cauliflower-like structures with multiple loops are produced at end of the reaction (Notomi et al. 2000). Moreover, the reaction mechanism is illustrated in the underneath figure1.



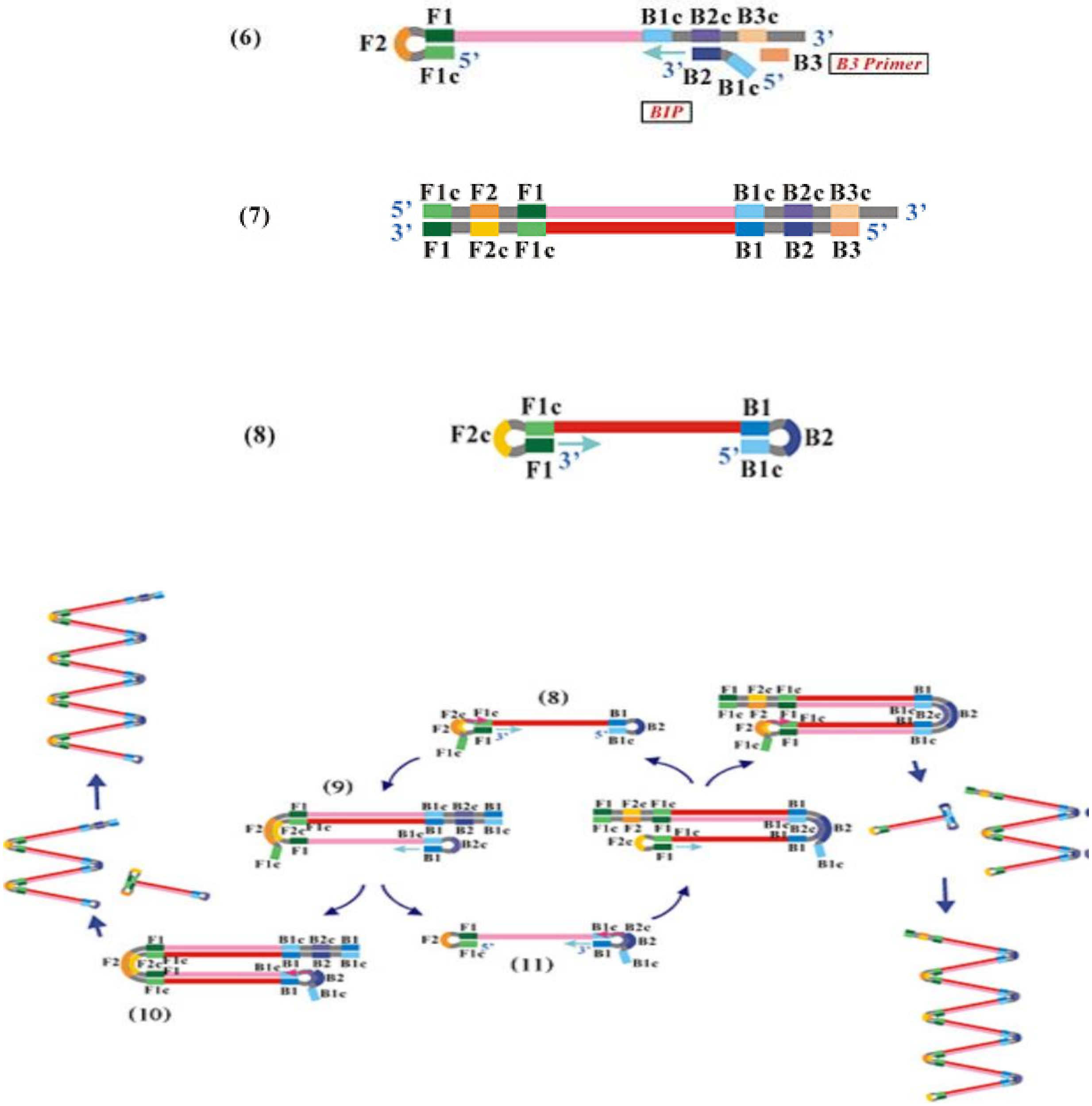


Figure 5: mechanism of loop mediated isothermal amplification (Notomi *et al.* 2000)

3.10.5 Lamp amplified product detection methods

Several detection methods can be used for detection of positive and negative result of Lamp reaction. The most common method is agarose gel electrophoresis. Gel staining agent such Ethidium bromide is used as intercalate in the DNA bases. Under UV illumination, the gel shows a ladder like structure from the minimum length of target DNA which are the various length stem-loop products of the LAMP reaction. Amplified LAMP reaction mixtures contained magnesium pyrophosphate (as a by-product), a white turbidity, which could be observed by the naked eye, emerged in the positive tubes. There is no turbidity in the negative tubes. Naked-eye detection is performed by SYBR Green II that is added to the reaction mixture, and the color change will be observed (Reza et al., 2016).

SYBR Green is one of a cyanine dye which closely binds to all double-stranded DNA present in the amplified product. During LAMP as well as PCR, polymerase amplifies the target sequence which creates the LAMP and PCR products. SYBR Green dye then binds to each new copy of double-stranded DNA. SYBR Green dye binds to all double-stranded DNA, so In case of RT PCR the result is an increase in fluorescence intensity proportioned to the amount of PCR product produced (Priyanka et al., 2016). SYBR Green also used as staining dye for southern analysis. Thus, it can be appropriate in primary clinics or field labs.

End point turbidity method detection uses spectrophotometer or turbidimeter which detects magnesium pyrophosphate precipitate produced by DNA polymerization. This method is rapid, expensive and not quantitative and detected by naked eye. Real time turbidity method is same as end point turbidity but it's rapid and quantitative. End point fluorescence is also other method of detection which increases in fluorescence of dsDNA intercalating dye in the presence of ds DNA. It uses fluorimeter, quantitative and result can detect with naked eye (Notomi et al, 2000). Real time fluorescence is mimic with real time turbidity but the difference is real time fluorescence uses fluorescence cation modulating dye (eg. Calcien) after precipitation of pyrophosphate salt. Lateral flow device is also other method of detection in which Lamplicons are biotinylated via biotinylated primer and hybridized to fluorescently labeled probe, the bid to avidin coated-coated test strip. It uses Lateral flow device and its sequence specific, rapid, inexpensive, nonquantitative, post amplification manual procedure of detection (Kiatpathomchae et al, 2008). The last detection method is ABC-LAMP (Alternately Binding quenching probe Competitive LAMP), target and synthetic competitive DNA are co-amplified; decrease in fluorescence of sequence specific fluorescently labeled ssDNA probe (up on binding to target) indicates amplification. This method is end point detection, quantitative, rapid detection and it uses fluorimeter. Moreover, based on the purpose, selection of Lamp detection method can be done; for instance endpoint turbidimetry and end point fluorimetry are inexpensive methods. For Sensitive, real time turbidimetry and real fluorescence can be used whereas for Sequence specific, lateral flow device and ABC-Lamp can be also used (Tani et al, 2007).

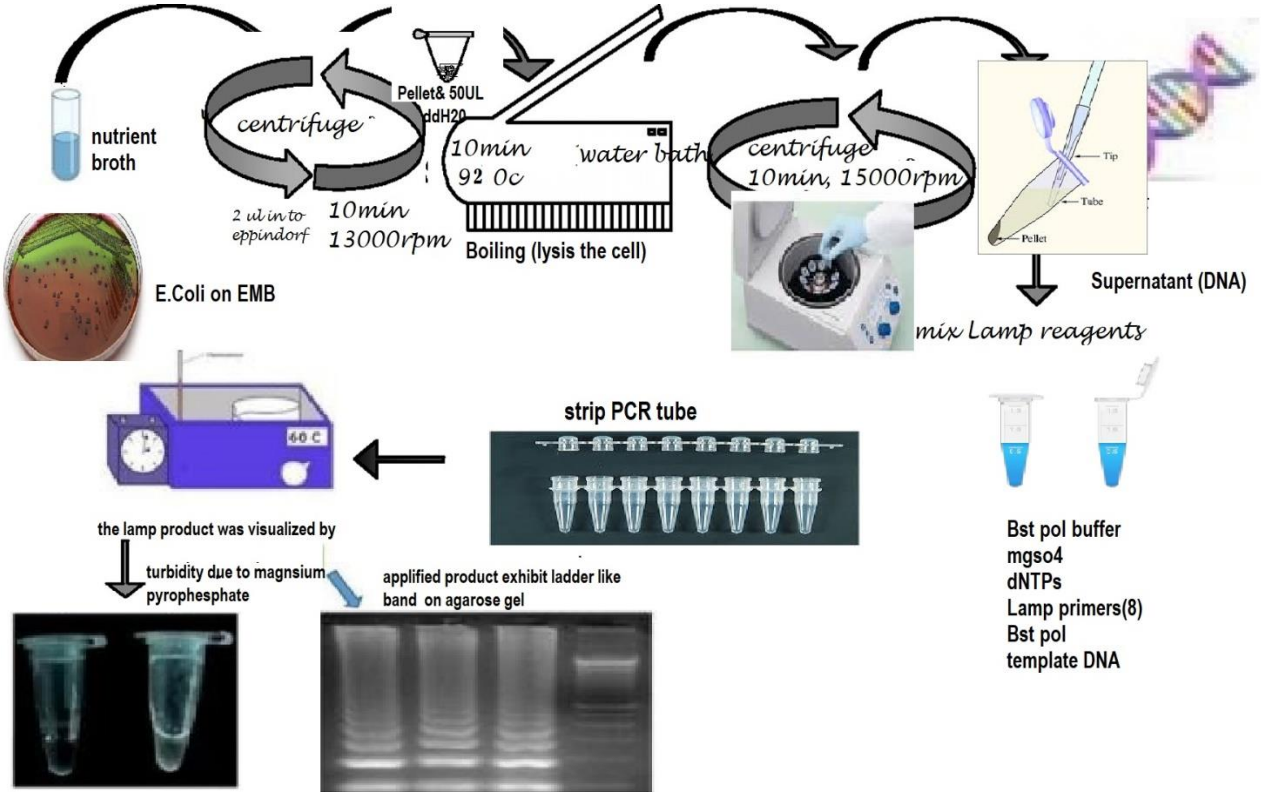


Figure 6: Simple DNA extraction through crude lysate DNA extraction for LAMP assay and LAMP procedure.

