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**ADDIS ABABA UNIVERSITY COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE**

# ISOLATION AND IDENTIFICATION OF CULTIVABLE AEROBIC PATHOGENIC BACTERIA FROM TICKS OF CATTLE IN CENTRAL ETHIOPIA

**BY**

**TEKABE GEBRE LIJE**

**AUGUST, 2021**

**BISHOFTU, ETHIOPIA**

# ISOLATION AND IDENTIFICATION OF CULTIVABLE AEROBIC PATHOGENIC BACTERIA FROM TICKS OF CATTLE IN CENTRAL ETHIOPIA

**A thesis submitted to College of Veterinary Medicine of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Veterinary Science in Veterinary Microbiology**

**BY**

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**DEPARTMENT OF VETERINARY MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH**

**MVSc: IN VETERINARY MICROBIOLOGY**

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As members of the examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by: **Tekabe Gebre** entitled, "**Isolation and Identification of Cultivable Aerobic Pathogenic Bacteria from Ticks of Cattle in Central Ethiopia**" and recommend that it be accepted as fulfilling the thesis requirements for the degree of Master of Science in Veterinary Microbiology.

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**DEPARTMENT OF VETERINARY MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH**

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## STATEMENT OF AUTHOR

First and foremost, I declare that this thesis is my original work, and that all sources of material used in this thesis have been properly acknowledged. This thesis was submitted in partial fulfillment of the advanced (MVSc) degree requirement at Addis Ababa University, College of Veterinary Medicine, and has been deposited at the University/College library to be available to borrowers in accordance with library rules. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of my academic degree, diploma or certificate.

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## LIST OF ABBERVIATIONS

| | |
|---|---|
| AAU | Addis Ababa University |
| API | Analytical Profile Index |
| CFU | Colony forming units |
| CI | Confidence Intervale |
| CLSI | Clinical Laboratory and Standards Institute |
| CSA | Central Statistics Agency |
| EMB | Eosin Methylene Blue Agar |
| FAO | Food and Agriculture Organization |
| GDP | Gross Domestic Product |
| Hr | Hour |
| ILRI | International Livestock Research Institutes |
| IND | Indole |
| LF | Lactose Fermenter |
| MR | Methyl Red |
| NLF | None Lactose Fermenter |
| OIE | Office of International des Epizooties |
| SIM | Sulfide Indole and Motility |
| Spp | Species |
| SPSS | Statistical Package for the Social Sciences |
| SS | Salmonella Shigella Agar |
| Subsp | Subspecies |

| | |
|---|---|
| TBD | Tick Borne Diseases |
| TDA | Tryptophane deaminase |
| TSB | Tryptic Soya Broth |
| TSI | Triple Sugar Iron Agar |
| USA | United States of America |
| USD | United States Dollar |
| VP | Voges Proskauer |
| WGS | Whole Genome Sequence |
| WHO | World Health Organization |
| XLD | Xylose lysine Deoxycholate Agar |

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## ABSTRACT

Ticks are well-known vectors of a variety of intracellular tick-borne pathogens associated with tick-borne diseases worldwide (TBD). There is, however, a scarcity of precise and up-to-date comprehensive information on cultivable aerobic pathogenic bacteria from ticks of cattle from Ethiopia. A cross-sectional study was conducted from November 2020 to July 2021 with the objectives to isolate and identify cultivable aerobic pathogenic bacteria from ticks infesting cattle in central Ethiopia, namely, Ada'a, Lome and, Ezha districts. All ticks used to study pathogenic bacteria were morphologically identified to species level under a stereomicroscope using standard taxonomic keys. During the study period, a total of 205 adult live ticks belonging to eight species, namely, *Hyalomma truncatum* (N=50; 24.4 %), *Amblyomma variegatum* (N=41; 20%), *Amblyomma cohaerens* (N=40; 19.5%), *Rhipicephalus decoloratus* (N=33; 16.1%), *Hyalomma rufipes* (N=29; 14.1%), *Rhipicephalus evertsi* (N=7; 3.4%), *Amblyomma gemma* (N=4; 1.9%), and *Rhipicephalus pulchellus* (N=1; 0.5%) were identified in decreasing order and collected for bacteriological examination. Bacterial identification was performed by using multiple biochemical tests and API-20E strips. Results of the study showed that out of the total of 205 ticks studied for the presence of bacteria, 107 (52.2%) ticks were positive and 98 (47.8%) ticks were found negative for bacterial isolation and a total of 107 isolates of different bacterial pathogen were identified from all the study areas. Out of the total of 107 bacterial isolates recorded, a total of 5 species of aerobic pathogenic bacteria were identified including, (N=39; 36.5%) *Citrobacter freundii, (N*=34; 31.8%) *Escherichia coli*, (N=18; 16.2%) *Staphylococcus aureus*, (N=8; 7.5%) *Proteus mirabilis*, and (N=8; 7.5%) *Morganella morganii.* Statistically significant differences between study districts as well as species of ticks with the isolation rates of the pathogenic bacteria were observed. In vitro efficacy evaluation of the most commonly used antibiotics demonstrated that majority of the cultivable aerobic pathogenic bacteria detected in ticks collected from cattle were susceptible to chloramphenicol, streptomycin, and gentamicin but resistance against the action of bacitracin, penicillin, and clindamycin was recorded on the disk diffusion test method. In conclusion, the high isolation rate of pathogenic bacteria in ticks collected from cattle in the current study most likely indicates that ticks play an active role in environmental contamination and increase the likelihood of pathogenic bacteria transmission to their hosts.

**Keywords:** *Tick species, pathogenic bacteria, Cattle, Ada'a, Lome, Ezha, Ethiopia*

## 1. INTRODUCTION

Ticks are blood-feeding ectoparasites that act as reservoirs for a wide range of emerging and re-emerging infectious diseases of medical and veterinary importance worldwide. Ticks rank second only after mosquitoes as vectors of human infectious diseases (Parola and Raoult, 2001). Approximately, 10% of the currently recognized tick species carry human and animal pathogens (Estrada-Pena and De la Fuente, 2014; Jongejan and Uilenberg, 2004). The parasitic lifestyle of ticks infesting livestock and companion animal hosts is of particular interest because it results in a wide range of pathogenic effects. Feeding activities of ticks are associated with direct damage to the skin and other subcutaneous tissues, inflammation, and significant blood loss. Pruritis, erythema, excoriation, papules, scale and crusting, and self-trauma are all common symptoms associated with this activity (Van den Broek *et al*., 2003). Ticks are the major vector for bacteria, protozoa, and viruses capable of causing serious and life-threatening infections in humans and animals, in addition to tissue damage, irritation, hypersensitivity, abscess, and anemia (Khoo *et al.,* 2016; Bell-Sakyi *et al.,* 2018). Among the bacterial species found within ticks, those in the genera of *Rickettsia*, *Anaplasma*, *Coxiella*, and *Ehrlichia* are already known to commonly associated with human and animal infections (Kumsa *et al.,* 2015a, b; Khoo *et al.,* 2016) and are obligatory intracellular pathogens that can't grow in artificial nutrient culture media. Tick bites are the means by which disease spreads from ticks to humans and animals (Loong *et al.,* 2018a).

In Ethiopia, several species of ticks are common and widely distributed throughout all regions (Tafesse, 1996). There are eight species of *Amblyomma*, two species of the subgenus *Boophilus*, four species of *Haemaphysalis*, nine species of *Hyalomma*, eight species of *Rhipicephalus*, and one species of *Ixodes* (Mekonnen *et al.,* 2001; Kumsa *et al.,* 2016). Ticks are regarded as having greater veterinary importance than the medical value in the country (Kumsa *et al.,* 2015a). *Anaplasmosis, babesiosis, cowdriosis,* and *theileriosis* have been reported as major tick-borne diseases affecting domestic animals (Mokonnen, 2001; Tomassone *et al.,* 2012; Teshale *et al.,* 2016). Among vector-borne pathogens of zoonotic importance obligate intracellular pathogens incapable of growing on an artificial nutrient, culture spotted fever *Rickettsia* spp., *Borrelia* spp., *Coxiella burnetii*, and *Bartonella* spp. have been documented from ticks, flies, lice, and fleas collected from

domestic animals in Ethiopia (Kumsa *et al.,* 2015a; Kumsa *et al.,* 2015b) using molecular tools.

The majority of research and reviews on isolation and identification of cultivable bacteria from various species of ticks were studied outside of Ethiopia. The major pathogenic bacteria isolated include *Escherichia coli, Klebsiella* species, *Salmonella* species from *Hyalomma* and *Rhipicephalus* tick species in Iraq (Khalaf *et al.,* 2018). *Shigella* species, *E. aerogenes*, *E. coli*, *P. aeruginosa*, *Bacillus cereus*, *S. aureus*, *C. freundii*, *Enterobacter* spp. from *Rhipicephalus annulatus*, and *Hyalomma turanicum* collected from sheep in Iraq (Jalil and Zenad, 2016). From nymphs of *Ixodes scapularis* mostly Gram-negative cocci and 11 *Bacillus* strains of Gram-negative and positive rods were isolated from adults in the US (Martin and Schmidtmann, 1998). Gram-positive bacteria including species of the genera *Staphylococcus*, *Bacillus*, and gram-negative *Pseudomonas* species were isolates reported from ectoparasites (fleas, ticks, and lice) in Australia (Murrell *et al.,* 2003). Nine strains of Gram-negative bacteria were detected *Ixodes ricinus* in which *Pasteurella* with the lowest prevalence of 0.7%-5.9% in Europe using three agar media (Stojek and Dutkiewicz, 2004).