3.10.6 Overview of diverse application for LAMP

Mostly published journals are talking about that application of LAMP on microbial detection. But LAMP is also employed in diverse fields. It can be successfully employed for the identification of genetically modified organisms (GMOs), cancer cells, food adulterations, molecular diagnosis, drug resistance, identification of medicinal plants, and allergens (Kundapur and Nema, 2016).

LAMP assay was developed by Maeda et al. (2009) for diagnosis of cancer. As Maeda et al. (2009), Lamp can detect of carcinoembryonic antigen-mRNA as a marker for detecting tumor cells in patients with non-small cell lung cancer. RNA isolated from tumor and lung node were used for comparison of lamp with RTPCR as diagnosis of cancer. The Lamp diagnostic assay was found to be 81% sensitive and 100% specific. Minnucci et al. (2012) also developed the LAMP assay for the diagnosis of chronic myeloproliferative neoplasms. This LAMP assay detected low levels of mutation which were undetectable by PCR. The other application of lamp is GMO detection event and it was proved to be sensitive than the routine PCR. Lamp assay is target mainly exogenous elements or foreign genes of GMO (Kundapur and Nema, 2016).

Table 2: Diverse applications of lamp

Purpose	**Detection**	**Gene**
GMO	Maize	35S Promoter
	Rice (TT51-1)	Sucrose phosphate synthase
	Soybean	Lectin, Nos: GTS 40-3-2: MON89788
	Wheat	(B73-6-1) B73-6-1
Diagnosis	Lymph node metastasis in lung	Carcinoembryonic antigen-mRNA
	Neoplasm (myeloproliferative neoplasm) JAK2V617F	JAK2V617F
	Gastric cancer cells	Cytokeratin-19
Allergen	Celery (*Apium graveolens*)	Mannitol dehydrogenase
Pesticide	Organophosphorus in agroproducts	Monoclonal antibody against OP
Medicinal plants	Ginger (Zingiber officinale)	RAPD amplicon
Adulteration	Ostrich meat	Cytochrome b
Drug resistance	Multidrug resistance gene	NDM-126590244/ Cfr
Species and sex identification	Formosa landlocked salmon	Growth hormone GH 1 and OtY2m;
Epigenetic study	Hypermethylated DNA	Promoters of CDKN2A, GATA5 and

Notes: GMO: genetically modified organisms, NDM-1: New Delhi Metallo-lactamase 1, cfr: chloramphenicol-florfenicol resistance, CDKN2A: cyclin-dependent kinase inhibitor 2A, GATA5: GATA binding protein 5, DAPK1: death-associated protein kinase1.

Source: (Kundapur and Nema, 2016). Review article Loop-mediated isothermal amplification: Beyond microbial identification

4. MATERIALS AND METHODS

4.1 Bacteria strains and bacterial culture

In this study, 36 *E. coli* strains among them 6 entrohemorrhagic (O157:H7) and 10 entropathogenic *E. coli*, 7 non diarrheic *E. coli* strains and 13 non entrohemorrhagic shiga toxic (stx) *E. coli* isolates and 4 non *E. coli* species (*Salmonella spp*, *Shigella spp*, *Staphylococcus aureus* and *Klebseilla pneumoniae*), available as glycerol stocks in the Dr. Tesfaye's laboratory, Institute of Biotechnology, Addis Ababa University were used. Four non *E. coli* bacteria were obtained from Ethiopian public health institution (EPHI), from the stock cultures of the Department of Microbiology. Eosin methyl blue (EMB 37.46 gm/l) was used for culture *E. coli* isolates; Mac Conkey Agar (Suspend 50 gm of powder in 1L distilled) and nutrient agar were used to culture non *E. coli* bacteria strains. Those *E. coli* and non *E. coli* strains were incubated aerobically overnight at 37 ^{o}C. The EHEC *E. coli* isolates were further tested in for their sugar fermentation in SMAC agar after growth over night at 37 ^{o}C.

4.2 DNA extraction

Boiling method of DNA extraction was carried out. Thus, pure colonies of bacteria were taken from overnight culture and placed in a test tube containing 5ml of nutrient broth (30 gm/L); 1.5 ml aliquot was taken in to 2ml eppendorf tube from the overnight cultured broth. Then, 1.5 ml of aliquot was centrifuged at 15,000 rpm for ten min. The supernatant was discarded and 50ul sterile distilled water was added to the bacterial pellet, boiled at 95 ^{0}C for 10min. Then, centrifugation was carried out at 15000 rpm for ten min. Then the supernatant to be used as template DNA was transferred to a new eppendorf tube. Bacterial culture, SMAC differential culture and DNA extraction from broth, serial dilution and spiked faece were carried out in Institute of Biotechnology laboratories, Addis Ababa University whereas PCR and LAMP assay were done in HIV/AIDS research laboratory, Ethiopian Public Health Institution.

4.3 Primers

Z3276 genes LAMP assay primers and PCR primers used in this study to detect entrohemorrhagic O157:H7 *E. coli* were used based on Ravan et al. (2016) and Li et al. (2017), respectively. The lamp assay primer and PCR primer sequences that were used in this study are listed in table below. The oligonucleotide primers were synthesized by Encaba biotech. Plc., South Africa.

Table3. Lamp and PCR primer sequences used for the study

Target gene primer		Sequence (5’–3’)	Reference
Lamp Z3276	Forward outer primer(F3)	5’cggcgaacagtaaggaag3’	Ravan et al. (2016)
	Backward outer primer(B3)	5’actggccatgactggtat3’	
	Forward inner primer (FIP)	5’tggtggttctgttagatccaacaagcatcgcggaatatgg3’	
	Backward inner primer (BIP)	5’ttcggccaacggcgatcatgaaactcctggtttatctg3’	
	Forward loop primer(FLP)	5‘caaaccctacaccattatctgt3’	
	Backward loop primer(BLP)	5’gcggcgacatcattatgga3’	
PCR Z3276 (Amplicon size 836bp)	Forward	5’tattccgcgatgcttgttttt3’	Li and Chen (2012) sited in Li et al. (2017).
	Reverse	5’attatctcaccagcaaactggcgg3’	

4.4 Optimization of LAMP Assay reaction components and temperature for Z3276 gene

The LAMP assay optimization was conducted as follows. Each reaction mixture (total volume of 25 µL) contained 1.6 uM of each FIP and BIP or 1 ul of 40 uM , 0.4uM of each F3 and B3 or 1 ul of 10 uM, 0.8 uM of each FLP and BLP primers or 1ul of 20uM , 3.5 ul deoxynucleotide triphosphate, 2 ul of MgSO4, 2.5 ul of 10X Bst ThermoPol Reaction Buffer, 1 ul (8 U) or 0.32 U/25ul of the Bst 2.0 DNA Polymerase(NEW ENGLAND BioLABS # M0538), ddH_2O and 2 µL of the target genomic DNA. Moreover, different concentrations of betaine (4 ul, 2 ul and 2.5 ul) and diminishing of betaine were used during LAMP assay optimization step. The reaction mixture was incubated at 65 ^{0}C for 60 min in a thermal cycler or water bath at the same temperature settings. Next, by decreasing the temperature to 4 ^{0}C for 5 min the reactions were terminated. Then, the detection of amplification products was performed; for this, 6ul of the amplification products was mixed with 6x loading dye and electrophoresed in 1.5% agarose gel and then stained with ethidium bromide for visualization by Gel Logic imaging system (GL 200) carestream health.INC.

4.5 PCR validation

PCR assay for Z3276 gene was performed in parallel to validate the results produced by LAMP assays. Hence, the reaction mix (25 µl) was composed of 2.5 µL 10× PCR buffer (100 mmol/L Tris-HCl, pH 8.3 and 15 mmol/L MgCl2), 1ul of deoxynucleoside triphosphate, 0.5 ul of 10uM each of the outer primer (F3 and B3), 1 U of Taq polymerase, and 2 µL of sample DNA. ABiometra PCR cycler was used for amplification with reaction conditions including heat denaturation by incubation at 95°C for 5 min, and then 30 cycles at 95°C for 40 s, annealing at 60°C for 40 s, and extension at 72°C for 40 s, followed by the final extension at 72°C for 7 min. The amplified products were subjected to gel electrophoresis on 1.5 % agarose gel; then the gels were stained with 7µl ethidium bromide. The fragmented bands were visualized via Gel documentation system (Gel Logic imaging system (GL 200) Kodak Company).

4.6 Detection Limit (Sensitivity) and specificity of LAMP Assay on Bacterial Culture

To confirm whether the Lamp assay was specific to enterohmorrhagic E. coli O157:H7, the following 36 E. coli isolates were used: 6 EHEC O157:H7 isolates, 10 entropathogenic E. coli, 7 E. coli isolates from non-diarrheic calves (non-pathogenic) and 13 non entrohemorrhagic shiga toxic producing E. coli (STEC), were used. On the hand, overnight broth cultures of *E. coli* O157:H7 were serially diluted 10^{-1} up to 10^{-8}. An aliquot of 1 ml of each dilution was used for DNA template preparation and 500 ul of each dilution was plated on plate count agar. In addition, 1gm of stool was also spiked with 10 ml of 10^{-4} serial dilution of overnight culture. An aliquot of 1.5 ml of the dilution was plated on the agar for viable count while another 100 μL was used for the extraction of DNA by using crude lysate method. Then both DNA templates prepared for sensitivity and spiking were subjected to Lamp assay and PCR method.

4.7 DNA extraction from *E. coli* O157:H7 spiked faecal samples

Faecal samples (1 g) were diluted in 9 ml d H2O. Samples were vortexed for 5 sec and allowed to settle for 2 min and then vortexed again; 1.5ml spiked faecal samples were transferred in to 50ul 10X EDTA contained eppendorf tube. Samples were vortexed for 30 seconds followed by centrifugation at 200 g for 30 sec; then, the supernatants from each 5 sample were transferred to new 2-ml eppendorf tubes. The supernatants were centrifuged at 14,900 rpm for 2 min. Supernatants were discarded and pellets washed 2 times with 1 ml of 1x TE buffer. Pellets were re suspended in 50 ul of 1x TE buffer and placed in boiling water for 10 min. Centrifugation was carried out at 15000 rpm for 10 min. Then the supernatant used as template DNA was transferred to a new eppendorf tube and DNA samples were stored at -20 ^{0}C until amplification reactions (PCR and LAMP) were performed.

4.8 Data analysis

Diagnostic performance matrix was performed to evaluate the sensitivity, specificity and efficiency of the LAMP assay. The calculation was carried out based on Baker (2009) as shown below. Additionally, in order to measure the agreement or relationship between PCR assay and LAMP assay results, Cohen's kappa test statistics were carried out.

Table 4. 2x2 contingency table

	Actual +ve	**Actual -ve**	**Total**
Test +ve	A	B	E
Test –ve	C	D	F
Total	G	H	T

1. Sensitivity = fraction of actual positive that tested positive

$$= \frac{Actual\ positive\ test\ positive}{Total\ positive}$$

2. Specificity = fraction of actual negative that tested negative

$$= \frac{Actual\ negative\ test\ negative}{Total\ negative}$$

3. PPV (+ve predicted value)= Deals about what fraction of time that result has the correct answer, if the result gives +ve.

4. Efficiency = fraction of all samples that test status matched actual status

5. RESULTS

5.1 Growth on SMAC Medium

From 36 *E.Coli* isolates, eight *E. coli* isolates were tested by SMAC: EHEC; H2, D18, 107N and 155N, non EHEC; 157N, 158N, 154N and 159N. This was done after isolates (intimin and shiga toxin positive from previous studies) were identified as EHEC by PCR (Z3276 primers) in order to check whether the SMAC agar could discriminate non EHEC from EHEC. Sugar fermentation characteristics of the EHEC O157: H7 isolates used in this study on SMAC media agar showed that none of the 8 samples (EHEC and non EHEC pathogenic *E. coli* were isolated from non-diarrheal calves) fermented sorbitol; thus they appeared as colorless colonies.

5.2 Optimization of PCR for amplification of Z3276 gene and identification EHEC O157:H7

Pathogenic *E. coli* strains which were isolated from diarrheic calves and children were identified and confirmed by PCR. Z3276 gene (putative fimbria protein coding gene) primers which are specific for EHEC O157:H7 were used for conformation of the enterohemorrheagic *E. coli*. Pooled DNA from stx positive and eae positive *E. coli* was examined using Z3276 specific primers. Pooled DNA, T004, h(hemolysin) and dd water as negative control were used For this experiment.

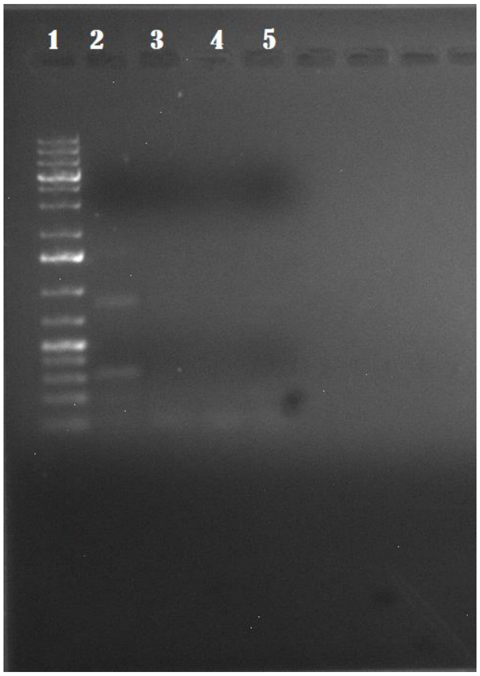


Figure 7. Optimization PCR and screening of EHEC O157:H7. Lane1: DNA ladder (100plus), lane 2: pooled DNA Z3276 gene amplification, lane3: T004, lane4: h and lane 5: negative control.

5.2.1 Optimization of cycle number and temperature

On the first optimization, multiple bands were observed from the pool DNA and the bands were not also clearly visible. To address this problem, the PCR cycle number was increased from 25 to 30 cycles. Additionally, in order to eliminate nonspecific band, annealing temperature was increased from 55^{0}c to 60 ^{0}c (Figure 8).

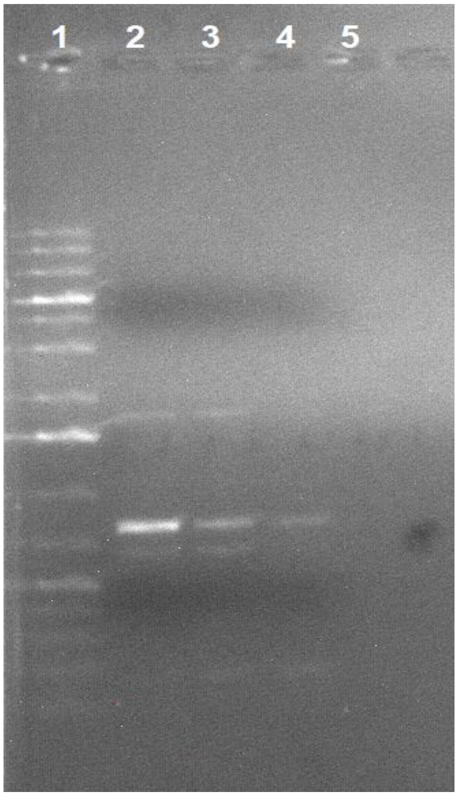


Figure 8: Optimization of PCR cycle number and temperature. Lane1: DNA ladder (100plus), lane 2: pooled DNA (Z3276 gene amplification, band size 836 bp), lane3: H2 isolate (EHEC), lane4: D18 isolate (EHEC), lane5: negative control.

Positive result was obtained from the pooled DNA samples and then separately each isolate was tested. Among eight stx(shiga toxin) and eae(intimin) positive *E. coli* isolates, six isolates showed positive amplification. Hence, those six isolates were confirmed as enterohemorrhagic *E. coli* O157:H7. Thereof, one of the six positive results (H2 *E. coli* isolate) was used as positive control for the subsequent experiments.