Recent reports show worldwide growing health risks associated with tick and vector-borne diseases in both humans and animals (Dantas-Torres *et al.,* 2012). There is a risk of transmission of other pathogens carried within the ticks between cattle and man, bacterial culture and isolation are essential as they have long been accepted as the gold standard for laboratory confirmation of bacterial infections (Loong *et al.,* 2016). However, there is a scarcity of precise and up-to-date comprehensive information on cultivable pathogenic bacteria from tick species infesting cattle from Ethiopia. Therefore, this study was conceived with the following general and specific objectives in the study areas.

**General objectives:**

- To provide baseline information on cultivable aerobic pathogenic bacteria from ticks infesting cattle in central Ethiopia.

**Specific objectives:**

- To isolate and identify cultivable aerobic pathogenic bacteria from ticks infesting cattle in central Ethiopia.
- To determine the prevalence of aerobic pathogenic bacteria from ticks infesting cattle in central Ethiopia.
- To determine the antimicrobial susceptibility profile of aerobic pathogenic bacteria from different tick species infesting cattle in central Ethiopia.

# 2. LITERATURE REVIEW

## 2.1. Ticks

Ticks (Acari: Ixodida) are obligate hematophagous ectoparasites that are well-known as vectors for several pathogens linked to tick-borne diseases (TBD) all across the world. Ticks belong to the Parasitiformes suborder Ixodida, which has a single superfamily, the Ixodidae, which is divided into three families: Argasidae (soft ticks), Ixodidae (hard ticks), and the Nuttalliellidae, which has a single species (Rodriguez-Vivas *et al.,* 2004). Ticks of the genus *Amblyomma* (8 species), subgenus *Boophilus* (2 species), *Haemaphysalis* (4 species), *Hyalomma* (9 species), *Rhipicephalus* (15 species), *Ixodes* (1 species), *Argas* (1 species), and *Ornithodorus* (1 species) have all been found in Ethiopia (Mokonnen *et al.,* 2007). which are reported to have great veterinary and medical importance in Ethiopia (Pegram *et al.,* 1981). Several types of research on ticks infesting cattle have been undertaken in various locations of Ethiopia (Pegram *et al.,* 2004; Yacob *et al.,* 2008; Kumsa *et al.,* 2016).

### *2.1.1. Life cycle of ticks*

The life cycle of ixodids consists of four stages: an embryonated egg followed by three active stages. The three active stages include the six-legged larva, an eight-legged nymph, and a sexually mature eight-legged adult (Sonenshine, 1991). Generally, the life cycle of an ixodid tick begins when an engorged female retracts from a host to lay eggs on the surrounding vegetation. After hatching, larvae emerge at the vegetation's tip and begin "questing" for a host, a process in which the tick detects cues emitted by potential hosts, such as carbon dioxide (Anderson *et al.,* 1998). After attaching to a host, the larval tick then feeds and molts into a nymph. Before the final 'questing' process and mating on the final host, the nymph detaches and molts into an adult. Depending on the number of host animals they attach to during their life cycle, Ixodidae can be one-, two-, or three-host species (Walker *et al.,* 2014). Ticks with a one-host life cycle, such as *Rhipicephalus microplus*, only require one host to complete their life cycle. A one-host tick feeds on a single host throughout its entire life cycle, with only the engorged adults dropping off. Ticks that are adults feed and mate on the host. Following mating, a

female tick imbibes an enormous amount of host blood to attain full engorgement and thereafter drops off from the host into the vegetation to lay eggs. The eggs hatch into host-seeking larvae. In two-host ticks such as *Rhipicephalus evertsi evertsi* (Acari: Ixodidae), the larvae attach to a host, feed to repletion, and molt into nymphs while on the host. The unfed nymphs reattach and feed to full engorgement on the same host. Having engorged, the nymphs drop off from the host to a sheltered microenvironment and molt into a male or female adult tick. The newly emerged adult ticks search for a suitable host to attach to and feed on. Three-host ticks, such as *A. variegatum*, require three hosts to complete their life cycle (Sonenshine, 1991). A complete tick life cycle generally takes one year, but it may take three to four years for some species that dwell in colder habitats (Estrada-Pena and de la Fuente, 2014).

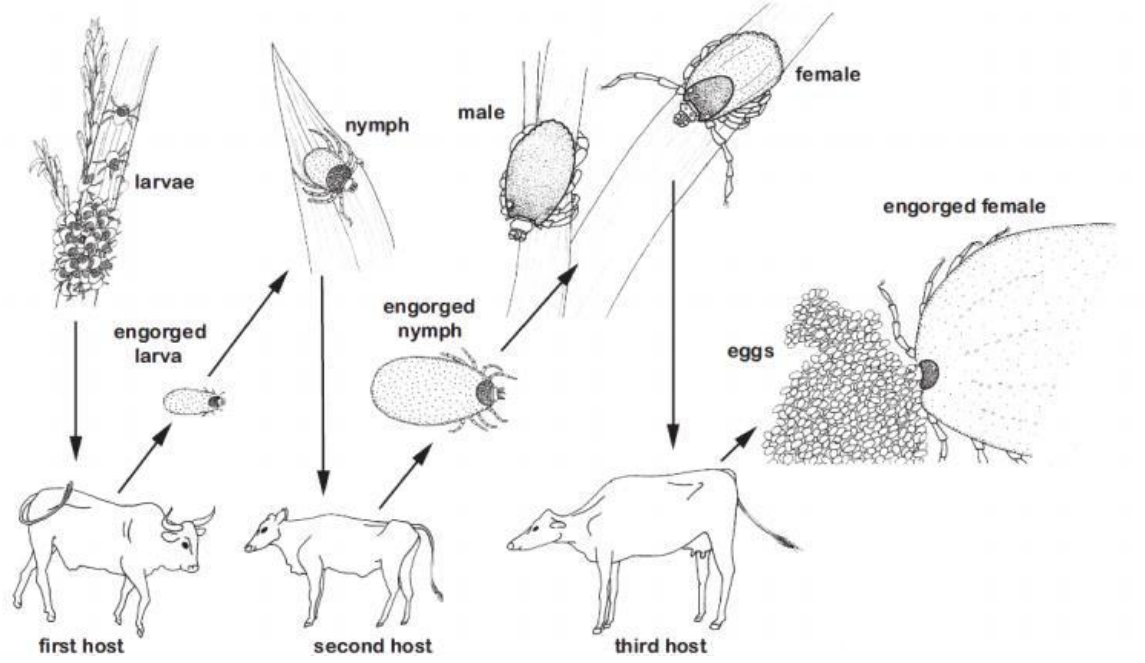


**Figure 1:** Three-host life cycle. An example is *Amblyoma varigatum* (Sonenshine, 1991).

*2.1.2. Morphological identification of ticks*

Tick morphology is divided into two sections: the mouthparts (capitulum) and the body (idiosoma). The mouthparts of hard ticks protrude in front of the body and are visible from above, whereas the body of soft ticks extends forward above the mouthparts,

making them visible only from beneath. Ticks' bodies contain the eyes, legs, as well as respiratory, digestive, and reproductive structures. Ticks are the largest and most visible members of the Acarina order; they feed exclusively on the blood of vertebrates, such as mammals, birds, reptiles, and amphibians (Wall and Shearer, 2001).

The mouthparts (capitulum) are composed of three specialized structures called palps, chelicerae, and a hypostome, all of which are attached to a base called the basis capituli (Houseman, 2013). Tick bodies (idiosoma) are not typically hardened to a great extent. The majority of the exterior cuticle in hard ticks is soft, with many internal folds that resemble grooves on the body's surface. Festoons are the uniform, rectangular folds found on hard ticks' rounded posterior end. Because of the unfolding and stretching of the soft cuticle along these grooves, immature and adult female hard ticks can consume massive amounts of blood and swell to 50 to 100 times their original weight (Houseman, 2013).

Ticks are sexually dimorphic, with males and females easily distinguishable. The side of the scutum, a sclerotized plate on the dorsal side of the tick body, is the main feature that distinguishes males from females. A male's scutum covers the entire body, whereas a female's scutum only partially covers the body. The female's shortened scutum allows the body to expand during engorgement (Barker and Murrell, 2004). The most direct and cost-effective method for identifying tick species is morphological identification using light stereomicroscope; however, accurate identification can be difficult in fully engorged ticks or ticks with damaged morphological features (Brahma *et al.*, 2014). As a result, morphological identification is sufficient to differentiate to the genus level with high confidence, making it more sensitive methods are required to distinguish to the species level (Kwak, 2017).