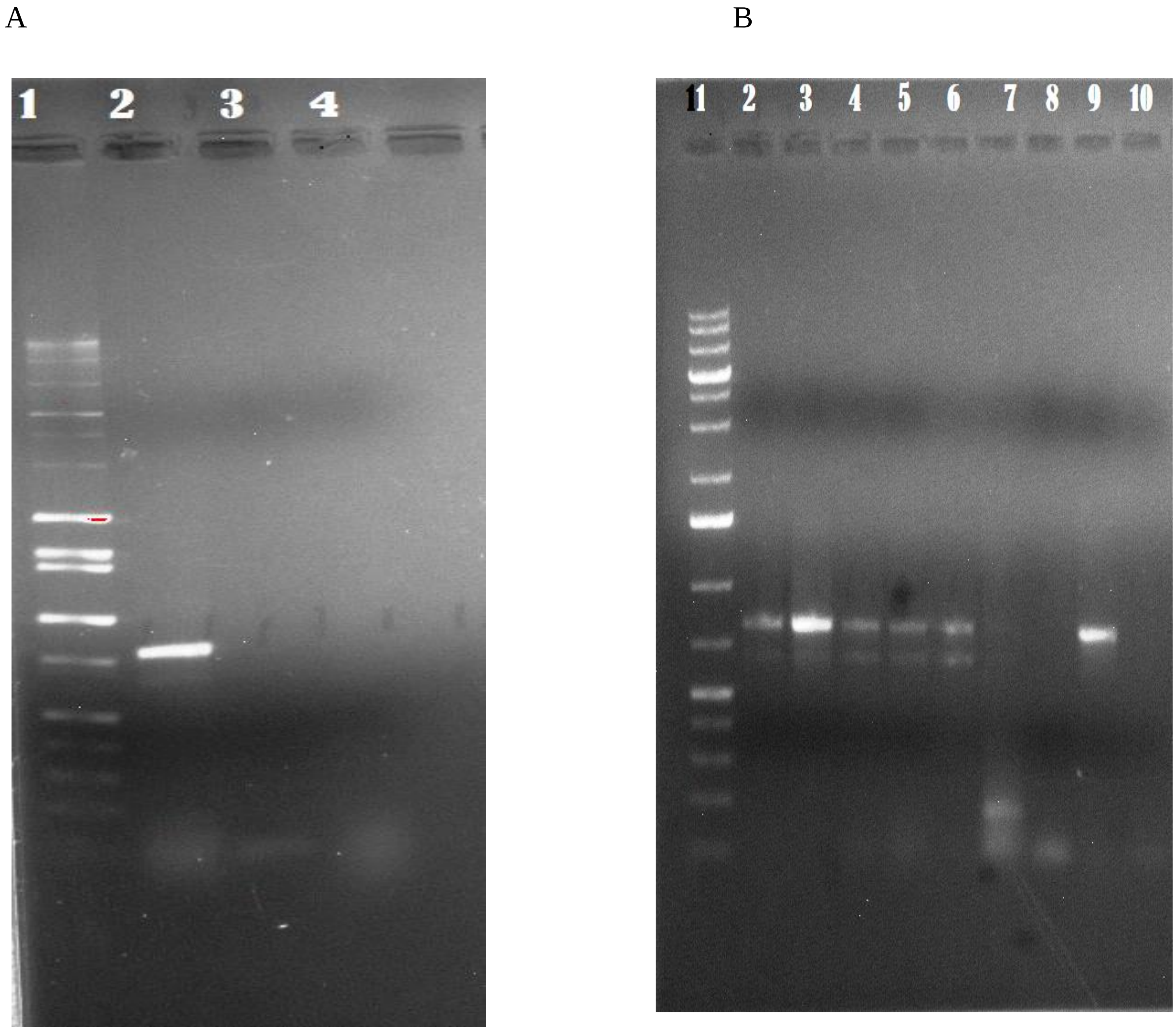


Figure9. Electrophoretic analysis of Z3276 gene amplification products, band size 836bp. (A) PCR detection of pooled DNA of different *E. coli* isolates; lane1: DNA ladder (100plus), lane 2: pooled DNA, lane3: negative control(T004 DNA), lane4: negative control(ddwater) (B) Different *E. coli* isolates; lane 1: DNA ladder (100plus), lane 2: D39, lane 3: H2, lane 4:155N, lane 5: 107N, lane 6:166N, lane 7: K33, lane 8: K51, lane 9: K51(Z3276 gene amplification: 836bp) and lane 10: negative control.

5.3 LAMP detection method

After the isolates were confirmed by PCR, one EHEC isolate (H2) was subjected to LAMP technique. A successful LAMP reaction was confirmed by observing turbidity due to formation of large amount of as pyrophosphate ion by-product, the yield of an insoluble white precipitate of magnesium pyrophosphate in reaction mixture. The white precipitate was easily observed under UV light so precipitation was easily seen rather than direct by naked eye (Figure 10 B). Further validation was carried out through Gel electrophoresis on a 1.5% agarose gel which exhibited smear in case of DNA amplification. LAMP result of positive isolate which was confirmed by PCR formed smear with bands of different sizes from up to dawn, whereas negative control did not show any turbidity and any bands on the agarose gel electrophoresis under similar conditions (Figure 10). LAMP optimization was carried out by changing bst buffer, magnesium phosphate and template DNA. In order to optimize the assay, the time for amplification was adjusted to 60 minutes at 65 ^{0}C temperature. During optimization bst buffer 2.5 ul, dNTPs 1ul, magnesium sulfate: 2, 1 and 1.5 ul, betain : 4, 2 and 2.5 ul, 1ul primer each, Bst polymerase 1ul and template 7 ul were used in the LAMP final volume of 25ul. Therefore, optimizations were carried out through changing magnesium sulfate and betain concentration in several trials. Nonetheless, no result was observed. Further optimization was carried out by removing betain (enhancer) and via changing the concentration of primers, and dNTPs. Thereof, 0.5 ul primer each, 2 ul of magnesium sulfate, 3.5ul of dNTPs were used in 25 ul of Lamp final volume.

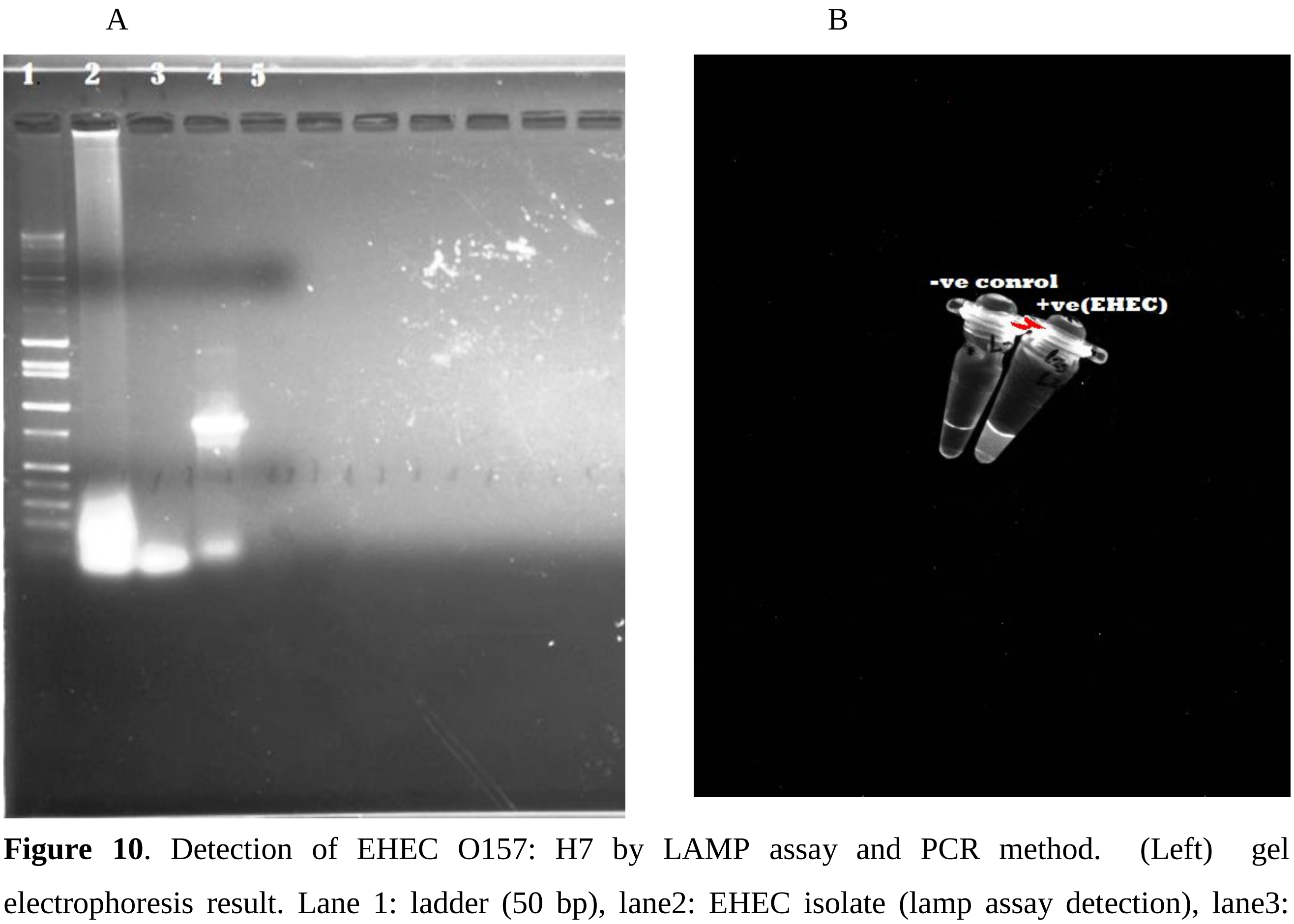


Figure 10. Detection of EHEC O157: H7 by LAMP assay and PCR method. (Left) gel electrophoresis result. Lane 1: ladder (50 bp), lane2: EHEC isolate (lamp assay detection), lane3: lamp negative(the lower band was indicated the primer dimer (the band size was less than 100bp): because of six primers were used in the reaction mix, lamp assay was prone for primer dimer than

PCR), lane 4: EHEC isolate (PCR amplicon), lane 5: PCR negative. (Right) precipitation under UV. Left PCR tube: Negative control, right PCR tube: EHEC(positive).

5.4 Specificity of LAMP assay

Among the 36 bacterial isolates tested by the EHEC LAMP assay, false-positive or false-negative results were not observed and these results were used for performance assay calculation (see sub topic 5.5). This indicates that the high specificity of the LAMP assay for enterohemorrhagic *E. coli* detection. To validate the smears which were observed from positive results, the template DNA was decreased from 7 ul to 2ul of 25 ul of Lamp reaction mix. Therefore, ladder-like pattern of LAMP amplification was demonstrated upon gel electrophoresis. Furthermore, the underneath figures (9, 10, 11) illustrate the specificity of Lamp assay.

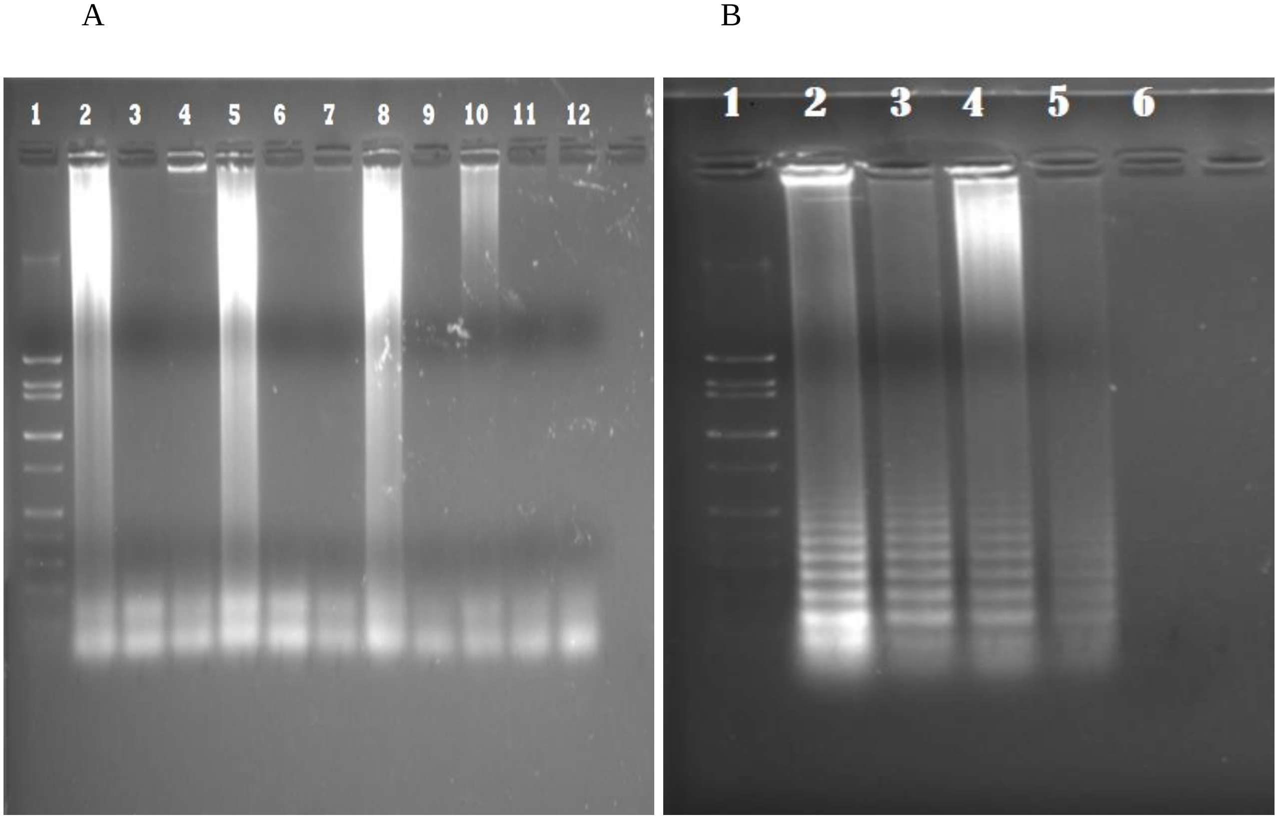


Figure 11. Specificity of lamp assay for detection of EHEC: (A) 4 EHEC isolates and 7 non diarrheic *E. coli* isolates. Lane 1: DNA ladder, lane 2: H2 (EHEC), lane 3: 158 N, lane 4: 154 N, lane 5: 155 N (EHEC), lane 6: 108N, lane 7: 159, lane 8: 107N (EHEC), lane 9: 157N, lane 10: 166N (EHEC), lane 11: 156N and lane 12: 160N. (B)Ladder like band pattern of EHEC (positive results) as observed by decreasing of template DNA to 2 ul.

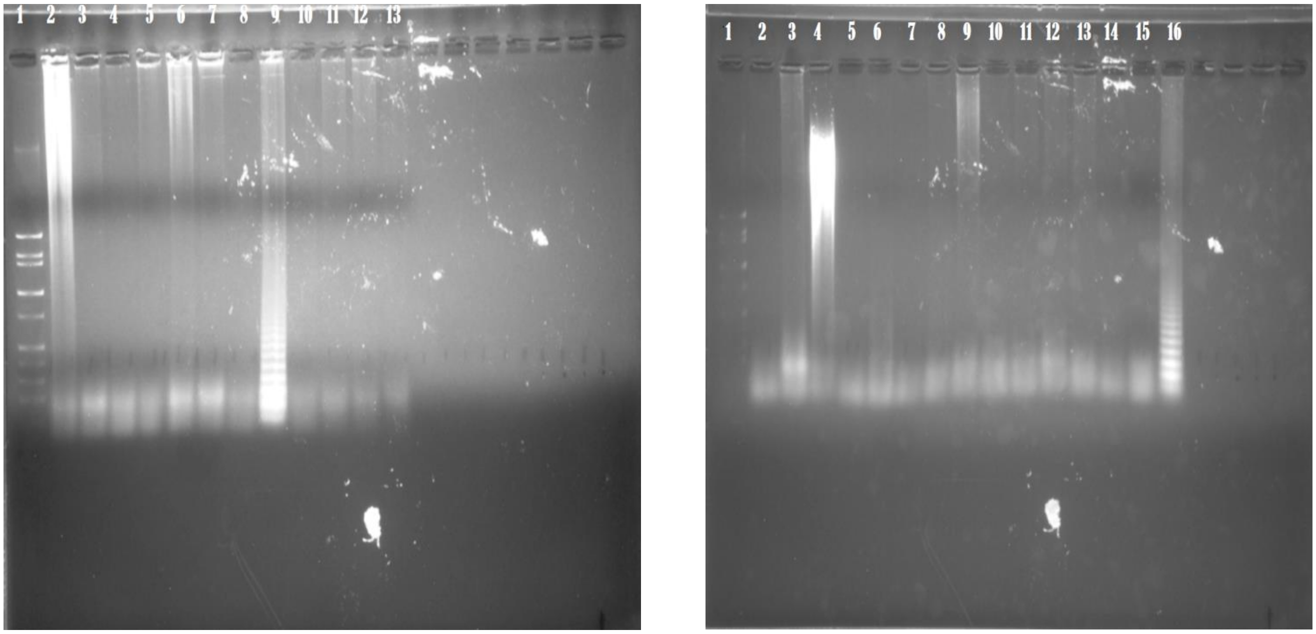


Figure 12. Specificity of LAMP assay: (left) 10 entropathogenic *E. coli* isolates. Lane 1: DNA ladder, lane 2: H2 (EHEC), lane 3: D33, lane 4: D27, lane 5: 56, lane 6: 38, lane 7: 25, lane 8: 34 A, lane 9: D 18(EHEC), lane 10: K33, lane 11: K51 and lane 12: 16, lane 13: 004. (Right) 13 non entrohemorrhagic shiga toxic (stx) producing *E. coli* isolates. Lane 1: DNA ladder, lane 2: negative control (K33), lane 3: D42, lane4: D39 (EHEC):, lane 5: D43 , lane 6: D29, lane 7: D27, lane 8: D24, lane 9: 13, lane 10: D 28, lane 11: 34T, lane 12: 39, lane 13: 008, lane14: D44, lane 15: h (hemolysin positive), lane 16: D18 (EHEC) and lane 17: ddH$_2$O.

Furthermore, specificity tests for non *E. coli* bacteria were also evaluated. Among four non *E. coli* spp one was false positive by Lamp assay. Thus, shigella, salmonella and *Staphylococcus aureus* bacteria were negative, whereas *Klebseilla pneumoniae* was false positive. False positive means that if LAMP assay was detected non EHEC as EHEC while it was not EHEC by PCR whereas false negative means that if the LAMP assay was not detected EHEC while it was EHEC by PCR.

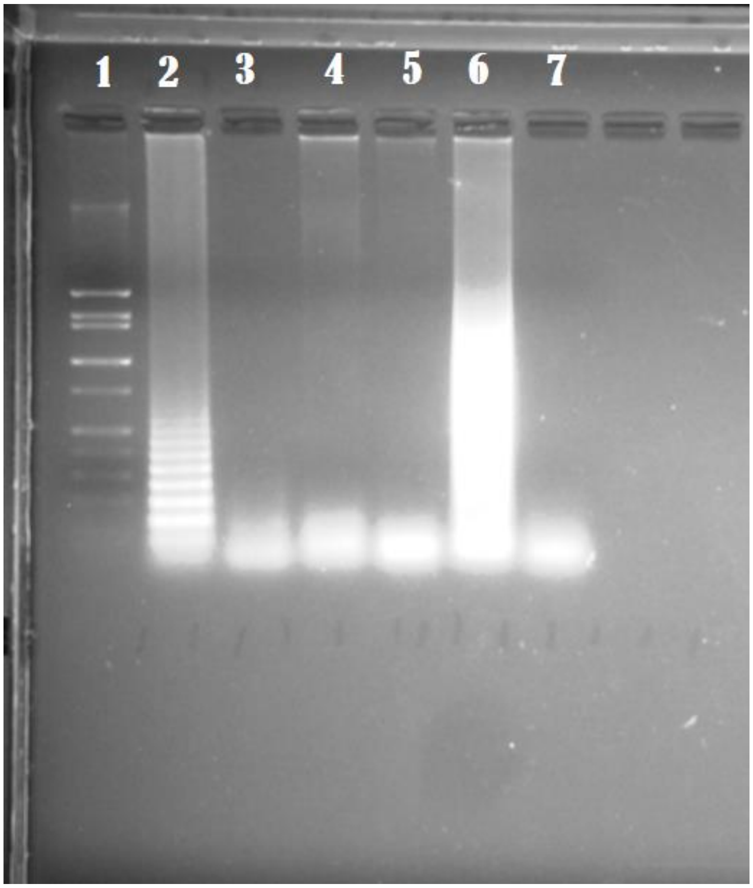


Figure 13. Specificity of LAMP assay: non *E. coli* bacteria. Lane 1: DNA ladder, lane 2: H2 (EHEC), lane 3: *Salmonella spp*, lane 4: *Shigella spp*, lane 5: *Staphylococcus aureus*, lane 6: *Klebseilla pneumonia*e , lane 7: negative control.