## 2.2. Economic and health impacts of the ticks in cattle

Ticks comprise veterinary problems because they transmit diseases, produce paralysis or toxicosis, and cause physical damage to livestock. Tick-borne diseases affect 80 percent of the world's cattle population and are found all over the world, particularly in the tropics and subtropics. They account for a significant proportion of all animal diseases affecting the livelihood of poor farmers in tropical countries. The complexity of vector-borne diseases limits the growth of the livestock industry, which is critical to rural people in sustaining not only their food supply but also their daily income and other agricultural activities (FAO, 2011). Ticks and tick-borne diseases cause significant economic losses in Ethiopia, ranking third among major parasitic diseases after trypanosomiasis and endo-parasitism (Pegram *et al.,* 1981). Cattle are free-range and graze extensively, making them vulnerable to diseases and parasites (Marufu, 2008). Ticks acquire pathogens from an infected host during a blood meal, maintain infested through multiple life stages by the means of transstadial passages, and transmit it to other hosts when feeding again (Klareenbeek, 2010).

Large numbers of ticks feeding cause a reduction in live weight, unrest, tick worry irritation, severe dermatitis, anemia, and serious physical damages, while direct injury reduces the quality of hides due to tick bites and blood loss due to tick feeding. These parasites have direct effects on milk production and weight gain in cattle (Peter *et al.,* 2005). Tick infestation causes significant economic losses, with a conservative estimate of one million USD lost in Ethiopia each year due to the rejection of downgraded hides and skins due to tick damage (Gashaw, 2005).

Ticks, in addition to sucking a large volume of blood, serve as reservoirs and vectors for a variety of human and animal pathogens around the world, injecting pathogens such as viruses, bacteria, protozoa, and toxins into their hosts. East Coast Fever, Redwater, anaplasmosis, and heartwater are among the most serious tick-borne diseases. Many other fatal and benign babesiosis and theileriosis are also transmitted by various tick species (Jongejan and Uilenberg, 2004).

The vectorial competence of ticks is essentially attributable to their capacity to allow, within their organs (e.g., mainly guts, salivary glands, etc.) the development of infective

forms of several animal and human pathogens that are then transmitted to susceptible vertebrate hosts via the tick feeding activity. Once in the vertebrate host, these pathogens' life cycle can continue, with or without a tangible impairment of the host's health (Jongejan and Uilenberg, 2004).

Ixodid ticks' blood-feeding consists essentially of two phases of ingestion, more distinctively identifiable in females, due to their larger blood intake than males (Sonenshine, 1991). Briefly, a first slow phase of feeding lasting for the 6-9 days after attachment is followed by a rapid 12–24-hour long ingestion of blood, during which these ticks take two-thirds of the total blood ingested, before detaching from the host (Sojka *et al.,* 2013). Besides the rapid ingestion, the final phase of feeding is also characterized by a concentration of the blood nutrients, made possible via the excretory activity of the salivary glands (Melhorn, 2008). This phase of the blood meal consists of an alternation of rapid ingestion and rapid regurgitation of tick saliva, allowing for the concentration of their blood meal as well as the expulsion of salivary molecules (such as antigens and allergens) and pathogens (Sonenshine, 1991). Ticks can become infected with microorganisms through several routes of transmissions, including: -

**Systemic transmission:** the host is the source of infection ('reservoir'), acquired by the tick during the blood meal. A latency period of days or even months usually intercurs from the time the host is infected until when it can be infectious to vectors. This period is needed for the pathogen(s) to replicate in the host, reaching sufficiently high blood load enabling the vector's infection (Voordouw, 2014).

**Co-feeding transmission (also known as "non-systemic transmission"):** from an infected ('donor') to an un-infected ('acquiring') tick feeding in spatiotemporal proximity to each other on the same host, in absence of a systemic infection (reviewed in Randolph *et al.,* 1996). This model of transmission may be particularly important for tick-transmitted pathogens because ticks, unlike other arthropod vectors, remain attached to their hosts for several days (immature stages) or even weeks (adults) to blood feed (Randolph, 1998).

**Transstadial transmission:** from a developmental stage to the next one (e.g., from larvae to nymph; from nymph to adult). When the newly infected tick stage feeds, it will pass the microorganism to its vertebrate host (Walker *et al.,* 2003).

**Transovarial transmission:** from an engorged mated female to her progenies (i.e., eggs, and therefore larvae). In this case, hatched larvae will be infected. This route of transmission is known to occur in the case of *Babesia bovis* and *Babesia bigemina* infections (Bock *et al.,* 2004).

**Intrastadial transmission:** between ticks of the same developmental stage of the same species during co-feeding or by the same individual (i.e., male) tick feeding on several individual hosts (Potgieter, 1981; Stiller *et al.,* 1983; Zaugg *et al.,* 1986; Dagliesh *et al.,* 1987; Andrew and Norval, 1989; Norval *et al.,* 1990; Kocan *et al.,* 1993).

The estimated annual global costs associated with ticks, and tick-transmitted pathogens in cattle amounted to between US$ 13.9 billion and US$ 18.7 billion (De Castro *et al.,* 1997). In 1989, the cost of purchasing acaricides was estimated to be 3 million Birr (US$ 1.5 million) (Newson, 1991). The economic losses caused by ticks and tick-borne diseases are significant when other costs such as fatalities, lower growth rate, and decreased milk output are taken into account.

### 2.3. Ticks and associated bacterial pathogens

Ticks are ubiquitous and are well recognized globally as vectors of several emerging pathogens, many of which pose significant threats to the health of humans and animals. The term ‘microbiome’ was defined by Nobel laureate Joshua Lederberg in 2001, as “the assembly of the microbes’ genome residing in a host body, including the commensal, symbiotic and pathogenic microorganisms” (Hooper and Gordon, 2001). Bacteria, protozoa, fungi, and viruses are examples of microbes. Understanding the host-microbe relationship and the role of microbes in host physiology can help with managing and controlling emerging infectious TBD and mitigating their negative economic and public health impacts (Narasimhan and Fikrig, 2015). There are increasing efforts to explore the tick microbiome to provide new insights into managing and controlling TBD and

transmission risks to humans. In the tick, the microbiome that has been investigated consists of bacteria, protozoa, and viruses (Andreotti *et al.,* 2011; Xia *et al.,* 2015; Bouquet *et al.,* 2017; Lado *et al.,* 2018).

### *2.3.1. Bacterial tick-borne diseases of cattle*

**Q fever** is a zoonosis tick born disease caused by *Coxiella burnetii*, an obligate intracellular parasite classified within the Rickettsiaceae family and classified into six genomic groups based on restriction fragment length polymorphism. Unlike the other members of *Rickettsiae*, *C. burnetii* is quite resistant to environmental influences and is not dependent upon arthropod vectors for transmission. *C. burnetii* exhibits two antigenic phases: phase I and phase II. Phase I organisms are more infectious. The organism has a worldwide distribution, although a large serological survey argues that it is not present in New Zealand (Maurin and Raoult, 1999).

**Rickettsiosis** is members of the family Rickettsiaceae have cell walls similar to those of other Gram-negative bacteria. Ultra-structural studies have shown that the Anaplasmataceae family has outer membranes but lacks an obvious peptidoglycan layer (Cowan, 2003). Rickettsiae are endothelial cell-targeting organisms from the Rickettsiaceae family. Although several new rickettsiae species have recently been identified in domestic animals using molecular techniques, their pathogenicity is unknown, and the only species of veterinary importance in the Rickettsiaceae family is *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever. Many Rickettsia species exist, including the typhus-causing agents (*R. prowazekii*), murine typhus (*R. typhi*), and scrub typhus (*R. tsutsugamushi*) are primarily human pathogens. These highly pathogenic organisms prefer the endothelial cells of small blood vessels, causing vasculitis and thrombosis in a variety of organs. Rickettsia species produce phospholipase, which damages phagosome membranes and allows the organisms to escape into the cytoplasm. *R. rickettsii* replicates in the cytoplasm as well as the nucleus of host cells, causing cytotoxicity (Walker and Raoult, 2000).

**Borreliosis,** which is longer and wider than other spirochaetes, has a helical shape similar to other spirochaetes. Borreliae have a linear chromosome, which is unique among bacteria, as well as linear and circular plasmids, some of which appear to be essential for the organism's growth and survival. Although these spirochaetes are capable of causing disease in both animals and humans, subclinical infections are also common. Borreliae are transmitted by arthropod vectors. Arthropod vectors are responsible for the transmission of *Borrelia* species in animals. Borreliae are obligate parasites that can be found in a wide range of vertebrate hosts. Although these organisms can survive in the environment for short periods, their long-term survival is dependent on vertebrate reservoir hosts and arthropod vectors. The associations of certain Borrelia species with specific arthropod vectors and reservoir hosts are important in determining the epidemiology of *Borrelia* species. Borreliae multiply and spread throughout the body after entering a susceptible host's bloodstream. Organisms can be seen in the joints, brain, nerves, eyes, and heart. It is unknown whether the disease is caused by active infection or by host immune responses to the organism. Persistent infection, which results in the production of cytokines, may contribute to the development of lesions. (Roberts, 1998). *Borrelia burgdorferi* genotypes may be linked to specific clinical syndromes in humans; *B. burgdorferi sensu stricto* (s.s.) is frequently linked to arthritis, *B. garinii* to neurological disease, and *B. afzelii* to skin disease (Van Dam, 1993; Gray, 1999).

**Ehrlichiosis and anaplasmosis** caused by *Ehrlichia (Cowdria) ruminantium*, a Gram negative intracellular rickettsial organism from the genus Ehrlichia. It grows in colonies or morulae and prefers the vascular endothelium, staining blue with Giemsa. The organism is coccoid and measures 0.2–0.5 in diameter. It can now be grown in vitro, and it can also be grown in mice. Tick intestinal and salivary epithelia are thought to undergo cyclical development. Bovine ehrlichiosis (also known as heartwater or *cowdriosis*) is an infectious and tick-borne disease of ruminants caused by the rickettsial organism *Ehrlichia ruminantium* (formerly *Cowdria ruminantium*) and transmitted by ticks of the genus Amblyomma, particularly *A. variegatum*, which is common in Ethiopia (Mekonnen *et al.*, 2001). The disease is common in Sub-Saharan African countries and has a significant detrimental impact on livestock productivity, with high morbidity and mortality rates in

vulnerable ruminants (up to 90%). European breeds are more susceptible than indigenous African breeds in general (OIE, 2011).

Limited data are available in the literature about the cultivable bacterial flora of ticks, only a few papers were found where authors tried to demonstrate cultivable bacteria from tick species. Earlier data show that approximately 80% of cattle populations of the world are at risk of tick infestation and tick-borne diseases (Kumsa and Mekonnen, 2011). In Ethiopia *anaplasmosis, babesoisis, cowdrosis,* and *theileriosis* have been reported as major tick-borne diseases affecting domestic animals (Mokonnen, 2001; Tomassone *et al.,* 2012; Teshale *et al.*, 2016). Among tick-borne pathogens of zoonotic importance spotted fever *Rickettsia* spp., *Borrelia* spp., *C. burnetii*, and *Bartonella* spp. have been documented from Ethiopia (Kumsa *et al.*, 2015a, b).

Researchers from the United States isolated 73 strains from 43 adults and nymphs of *Ixodes scapularis.* Gram-negative cocci were isolated predominantly from nymphs, while Gram-negative and positive rods were obtained from adults. Bacillus was the most abundant genus, with 11 strains (Martin and Schmidtmann, 1998). In an Australian investigation, the majority of ectoparasite isolates (fleas, ticks, and lice) were Gram-positive, with Staphylococcus and Bacillus genera being the most common, whereas gram-negative *Pseudomonas* species were the most frequent (Murrell *et al.*, 2003).

In Europe, two investigations were conducted in the continent's central-eastern region. In eastern Poland pooled samples of 372 *Ixodes ricinus* individuals were checked for Gram-negative bacteria, on three agar media 9 strains were found (*Pasteurella*) with low 0.7%-5.9% incidence (Stojek and Dutkiewicz, 2004). Three tick species were sampled at three sampling sites in a large study in Chechia, and 151 strains were identified (Rudolf *et al.*, 2009). 87% of the bacteria were Gram-positive (12 genera, most frequent were the *Bacillus* and *Paenibacillus*) the rest, were the Gram-negatives (5 genera). Some strains with potential medical significance were also mentioned.

Chinese researchers have isolated 15 species of bacterial flora belonging to 12 genera from the midgut of laboratory-reared *Haemaphysalis longicornis, Hyalomma asiaticum* and *Rhipicephalus haemaphysaloides* based on their morphological and biochemical

characteristics, which were combined with the 16S rDNA sequence analysis. Strains identified as belonging to *Kocuria* sp. and *Staphylococcus* sp. proved to be the most common Gram-positive bacteria in all three Ixodes ticks. *Brevibacterium* sp., *Staphylococcus cohnii* and *Staphylococcus saprophyticus/xylosus* were recovered from both *H. longicornis* and *H. asiaticum*. *Staphylococcus lentus* and *Alcaligenes* sp. were detected in both *R. haemaphysaloides* and *H. asiaticum*. No similar strains were isolated other than *Kocuria* sp. from *H. longicornis* and *R. haemaphysaloides*. However, most of the strains were isolated rarely and only in one of the ticks, such as *Caryophanon* sp., *Microbacterium* sp., *Pseudomonas* sp., *Moraxella oslo, Tetrathiobacter kashmirensis, Corynebacterium* sp., *Streptomyces* sp., and *Dietzia* sp. (Li, C. H *et al.,* 2014).

Using 16S rDNA gene sequencing, researchers in India identified 9 bacterial species from two genera (Bacillus and Staphylococcus) in the midgut of both types of *Hyalomma anatolicum* ticks (post-blood meal and digested blood meal). The majority of the midgut bacterial species (90%) were *Staphylococcus* sp., and there was a high frequency of three *Staphylococcus* species (*S. chromogenes, S. epidermidis,* and *S. gallinarum*). Phylogenetic analysis of 16S rDNA gene sequences revealed that all midgut isolates were monophyletic and shared high-frequency relationships with *S. chromogenes* and *S. epidermidis* (Anbalagan, S *et al.,* 2014). These are exhibited with the summaries are given in below table 1.

**Table 1:** Bacterial associations with different species of ticks.

| No | Tick Species | Bacteria Isolated | References |
|---|---|---|---|
| 1 | *Ixodes scapularis* | *Bacillus* species<br>*Corynebacterium* species<br>*Pseudomonas* species<br>*Pastuerella* species | (Martin and Schmidtmann, 1998) |

| | | | |
|---|---|---|---|
| 2 | *Ixodes ricinus* | *Pasteurella pneumotropica/haemolytica*<br>*Serratia marcescens,*<br>*Pseudomonas aeruginosa* | (Stojek and Dutkiewick, 2004) |
| 3 | *Ixodes holocyclus, Amblyomma triguttatum, Amblyomma fimbriatum, Boophilus microplus, Haemaphysalis longicornis* | *Stenotrophomonas* species, *Pseudomonas* species, *Acinetobacter* species, *Staphylococcus* species, *Citrobacter* species, *Klebsiella* species, *Salmonella bongori, Serratia proteamaculan, Enterobacter* species | (Murrell *et al.*, 2003) |
| 4 | *Ixodes ricinus*<br>*Dermacentor reticulatus*<br>*Haemaphysalis concinna* | *Staphylococcus* species<br>*Corynebacterium, Micrococcs* species<br>*Bacillus* species | (Egyed and Makrai, 2014) |
| 5 | *Haemaphysalis longicornis*<br>*Hyalomma asiaticum*<br>*Rhipicephalus haemaphysaloide* | *Staphylococcus* species<br>*Microbacterium* species<br>*Pseudomonas* species | (Li, C. H *et al.*, 2014) |
| 6 | *Hyalomma anatolicum* | *Staphylococcus* species | (Anbalagan, S *et al.*, 2014) |

| | | | |
|---|---|---|---|
| 7 | *Rhipicephalus microplus*<br>*Haemaphysalis bispinosa*<br>*Ixodes granulatus*<br>*Dermacentor* sp. | *Bacillus* species, *Enterococcus faecalis, E. coli, K. pneumoniae*<br>*Lysinibacillus* species<br>*Corynebacterium* species<br>*Rothia* species, *Staphylococcus* species | (Loong *et al.*, 2020) |
| 8 | *R. microplus* | *Staphylococcus simulans, Bacillus subtilis and Bacillus flflexus* | Xu, X.-L *et al.*, 2015) |
| 9 | *Haemaphysalis wellingtoni, Haemaphysalis hystricis, Haemaphysalis bispinosa* | *Bacillus* species, *Staphylococcus* species, *Mycobacterium* species, *Acinetobacter, Corynebacteterium, Streptococcus, Klebsiella, Serratia, Clostridium, Stenotrophomonas* species, *Pseudomonas* species | (Khoo *et al.*, 2016) |
| 10 | *Rhipicephalus* species | *Klebsiella* species<br>*Staphylococcus aureus.* | (Rahuma *et al.*, 2005) |
| 11 | *Hyalomma* species | *Staphylococcus aureus.* | (Myahi *et al.*, 2019) |

| | | | |
|---|---|---|---|
| 12 | *Hyalomma* species<br>*Rhipicephalus* species | *Escherichia coli*<br>*Klebsiella* species<br>*Salmonella* species<br>*Serratia* species | (Kirecci *et al.*, 2015) |
| 13 | *Rhipicephalus annulatus*<br>*Hyalomma turanicum* | *Shigella* species, *E. aerogenes, E. coli, P. aeruginosa, Bacillus cereus, S. aureus, C. freundii, Enterobacter* spp. | (Jalil and Zenad, 2016) |
| 14 | *Hyalomma* species<br>*Rhipicephalus* species | *Escherichia coli, Klebsiella* species, *salmonella* spp. | (Khalaf *et al*., 2018) |

## 2.4. Microbiological diagnostics tools of cultivable aerobic pathogenic bacteria from ticks

### *2.4.1. Preparation of ticks for isolation and identification of aerobic pathogenic bacteria*

All the collected ticks from the same cattle should be counted and put in the same sterile vial, labeled, and transported live in plastic cups covered by cotton net gauze to allow the free circulation of air. In the laboratory, collected ticks should surface sterilized with 70% ethanol and rinsed thoroughly with sterile saline water to remove all possible environmental contaminants (Loong *et al.*, 2018a). Adult ticks were then sorted according to species, sex, and study districts into pools of between 1 and 10 individuals, before pulverization using a chilled mortar and pestle (Khoo *et al.*, 2016). The resulting homogenates should be cultured using Peptone Water Broth media and incubated aerobically at 37°C for 24-48 hr.