In order to check and confirm whether the LAMP primers bind to the DNA of *Klebseilla pneumoniae* genome, BLAST nucleotide was done beside LAMP assay result. The BLAST alignment between *Klebsiella pneumoniae* subsp. pneumoniae HS11286 chromosome and the eight Lamp primer sequences has shown that 94 % identity.

5.5 Sensitivity and specificity of LAMP assay

Sensitivity is the ability of a LAMP test to correctly identify those EHEC with the disease while specificity is the ability of a LAMP test to correctly identify those who do not have the disease. The 36 *E.Coli* isolates were identified through PCR from previous studies which were done in institution of Biotechnology. Additionally, through PCR, those strains were further tested parallel with LAMP for validation of LAMP results. Therefore, all LAMP results were validated via PCR. Thus regarding on PCR results the test isolates were declared as actual positive if they were EHEC and others from 36 *E.Coli* isolates were declared as actual negative if they were not EHEC. On the other hand test negative means that if the isolate was negative by LAMP assay and test positive reveals that if the isolate was positive by LAMP assay. For this study the four non *E.Coli* pathogenic bacteria were also used as actual negative. Hence, to measure the sensitivity and specificity of Lamp assay, performance of assay calculation was performed via two by two (2x2) contingency matrix method as mentioned below.

Table 5. Over all summary of SAMC, PCR and LAMP results of this study

Isolates	Bacteria category	SMAC sorbitol fermentaion	PCR assay for Z3276 gene	LAMP assay for Z3276 gene
H2	EHEC	-	+	+
D18	EHEC	-	+	+
D39	EHEC		+	+
155N	EHEC	-	+	+
166N	EHEC		+	+
107N	EHEC	-	+	+
158N	Nonpathogenic *E.coli*	-	-	-
154N	Nonpathogenic *E.coli*	-	-	-
108N	Nonpathogenic *E.coli*		-	-
159N	Nonpathogenic *E.coli*	-	-	-
157N	Nonpathogenic *E.coli*	-	-	-
156N	Nonpathogenic *E.coli*		-	-
160N	Nonpathogenic *E.coli*		-	-
D33	EPEC		-	-

D27	EPEC		-	-
56	EPEC		-	-
38	EPEC		-	-
38	EPEC		-	-
25	EPEC		-	-
34A	EPEC		-	-
K33	EPEC		-	-
K51	EPEC		-	-
16	EPEC		-	-
004	EPEC		-	-
D42	STEC		-	-
D43	STEC		-	-
D29	STEC		-	-
D27	STEC		-	-
D24	STEC		-	-
13	STEC		-	-
D28	STEC		-	-
34T	STEC		-	-
39	STEC		-	-
008	STEC		-	-
D44	STEC		-	-
H	STEC		-	-
Salmonella spp.	Non *E.Coli*		-	-
Shigella spp.	Non *E.Coli*		-	-
Staphylococcus aureus	Non *E.Coli*		-	-

Klebseilla pneumonia	Non *E.Coli*		-	+

Table 6: 2x2 contingency matrix table

	Actual +ve	**Actual -ve**	**Total**
Test +ve	6	1	7
Test –ve	0	33	33
Total	6	34	40

Sensitivity = fraction of actual positive that tested positive

$$= \frac{Actual\ positive\ test\ positive}{Total\ positive} = \frac{6}{6}\ x\ 100\% = 100\%$$

Specificity = fraction of actual negative that tested negative

$$= \frac{Actual\ negative\ test\ negative}{Total\ negative} = \frac{33}{34}\ x\ 100\% = 97.05\%$$

PPV (positive predicted value)

Deals about what fraction of time that result has the correct answer, if the result gives +ve

PPV$= \frac{Actual\ positive}{Total\ test\ positive} = \frac{6}{7}\ X100\% = 85.7\%$ NPV (Negative predicted value)

NPV (negative predicted value)

Deals about what fraction of time that result has the correct answer, if the result gives -ve

$$NPV = \frac{Actual\ negative}{Total\ Test\ negative} = \frac{33}{33}\ \text{X}\ 100\% = 100\%$$

Efficiency = fraction of all samples that test status matched actual status

$$= \frac{Actual\ positive\ test\ positive + Actual\ negative\ test\ negative}{Total}$$

$$= \frac{6+33}{40} x\ 100\% = 97.5\%$$

The study showed a sensitivity of 100%. This means that 100% of the bacteria isolates (*E. coli* and non *E. coli*) were declared to be enterohemorrhagic *E. coli* and none of isolates was falsely declared as enterohemorrhagic *E. coli* by the assay. The study showed a specificity of 97.05 %, which means that 97.05% of the study isolates were declared none enterohemorrhagic *E. coli*, while 2.95% was falsely declared enterohemorrhagic *E. coli* by LAMP assay. The study also showed a positive predicted value of 85.7%. This implies that when LAMP has a positive result, we are 85.7 % certain that the isolates are EHEC O157: H7 and if apositive result is received. On the other hand, the study showed negative predicted value of 100%, we were 100% certain that the isolates were not enterohemorrhagic *E. coli*. The efficiency of this assay was 97.5%, thus 97.5% sample tests were matched with actual status of the all samples.

Cohen's kappa test statistics

The relationship or agreement between the LAMP assay and PCR assay was evaluated by Cohen's kappa test statistics as showed below.

Table 7: kappa test statistics

LAMP assay	PCR assay			
		positive	negative	Total
	positive	6	1	7
	negative	0	33	33
	Total	6	34	40

$$k = \frac{p-pe}{1-pe} \qquad p = \frac{6+33}{40} = 0.975 \qquad pe = \frac{\frac{7+6}{40} + \frac{34+33}{40}}{40} = 0.05$$

$k = \frac{0.975-0.05}{1-0.05} = 0.97$, thereof, there was high agreement or near to perfect agreement between Lamp assay results and PCR assay results. Thus k is not like correlation coefficient that lying between 1 and -1 rather than it lay between 1 and 0. Hence, if the k value is near to 0, it reveals the poor relationship between the pairs of observations and the reverse is also true.

5.6 Detection limit LAMP versus PCR

The sensitivity comparison of the Z3276-LAMP assay and PCR was determined using serial dilution of overnight culture of E. coli O157:H7 strains. 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} serial dilutions were done. From the serial dilutions, 10^{-1}, 10^{-2}, 10^{-3} serial dilutions were detected by LAMP assay. This result was confirmed by gel electrophoresis. The LAMP method result showed a high sensitivity, with a positive detection rate of amplification of the DNA of EHECO157:H7 diluted to a minimum equivalent concentration of DNA which was the result of 10^{-3} serial dilution (figure B). On PCR amplification, the positive result was shown with the minimum equivalent concentration 10^{-1} serial dilution of EHEC O157: H7 (figure A). Negative control did not show any amplification in both LAMP and PCR method. Under the conditions established in this study, the comparative sensitivity of LAMP and PCR indicated that LAMP is 10-fold more sensitive than PCR for the detection of EHEC O157:H7.