*2.4.2. Culture and morphological staining of aerobic pathogenic bacteria*

Isolation and identification of colonies in various culture media should be carried out according to standard bacteriological procedures as described by (Quinn *et al.,* 2002; Swayne *et al.,* 1998). Gram stain, as described by Merchant and Packer, should be used to characterize morphological characteristics of representative bacterial colonies in any clinical materials (1967).

*2.4.3. Hemolytic activity*

To characterize the hemolytic patterns isolated strains should be tested for hemolysis on sheep blood agar plate by incubating them at 37°C for 24 hours. Hemolytic patterns should categorize as Alpha (α) hemolysis: a zone of greenish discoloration around the colony manifested by partial hemolysis. (β) Hemolysis; complete clear zone of hemolysis around the colony and Gamma (γ) hemolysis: no detectable hemolysis (Cheesbrough, 2006).

*2.4.4. Reactions of the organisms in TSI agar slants*

Triple sugar iron agar (TSI agar) should perform to detect the lactose, sucrose, and dextrose fermenter and also the bacteria which produce hydrogen sulfide. The organisms seeded over the surface of the slants and stabbed into the butt where the cases change after an incubation of 24 hours at 37°C (Cheesbrough, 2006).

*2.4.5. Citrate Utilization Test*

Simmons Citrate Agar should perform for the differentiation of Enterobacteriaceae based on the utilization of citrate as the sole source of carbon. Citrate utilization test used to detect whether the bacterial strain utilizes citrate or not, citrate utilization is called citrate positive and that without citrate utilization was called citrate negative. The isolated bacterial strain was inoculated on the Simmons citrate media plates, by taking a loop full of bacteria from each plate. The plates were then incubated in the incubator at $37^0$c for 48-72 hours after proper wrapping. The green color of media turned blue is called citrate positive others that don’t cause color change are citrate negative (Cheesbrough, 2006).

*2.4.6. Catalase, oxidase and coagulase test*

Slide catalase, coagulase, and commercially prepared paper disk oxidase tests were used to perform to differentiate the isolated bacteria whether coagulase/catalase/oxidase-positive or negative samples should be recorded according to the procedure described by Cheesbrough (2006).

*2.4.7. Indole test, Methyl red test and Voges-Proskauer test*

These tests were used to differentiate the isolated bacteria from various bacteriological samples collected. The test should perform and the result should be interpreted according to the standard procedure described by Cheesbrough (2006).

*2.4.8. Biochemical Tests by using API 20E*

The bacteria were should characterized biochemically by using the API 20E kit. The standard procedure that is undertaken for the biochemical characterization of bacteria includes 20 miniaturized tests for the identification and characterization of bacteria. It contains 20 microtubes that constitute dehydrated substrates. Bacteria to be identified was first to be isolated on a suitable culture medium according to standard microbiological techniques. The bacterial suspension was added in the microtubes after that they were placed for incubation which led to the color change that might be spontaneous or can be observed after some time. First, tryptic soy broth [TSB] cultured media was prepared for the API kit test. Then 1ml of media was transferred in the striped hole by using the pipette. All introduced tests except VP, TDA, and IND gave the result within five minutes, which was recorded and interpreted. However, in these three tests after 24 hours the reagents were introduced, TDA reagent and VP1 and VP2 reagent in TDA and VP test respectively. The results were recorded after 5 minutes. It takes 3-5 minutes to change color which is an indication of record results (Pava-Ripo *et al.*, 2015).

*2.4.9. Maintenance of stock culture*

The pure culture of isolated bacterial species should be inoculated into the tubes containing Tryptone Soya Broth and incubated at $37^0$C for 24 hours. After the growth of organisms, the tubes should be sealed with liquid paraffin (light) and kept in the refrigerator at $+4^0$C for further studies (Quinn *et al.,* 2002).

## 3. MATERIALS AND METHODS

### 3.1. Study area

The present study was conducted from November 2020 to July 2021 in three selected districts of central parts of Ethiopia. Study tick samples were collected from Lume, Ada'a, and Ezha districts found in central Ethiopia.

Lume district, the first study site, is located in East Shoa Zone, Oromia regional state, central Ethiopia. Lume district is located in the Great Rift Valley, is bordered on the South by the Koka Reservoir, on the West by Ada"a Chukala, on the North West by Gimbichu and on the North by East Adama town. The capital town of the district is Modjo which is located 70 km Southeast of Addis Ababa $8^0$35'N and $39^0$10' E. The altitude of the district ranges from 1500 to 2300 meter above sea level and the annual rainfall range from 500-1200 mm. The minimum and maximum annual mean temperatures are 14 and $27^0$C respectively (ILRI, 2005).

Ada'a, the second study site, is part of the East Shoa zone of Oromia regional state which is located 47 km SouthEast of Addis Ababa and lies between longitudes 38º51' to 39º04' East and latitudes 8º46' to 8º59'North covering a land area of 1750 $km^2$. The district has a maximum altitude of 2300 m.a.s.l and minimum altitude of 1500 m.a.s.l. This study site has an annual rainfall of a maximum of 1,200 mm and minimum of 800 mm. It has bimodal rainfall patterns, with the main rainy season lasting from June to September. A short rainy season occurs between March and May with an average rainfall of about 800mm. The mean annual maximum and minimum temperatures are $30^0$C and $8.5^0$C respectively and the mean relative humidity is 61.3% (NMSA, 2015).

Ezha district, the third study site, is located in the South Nation Nationality and People's Regional State (SNNPRs) of Gurage Zone. It is located 200km southwest of Addis Ababa, the Capital City of Ethiopia. Geographically, the study area is located within latitudes of $7^0$59' 30" to $8^0$16' North and longitudes of $37^0$53' 30" to $8^0$10' 00" East. The elevation of Ezha district ranges from 1950 meter above sea level to 3200 meter above sea level (NMSA, 2015).

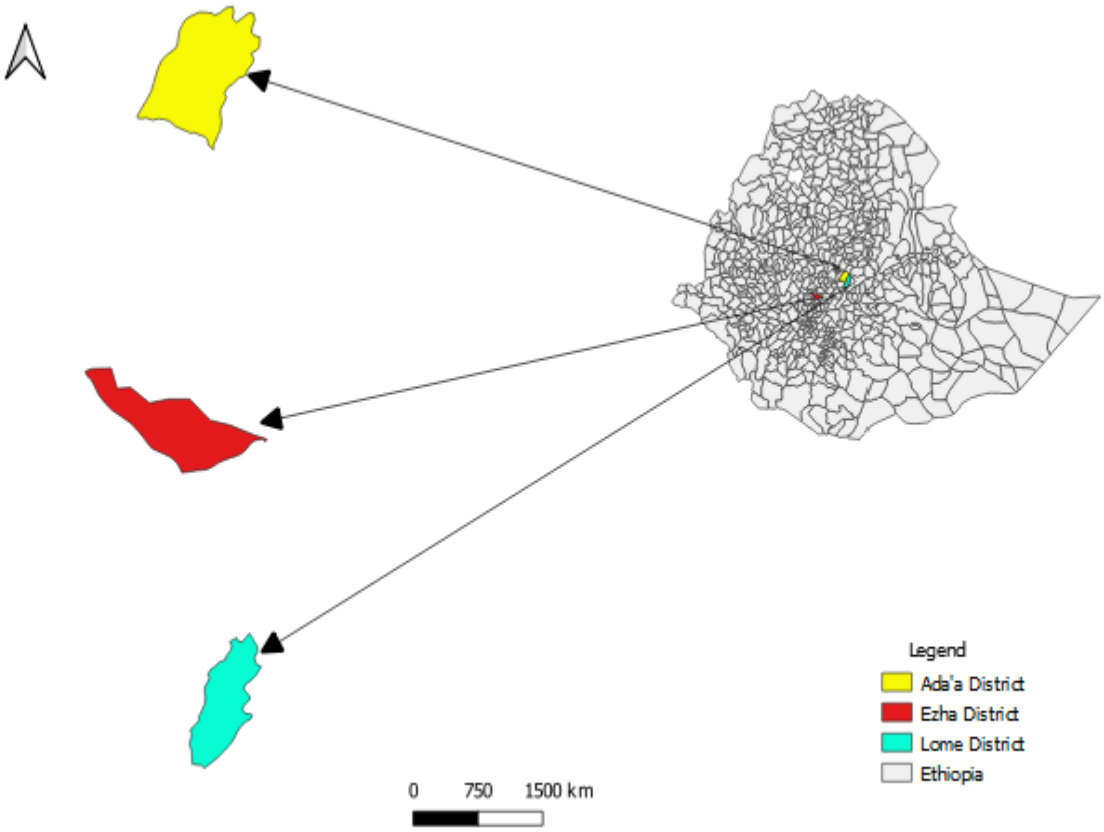


**Figure 2:** Map of the study areas. The study area map was created using Q GIS software as indicated above.

### 3.2. Study Design and Animals

A cross-sectional study design was conducted from November 2020 to July 2021, on isolation and identification of the cultivable aerobic pathogenic bacteria from ticks collected from cattle in central parts of Ethiopia namely, Ada'a, Lume, and Ezha districts. The tick samples were collected purposively from ticks-infested cattle. The skin was palpated across all parts of the cattle for the presence of ticks, and gross lesions suggestive of a clinical form of tick infestations and cattle found infested was considered as positive. Visual inspection of the skin was conducted to detect the ticks. Then the ticks were collected manually according to the standard protocol Walker *et al.* (2014).