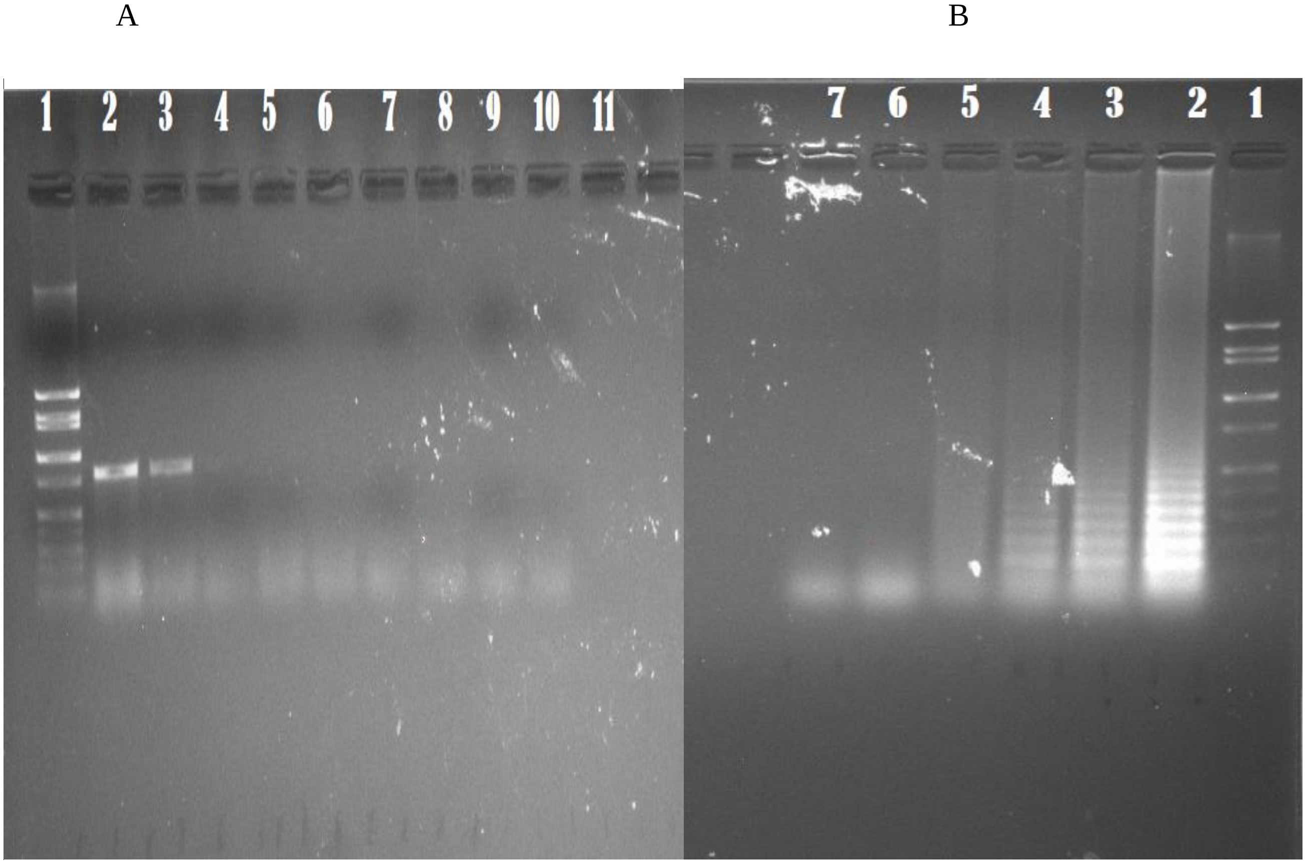


Figure 14. Comparison of sensitivity (serial dilution) of LAMP assay with PCR in detection of EHEC O157:H7: (A) Electrophoretic analysis of EHEC O157:H7 polymerase chain reaction (PCR)–amplified products. Lane 1: DNA ladder, lane 2: 10^{0}, lane 3: 10^{-1}, lane 4: 10^{-2}, lane 5: 10^{-3}, lane 6: 10^{-4}, lane 7:10^{-5}, lane 8: 10^{-6}, lane 9: 10^{-7}, lane 10: negative control. (B) Sensitivity of LAMP assay detection analyzed by gel electrophoresis. Lane 1: DNA ladder lane 2: 10^{0}, lane 3: 10^{-1}, lane 4: 10^{-2}, lane 5: 10^{-3}, lane 6: 10^{-4}, lane 7: negative control.

5.7 Detection of *E. coli* O157:H7 in artificially contaminated faecal samples

To evaluate the LAMP assay in fecal sample, 1 gm of each faecal sample was homogenized in 9 mL 10^{-4} serially diluted EHEC broth. Based on (Stabel et al., 2004) protocol with slightly modification, boiling method DNA extraction from faecal sample was carried out for each spiking faecal samples. Extracted EHEC DNA was used for PCR and LAMP. However, no positive result was observed by both PCR and LAMP detection method of EHEC. Nonetheless the positive control which was not extracted from spiked faecal sample was positive.

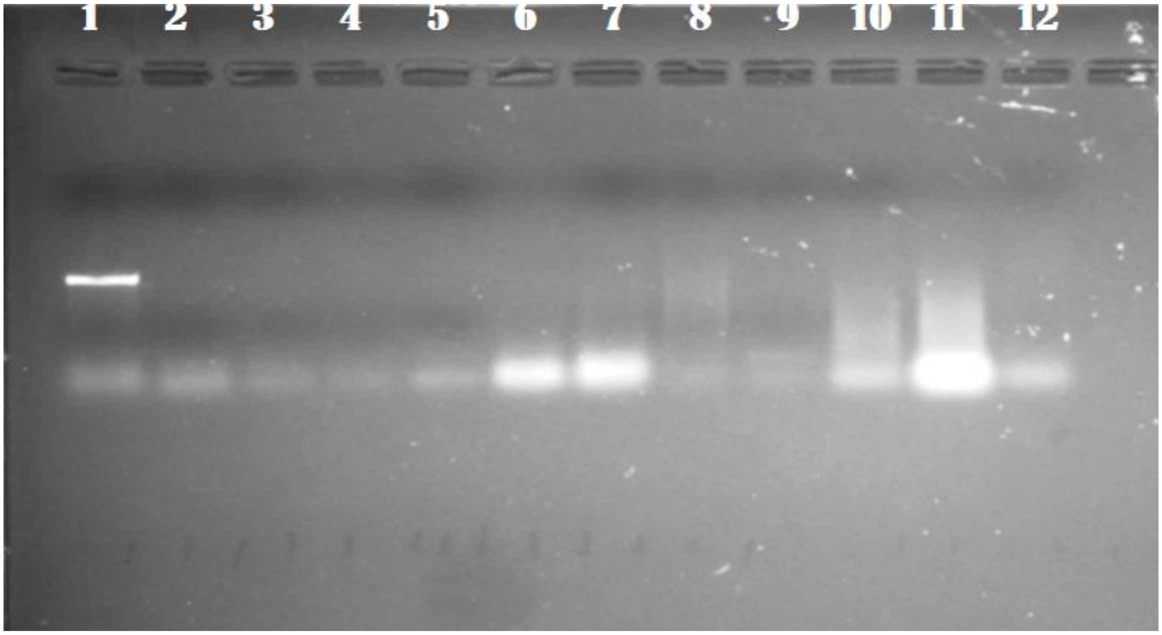


Figure 15. PCR method detection of *E. coli* O157:H7 in artificially contaminated faecal samples. Lane 1: positive control (nonspiked), lane 2: sample1, lane 3: sample 2, lane 4: sample 3, lane 5: sample 4, lane 6: sample 5, lane 7 - 11: sample replication (sample1-5), lane 12: negative control.

6. DISCUSSION

Escherichia coli O157:H7 (*E. coli* O157:H7) is one of the most important foodborne pathogen that causes illnesses such as bloody diarrhea, hemolytic-uremic syndrome (HUS) and kidney failure in human, and mainly affects children. *Escherichia coli* O157:H7 caused significant losses among the human population in the past two decades (Ashgan et al., 2015). Infections of this pathogen are worldwide. However, the lineages of this organism are reported to differ between regions, potentially influencing the incidence and severity of human disease. In case of Ethiopia, diarrhea outbreak occurred in Addis Ababa when 1,608 new diarrhea cases with 49 deaths were reported within three months; whereas across the country, a total case number of 19,176 with 182 deaths were reported (WHO, 2016). Tizeta et al. (2014) also reported the existence of *E. coli* O157:H7 from raw beef, sheep meat and goat meat of human consumed foods in Addis Ababa which leads to food born diarrheic disease. Proper detection of this human and animal pathogen (EHEC O157:H7) is needed. Different diagnostic methods of O157: H7 have been developed throughout the world and rely on basic techniques which include: isolation of bacteria on selective media, Gram-staining, biochemical characterization of the isolated bacteria, and confirmatory assays such as ELISA and PCR. Selective culture media is not sensitive and specific for EHEC, and do not identify EHEC O157: H– or non-O157 EHEC, which are biochemically similar with sorbitol fermenter bacteria. These techniques are labor-intensive, not widely available, tiresome and typically require up to 72 h (Reza et al., 2016). Rapid immunological tests that detect O and H antigens or various genes associated with EHEC are rapid detection methods, the techniques based on the detection of O157 somatic and H7 flagellar antigens also are inadequate due to their lack of specificity (Paton et al., 1998). Additionally, there are limited numbers of antibodies that are developed for the purpose of detection. Such methods have failed to differentiate between O157:H7 and other less-virulent variants (Paton et al., 1998). EHEC and several Shiga toxins may not be detected at all. Molecular methods, such as PCR and real-time PCR assay, have been used during the past decade to detect *E. coli* O157:H7. However, the need for trained staff, operating space and sophisticated instrument to maintain discrepancy, post amplification process like electrophoresis, takes more time(3-4 h) to know the result, requires extracted DNA from sample and sensitive for contaminants(PCR inhibitors) has impeded its usefulness (Dhama et al., 2014; Reza et al., 2016). Hence, there has been an increasing demand for simple and cost-effective molecular tests. Lamp can overcome and alleviate the problem of the above diagnosis methods.

Biochemical method has been used to distinguish EHEC O157:H7 from other non-pathogenic *E. coli* strains. On standard MacConkey Agar containing lactose, this strain is indistinguishable from other lactose-fermenting *E. coli*. Thereof, Sorbitol is selective and differential media for the detection of sorbitol-non fermenting Escherichia coli serotype O157:H7. EHEC O157:H7 slowly ferment sorbitol or not at all. However, from 8 samples (EHEC and non EHEC pathogenic *E. coli*) which were used in this study, did not ferment sorbitol. No color change was observed on all plated samples. Thus, the result of this study indicated that SMAC couldn't differentiate EHEC O157:H7 with non EHEC O157:H7 *E. coli*. This is because of the emerge of serotypes of sorbitol non fermenting nonO157 and O157 STEC. This reveals that like EHEC O157:H7, non EHEC O157:H7 strains are loosed their gene which was used to ferment sorbitol. To overcome the drawback of SMAC for isolation of EHEC O157:H7 by the use of chromogenic medium (CHROMagar) which has high efficiency for EHECO157:H7 isolation. Nonetheless, CHROMagar is not sensitive for all EHEC O157:H7 strains (Priyanka et al, 2016).