## 3.3. Study Methods

### *3.3.1. Collection, transportation, and morphological identification of ticks*

Ticks were collected purposively from 100 clinically diseased cattle for isolation and identification of cultivable aerobic pathogenic bacteria from ticks of cattle in central Ethiopia, namely, Ada'a, Lome and, Ezha districts. Following proper restraining of the cattle clinical examination was performed as described by (Kumsa *et al.,* 2012). The skin was palpated across all parts of the cattle for the presence of ticks, and gross lesions suggestive of a clinical form of tick infestations and cattle found infested were considered as positive. To detect the ticks, a visual inspection of the skin was performed. To avoid any harm to the body, all visible ticks were carefully removed manually, either with forceps or by hand (Walker *et al.,* 2014). All the collected ticks from the same cattle were counted and put in the same sterile vial, labeled, and transported live in plastic cups covered by cotton net gauze to allow the free circulation of air. The ticks were then transported to the Parasitology Laboratory of the College of Veterinary Medicine and Agriculture of Addis Ababa University located at Bishoftu, for identification. Ticks were identified to the species level according to their morphological key structures such as the shape of the scutum, leg color, scutum ornamentation, body grooves, punctuations, basis capitulum, coaxes, and ventral plates. During tick identification in the laboratory, the samples were put on Petri-dish and adult ticks were identified to species level under a stereomicroscope using the standard identification keys of Pegram *et al*. (1987); Taylor (2007), and Walker *et al.* (2014). For each tick, photographs of the dorsal view of adult male and female ticks were captured and depicted in Figure (3) in the result section.

### *3.3.2. Isolation and identification of aerobic pathogenic bacteria from ticks*

Bacterial isolation and identifications have conducted at Addis Ababa University, College of Veterinary Medicine and Agriculture in Microbiology laboratory located at Bishoftu. The ticks were bacteriologically examined using the standard procedure outlined below (Khoo *et al.,* 2016; Quinn *et al.,* 2002). Each tick was washed in sterile salty water (0.85% NaCl) and later their external body surface was disinfected with 70% ethyl alcohol, and was washed in sterile salty water again. Each tick was placed inside tubes and washed three

times in sterile salty water. Adult ticks were then sorted according to species, sex, and study areas into pools of between 1 and 10 individuals, before pulverization using a chilled mortar and pestle (Khoo *et al.*, 2016). The resulting homogenized tick samples were put in the Peptone Water Broth media and incubated aerobically at $37^0$c for 24 hrs. Then, a loopful of the broth culture was taken and streaked over an identified Petri-plate containing Blood Agar base (Oxide, Hampshire, England) supplemented with 7% sheep blood, MacConkey Agar (Oxide, Hampshire, England), and Mannitol Salt Agar (Oxide, Hampshire, England) for first isolation of the aerobic bacterial pathogen to rule out shown growing bacteria and morphologic features that is a colony, size, shape, color and hemolytic characteristics (Arıkan *et al.,* 2009; Stojek and Dutkiewicz, 2004; Quinn *et al.*, 2002). The plates were labeled and incubated aerobically at 37°C for 24 hours with further re-incubation for 36–72 hours if no growth observed after 24 hours (Ajuwape and Aregbesola, 2002).

The first step was Gram staining of randomly selected representative pure cultures to study staining reaction and cellular morphology under the light microscope, at 100X magnification. Potassium hydro-oxide (KOH) was used to further confirm the Gram reaction whenever there was doubt on the Gram staining. Based on the macroscopic and microscopic appearance of pure colony, Gram-negative bacteria colonies were selected from each sample and sub-cultured on MacConkey Agar (Oxide, Hampshire, England), Eosin Methylene Blue (EMB) Agar (Oxide, Hampshire, England), Salmonella-Shigella (SS) Agar (Oxide, Hampshire, England) and XLD Agar (Oxide, Hampshire, England) to get pure cultures of the bacteria and incubated at 37°C for 16 to 40 hours (Duerden *et al.*, 1998).

Based on the above results suspect colonies were sub-cultured to nutrient agar plates for further investigation, then isolated pure colony organisms were subjected to different biochemical tests. Pure cultures of single colony type were subjected to a series of secondary biochemical tests (TSI, Indole, MR-VP, Citrate, SIM medium) (Annex 8) for final identification, following standard procedures (Carter, 1984; Quinn *et al.*, 2002).

### *3.3.3. Validation of Biochemical Tests by using API 20E*

The bacteria were also characterized biochemically by using the API 20E kit. The standard procedure that is undertaken for the biochemical characterization of bacteria includes 20 miniaturized tests for the identification and characterization of bacteria. It contains 20 microtubes that constitute dehydrated substrates. Bacteria to be identified was first to be isolated on a suitable culture medium according to standard microbiological techniques. The bacterial suspension was added in the microtubes after that they were placed for incubation which led to the color change that might be spontaneous or can be observed after some time. First, tryptic soy broth [TSB] cultured media was prepared for the API kit test. Then 1ml of media was transferred in the striped hole by using the pipette. All introduced tests except VP, TDA, and IND gave the result within five minutes, which was recorded and interpreted. However, in these three tests after 24 hours the reagents were introduced, TDA reagent and VP1 and VP2 reagent in TDA and VP test respectively. The results were recorded after 5 minutes. It takes 3-5 minutes to change color which is an indication of record results (Pava-Ripo *et al.,* 2015).

### *3.3.4. Antimicrobial susceptibility*

Antibiotic susceptibility tests were done on the cultivable aerobic pathogenic bacteria isolated from ticks collected from cattle. The susceptibility test was carried out to assess the efficacy of 9 antimicrobial drugs (Oxoid OR TM media, New Delhi, India) using the disk diffusion (Kirby Bauer's) technique following the CLSI guidelines (CLSI, 2020) against cultivable aerobic pathogenic bacteria isolated from ticks collected from cattle. Pure bacterial colonies were inoculated into non-selective tryptone soya agar (Oxide, Hampshire, England) and incubated at $37^{O}$C overnight. Several distinct colonies (3-5 morphologically similar colonies) were harvested from the freshly grown plate culture and transferred to a tube containing 4-5ml sterile distilled water and inoculum visually compared with 0.5 McFarland turbidity standards. A sterile cotton swab was used to inoculum uniformly over the surface of the Muller Hinton agar plate (Oxide, Hampshire, England). The plates were kept at room temperature for 30 min to allow drying with subsequent application of antibiotic disc using sterile forceps, gently pressed with the point of the forceps for

ensuring complete contact with the agar surface and incubation at $37^{O}$C for overnight. Clear zones of bacterial growth inhibition were measured in mm using a measuring caliper. The zone of inhibition results was interpreted according to the Clinical and Laboratory Standard Institutes (CLIS, 2020) guideline. The list of panels of antimicrobial utilized, their symbols, and concentrations and breakpoints are shown in (Annex 6).

### 3.4. Ethical clearance

Ethical clearance for this study was obtained from the animal research ethical review committee of Addis Ababa University College of Veterinary Medicine and Agriculture for collecting ticks from the cattle with an approved certificate reference number of VM/ERC/06/13/021. All activities done on animals were conducted in accordance with animal research ethics.

### 3.5. Data management and analysis

The data (species of ticks, sex of ticks, and districts) collected and species of aerobic pathogenic bacteria isolated and identified from ticks collected from cattle of the study area were entered into Microsoft Excel 2010 spreadsheet and transferred to SPSS® Version 20 software for statistical analysis. The study's findings were summarized using descriptive statistics. Percentages were used to express the relative abundance of each species of ticks to the total number of isolates of bacteria. Furthermore, the chi-square test was computed to observe the relationship between the variants. A p-value of $< 0.05$ was considered indicative of a statistically significant difference.

## 4. RESULTS

### 4.1. Morphological identification of ticks

Out of the total of 205 ticks examined, an overall of eight species belonging to three ixodid tick genera (*Amblyomma*, *Rhipicephalus,* and *Hyalomma*) and subgenus *Rhipicephalus* (*Boophilus*) were identified (Table 3). An overall of 152 males and 53 females' ticks were identified and 89 (43.4%) from Ada'a district, 31 (15.1%) from Lome district and 85 (41.5%) from Ezha district were collected during the dry seasons from cattle. Overall, *Hyalomma truncatum* (N=50; 24.4 %), *Amblyomma variegatum* (N=41; 20%), *Amblyomma cohaerens* (N=40; 19.5%), *Rhipicephalus decoloratus* (N=33; 16.1%), *Hyalomma rufipes* (N=29; 14.1%), *Rhipicephalus evertsi* (N=7; 3.4%), *Amblyomma gemma* (N=4; 1.9%), and *Rhipicephalus pulchellus* (N=1; 0.5%) were identified in decreasing order of proportion.

**Table 2:** Overall frequencies of tick species collected from Ada'a, Lome, and Ezha districts.

| Tick species | District | | | Sex | | Overall count |
|---|---|---|---|---|---|---|
| | Ada'a Count | Lome Count | Ezha Count | Male | Female | |
| *Hy. truncatum* | 36 | 14 | 0 | 42 | 8 | 50 (24.4%) |
| *Am. variegatum* | 19 | 5 | 17 | 38 | 3 | 41 (20%) |
| *Am. cohaerens* | 0 | 1 | 39 | 36 | 4 | 40 (19.5%) |
| *Rh. decoloratus* | 10 | 0 | 23 | 0 | 33 | 33 (16.1%) |
| *Hy. Rufipes* | 20 | 5 | 4 | 24 | 5 | 29 (14.1%) |
| *Rh. evertsi* | 0 | 5 | 2 | 7 | 0 | 7 (3.14%) |
| *Am. gemma* | 4 | 0 | 0 | 4 | 0 | 4 (1.95%) |
| *Rh. pulchellus* | 0 | 1 | 0 | 1 | 0 | 1 (0.5%) |
| Total | 89 | 31 | 85 | 152 | 53 | 205 |


**Figure 3:** Ticks identified from cattle of all study areas. (A) Full dorsal view of a male *Am. variegatum* (B) Full dorsal view of a male *Am. cohaerens* (C) Full dorsal view of a male *Am. gemma* (D) Full dorsal view of a male *Hy. truncatum* (E) Full dorsal view of a male *Hy. rufipes* (F) Full dorsal view of a male *Rh. pulchuellus* (G) Full dorsal view of a male *Rh. evertsi* (H) Full dorsal view of a female *Rh. decoloratus* (I) Full dorsal view of a female *Am. variegatum* (J) Full dorsal view of a female *Am. cohaerens* (K) Full dorsal view of a female *Hy. rufipes* (L) Full dorsal view of a female *Hy. truncatum* (Walker *et al.*, 2014).

### 4.2. Isolation and identification of aerobic pathogenic bacteria from ticks of Cattle

Physical properties of bacterial colonies were also characterized based on size, color, and consistency on the agar media. The colonies grown on differential media were sub-cultured until isolated colonies (Annex 2). After pure culture was obtained the results from a few comparative primary tests were used to identify the organism to a genus level (Annex 3). Secondary biochemical tests and API 20E tests were carried out for the identification of the bacteria to the species level (Annex 4). So based on the above activities *E. coli, C. freundii, S. aureus, P. mirabilis,* and *M. morganii* aerobic pathogenic bacteria were identified from 107 bacterial isolates (Figure 4,5,6,7 and 8).

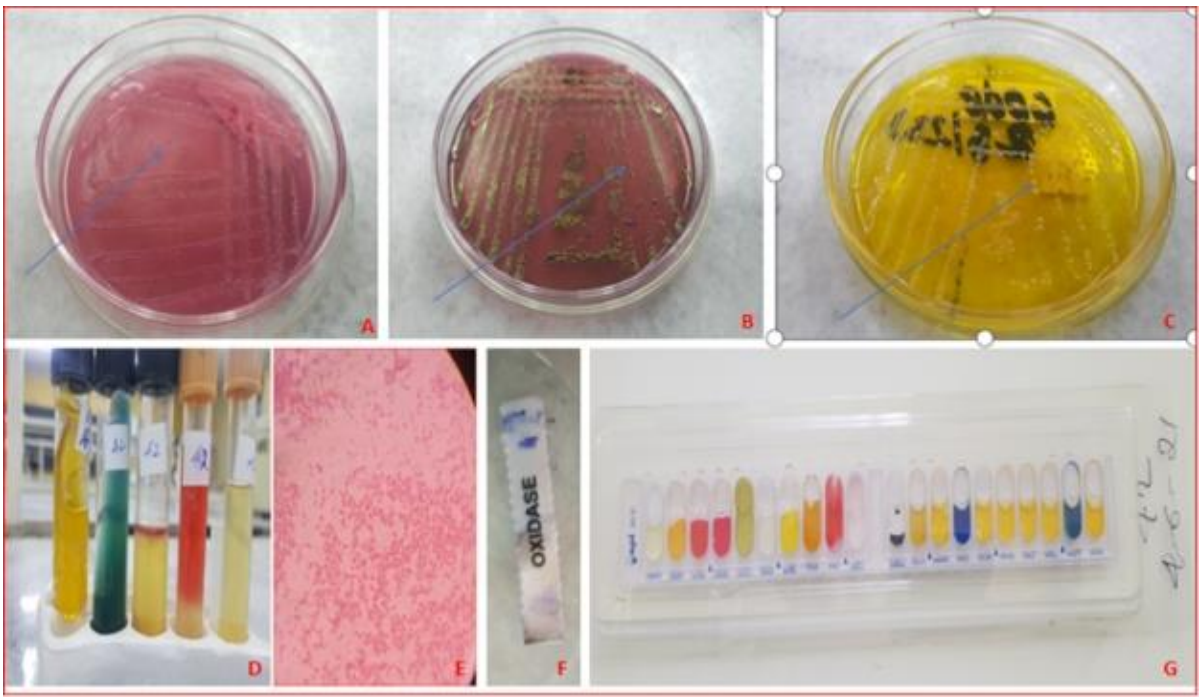


**Figure 4:** *E. coli* isolated and identified test results. (A) On MacConkey agar pink colony as a result of fermentation of lactose. (B) On EMB agar metallic sheen colony. (C) On XLD agar yellow circular colon. (D) Biochemical test results from left: TSI: A/A/Gas +ve/Citrate: -ve/Indole: +ve/MR: +ve/ VP: -ve. (E) Gram-negative rod. (F) Oxidase negative. (G) The API-20E miniaturized identification system showing the reaction of *E. coli.*

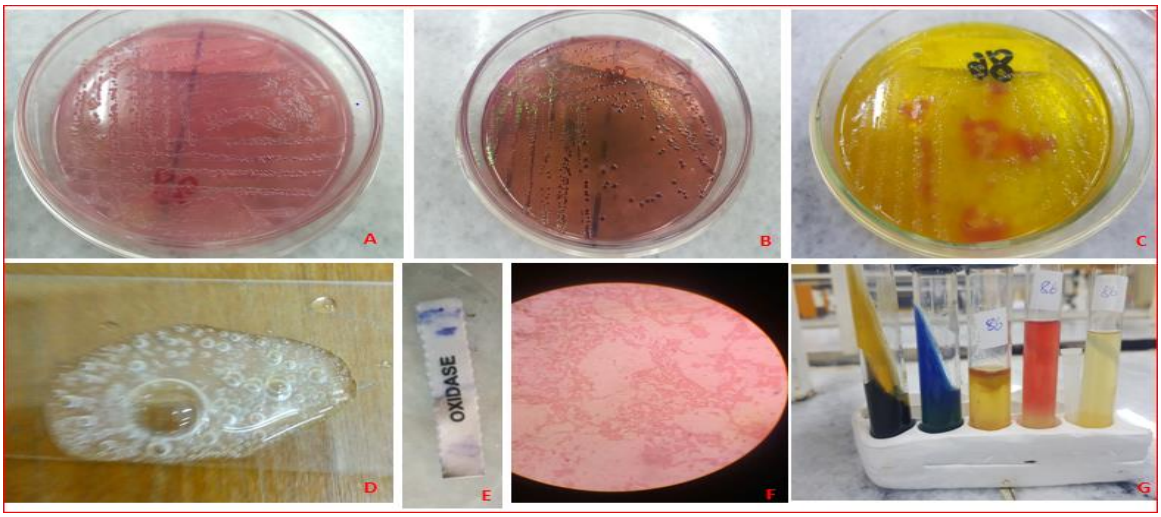


**Figure 5:** *C. freundii* isolated and identified test results. (A) On MacConkey agar pink colony as a result of fermentation of lactose. (B) On EMB agar brown dark colony. (C) On XLD agar yellow colony. (D) Catalase positive. (E) Oxidase negative. (F) Gram-negative rod. (G) Biochemical test results from left: TSI: A/A/ H2S: +ve /Citrate: +ve/Indole: -ve/MR: +ve/ VP: -ve.