Loop-mediated Isothermal Amplification (LAMP), a novel nucleic acid amplification method that relies on an auto-cycling strand displacement DNA synthesis, is performed by Bst DNA polymerase (Reza et al., 2016). Several LAMP assays have been developed for the detection of *E. coli* O157 by targeting O antigens, including stx2, flic, stx, rfbE, wzx, and wzy genes (Qin et al., 2018; Ranjbar et al., 2016; Ravan et al., 2015; Wang et al., 2014). However, they are not unique genetic marker for EHEC O157:H7. Hence, this impend discriminatory power for identification of genetic marker (Li, et al, 2017). Thus, Li et al.(2017) reported that they identified Z3276 gene as a unique genetic marker for detection of *E. coli* O157:H7 rather the above genes. Z3276 could be used as an appropriate alternative gene for the specific detection of *E. coli* O157:H7 (Ravan et al, 2015). In this study, lamp primers were used based on Z3276 gene which is unique putative fimbriae protein coding gene of O157:H7 bacteria. The devised six primers have strengthened specificity of Lamp detection to O157:H7 *E. coli*. This is because they recognize eight regions in the target DNA in LAMP (Notomi et al, 2000). The concentration of Lamp primers were 1.6 mM of each inner primer, 0.4mM of each outer primer and 0.8 mM of each loop primer. Thus, the concentration of inner, outer and loop primers should be 4:1:2 in ratio (Nagamine et al, 2002). The outer primer must be ¼ of the inner primer (Notomi et al, 2000) so this rendered the inner primer (FIP) attach primarily to the template DNA before outer primer for initiate complementary strand synthesis. DNA extraction was done through crude lysate method from overnight culture; simple extraction method was used because of LAMP assay is very sensitive and less prone for DNA contaminants (Ju Teh et al, 2014). LAMP assay enhancer chemical, Betaine (N,N,N trimethylglycine, 0.5-1.5 M) was used but no result was observed during LAMP optimization step. Most of the studies used this enhancer to stimulate the overall reaction (Notomi et al, 2000; Saleh et al, 2008; Ravan et al, 2015; Qin et al, 2018). Perhaps betaine (powder form) which was used in this study was not molecular standard chemical. Therefore, in absence of betaine, the designed LAMP assay was performed well or successful. Ferna´ndez-Soto et al (2014) and Ju Teh et al (2014) reported that optimizations can also carry out without the use of betaine for LAMP reaction. LAMP assays were performed by incubation of the reaction mixtures at a constant temperature of 65°C in a regular water bath or heating block, or thermal cycler for 1hr. Most of previous studies also used LAMP assay incubation temperature of 65 ^{0}C (Ju Teh et al, 2014; Mahony et al, 2016; Abbasi et al, 2016). This is because of the fact that the optimum activity of Bst polymerase ranges between 63 ^{0}C and 70 ^{0}C. Then, after amplification incubation at 4 ^{0}C (cool temperature) for 4 minute was needed to terminate the reaction. It has been reported that in order to stop the LAMP reaction, at the end of the reaction, temperature should be lowered to 4 ^{0}C for 2- 4 minutes or the temperature should rise to 80 ^{0}C (Ferna´ndez-Soto et al, 2014). At those temperatures the Bst polymerase has no activity and has been called heat or cold inactivation of Bst polymerase.

The mechanism of loop mediated isothermal amplification is similar to cascade rolling circle amplification, and is based on the principle of autocycling strand displacement DNA synthesis (Notomi et al., 2000). Amplification products were detected by agarose gel electrophoresis and magnesium pyrophosphate precipitation. Optimization of the LAMP reaction was the main thing to get the right amplification result. The right amount or concentration of reagents could be used to come up with result. For instance in this study after separating the LAMP product on gel electrophoresis smear was observed; smear indicates high amplification result, and this was improved by decreasing the concentration of template DNA. Hence, less than 10kb bands which were various in length stem-loop products of the LAMP reaction, were observed after the smear (Nagamine et al, 2002). This is because of at the end of LAMP reaction, mixture of stemloop DNA with numerous stem length and cauliflower-like structures with multiple loops are produced (Nagamine et al., 2002, Ravan et al, 2016).

Assay sensitivity and specificity were assessed using DNA samples from other related bacteria; thus a sensitivity of 100% and specificity of 97.05 % were recorded in this study. Hence, 97.05 specificity means 2.95% was falsely declared to be enterohemorrhagic *E. coli* by LAMP assay while it was non EHEC. In this study the assay was not specific merely for *Klebseilla pneumoniae* out of 36 nonEHEC bacteria. Perhaps, this was happened due to similar DNA segment that was recognized by Z3276 primer found in *Klebseilla pneumoniae* genome. The BLAST result showed that the LAMP primers aligned and 94% identity with *Klebsiella pneumoniae* subsp. pneumoniae HS11286 chromosome (Accession:NC_016845.1). Therefore those LAMP primers are not specific with Klebseilla pneumoniae genome. Fortunately, Unlike *Salmonella* and *Shigella*, this pathogen does not cause diarrhea and rarely found in human faeces. The developed assay worked well in the identification of *E. coli* strains bacterial spp common from faecal, food, soil, and water samples (eg. *Shigella, Salmonela and Staphylococcus*). *E. coli* and *Shigella* are closely related, shared greater than 90% homology by DNA-DNA association analysis (Ju Teh et al., 2014). This study showed that Z3276 gene LAMP assay discriminated two related bacteria. Other pathogenic *E. coli* bacteria (entropathogenic and shiga toxin producing *E. coli*) were not detected with this EHEC specific assay. Results showed that LAMP assay targeting Z3276 gene detects 10^{-2} and 10^{-3} serially diluted EHEC O157:H7, but PCR method did not. Therefore, this indicates that LAMP was at least 10 times more sensitive than PCR, due to eight distinct sequences that were required for recognition in the LAMP assay. Similar to this study, various reports indicated that LAMP was 10 fold sensitive than PCR (Ranjba et, al., 2016; Qin et al., 2017; Ravan et al., 2015). The agreement between the LAMP assay and PCR assay was also measured through Cohen's kappa statistics (k = 0.97). The result implies that they had very high agreement among themselves. In a sense, if the LAMP assay detects the isolate or a sample, with 97% confidence PCR assay also detect that isolate. Hence, like correlation test, Cohen’s kappa test statistics reveals the relationship between the two assays.

Efficiency of this assay was found to 97.5%; this means 97.5% sample tests matched with actual status of the all the positive and negative samples. Thus, 2.5 % tests did not match with the actual status of the sample. This indicated that the specificity and amplification efficiency of the LAMP assays are high. In this regard, LAMP proceeds more rapidly than regular PCR as there is no time required for thermal cycling, and inhibition reactions at later stages are less likely to occur (Notomi et al. 2000; Nagamine et al. 2001).

For direct testing of faecal samples by the developed LAMP assay, faecal samples were spiked with 10^{-4} serially diluted EHEC O157:H7. However, no result was observed by both LAMP and PCR. This might be due to inefficient DNA extraction method; DNA was extracted directly from the spiked faece sample through crude lysate (boiling) method. This method mainly relies on high concentration of bacteria at pellet after centrifugation. But if the concentration of bacteria decreases by serial dilution, it’s difficult to get concentrated bacteria at the pellet. Ju Teh et al. (2014) reported boiling method was not effective for direct DNA extraction from faecal samples. Wang et al. (2014) also suggested the need of enrichment time from 8 h to 10 before DNA was extracted from spiked vegetables. Additionally, specificity of detection (*Mycobacterium paratuberculosis*) by PCR, crude lysate method was less than other commercial available DNA extraction kits (Stabel et al., 2004). Nonetheless, the developed assay overcomes the use of expensive PCR, time, sensitivity problem of PCR. This study came up with simple, cost effective, rapid and sensitive diagnosis assay for detection of EHEC O157:H7. Many studies on *E. coli* O157:H7 detection showed the feasibility of using the LAMP method in developing countries (Hill et al., 2008). Therefore, such like assays enhance for physicians to order the right drug or specific antibiotics and monoclonal antibody for patients who suffer with manifestation of HUC which is lead to death and mainly caused by EHEC O157:H7 strains (Paul and Karl, 2012).

Limitations of this work were; 1. Bst polymerase was not available in the country (Ethiopia) chemical suppliers because it is not familiar like Taq polymerase for molecular study or researches. 2.

Detection dyes or DNA dyes such as SYBR green and calcien were not used for this study. This was due to that the high cost and unavailability of the dyes.

7. CONCLUSION AND RECOMMENDATION

Enterohemorrhagic *Escherichia Coli* O157:H7 bacteria are recently evoluted pathogenice bacteria which are characterized by their Shiga toxin, intimin, and enterohemolysin producing of key virulence factors for the pathogenesis. Cost effective, simple, rapid and sensitive detection methods are required for developing countries like Ethiopia. Various detection methods of EHEC O157:H7 have been developed throughout the world. Hence, Loop mediated isothermal amplification is an emerging nucleic acid amplification method. In this study, loop mediated isothermal amplification was devised for detection of EHEC OH157:H7. LAMP assay was very rapid; the amplification of target gene was accomplished within an hour at constant temperature(65 ^{0}C) , it is at least ten times more sensitive, simple and cost effective; the reaction can carried out with simple instruments such as water bath or heat block than conventional PCR.

In conclusion, based on Z3276 gene which codes putative fimbriae protein; responsible for bacteria adhesion, LAMP diagnosis assay for EHEC O157:H7 bacteria was devised and optimized. This assay was showed 100% sensitive, 97.05 % specificity and 97.5% efficiency. In comparison with performance of the most common molecular diagnosis tool (PCR).

Based on the findings of the present research work, we recommend that

1. Differential SMAC agar should not be used as guarantee for EHEC O157:H7 identification with other non EHEC O157:H7 bacteria.
2. LAMP assay can be used in hospitals and clinics as well as food microbiology laboratories for detection EHECO157:H7 bacteria. Hence, developing countries like Ethiopia may adopt such methods to overcome the scarcity of efficient assays, to reduce time and cost for diagnosis of EHEC.
3. Further study must need to be carried out to evaluate other direct DNA extraction method (rather than using crude lysate) from diarrheic faeces for Lamp assay.
4. The specificity of the assay should be further improved.

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