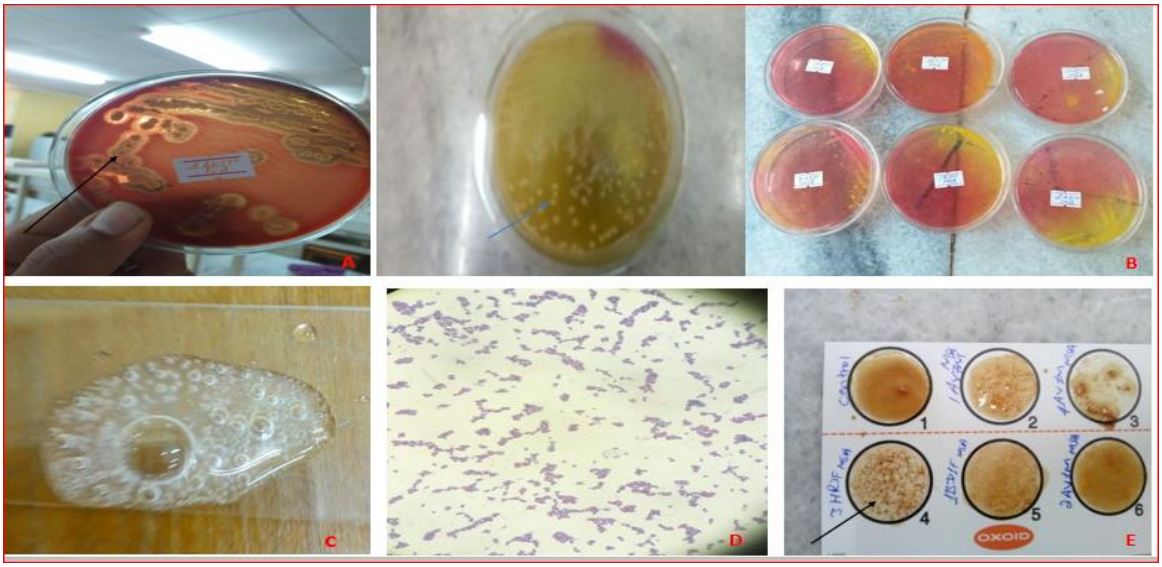


**Figure 6:** *S. aureus* isolated and identified test results. (A) On Blood agar golden yellow (beta hemolysis) colony. (B) On Mannitol salt agar yellow colony. (C) Catalase positive results. (D) Gram-positive cocci. (E) On Coagulase test-test kits Coagulase positive.

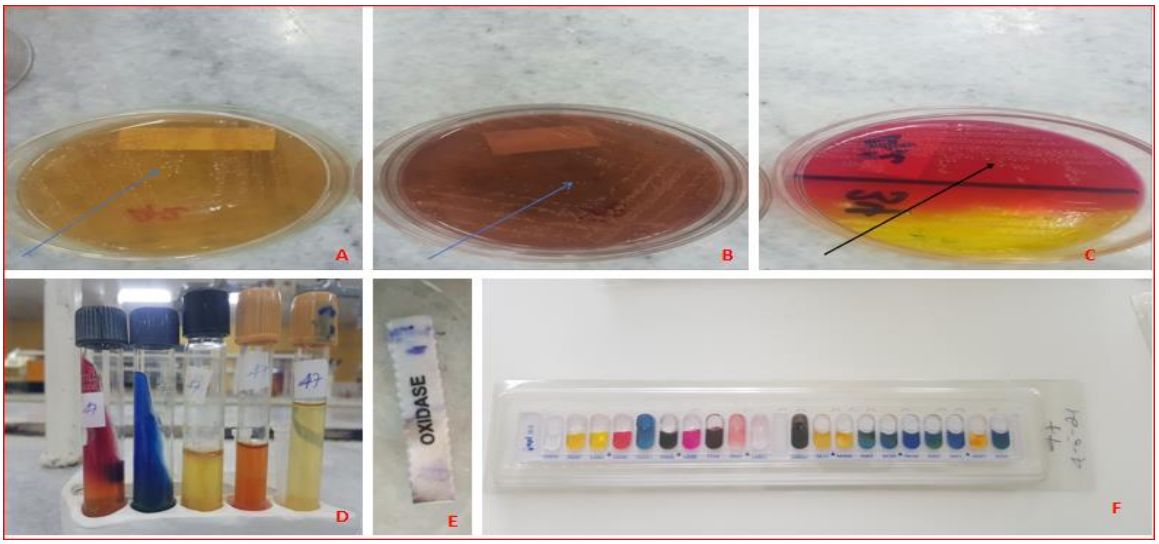


**Figure 7:** *P. mirabilis* isolated and identified test results. (A) On MacConkey agar non-lactose fermenter flat colorless colony. (B) On EMB agar colorless colony. (C) On XLD agar colorless colony. (D) Biochemical test results from left: TSI: K/A/ H2S: +ve /Citrate: +ve/Indole: -ve/MR: +ve/ VP: -ve. (E) Oxidase negative. (F) The API-20E miniaturized identification system showing the reaction of *P. mirabilis*.

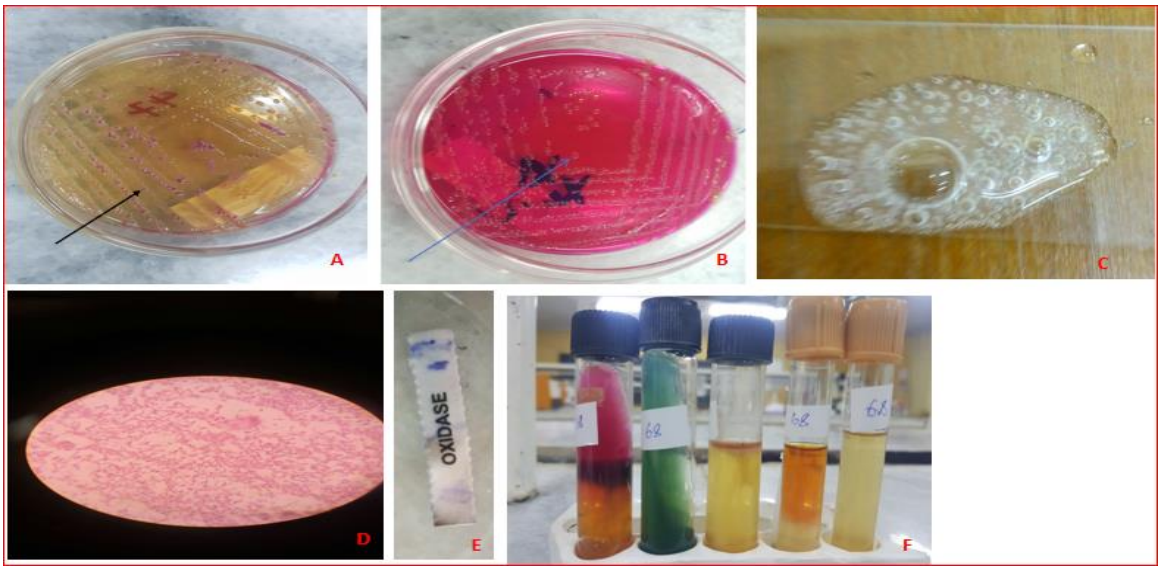


**Figure 8:** *M. morganii* isolated and identified test results. (A) On MacConkey agar non lactose fermenter flat colorless colony. (B) On XLD agar colorless colony. (C) Catalase positive test. (D) Gram-negative rode. (E) Oxidase negative. (F) Biochemical test results from left: TSI: K/A/ H2S: +ve /Citrate: -ve/Indole: -ve/MR: +ve/ VP: -ve.

### 4.3. Isolation frequency of aerobic pathogenic bacteria from ticks of cattle

Out of the total of 205 identified ticks, 107 (52.2%) ticks were found positive and 98 (47.8%) ticks were found negative for bacterial isolation and a total of 107 bacterial isolates of different species were identified based on the bacterial identification and biochemical tests from Ada'a, Lome and Ezha districts. More than one type of bacteria species was isolated from all tick species revealing that mixed bacterial species isolates were a common scenario. An overall of 107 bacteria isolates from positive ticks for pathogenic bacteria were differentiated in to (N=39; 36.5%) *Citrobacter freundii, (N*=34; 31.8%) *E. coli*, (N=18; 16.8%) *Staphylococcus aureus*, (N=8; 7.5%) *Proteus mirabilis*, and (N=8; 7.5%) *Morganella morganii* (Table 3).

**Table 3:** Pathogenic bacteria isolated from male and female ticks in the study districts

| | Study districts | | | Sex of ticks | | |
|---|---|---|---|---|---|---|
| Bacteria isolated | Ada'a district | Lome district | Ezha district | Female | Male | Total |
| *E. coli* | 29 (27.10%) | 5 (4.67%) | 0 (0%) | 30 (28.84%) | 4 (3.74%) | 34 (31.77%) |
| *S. aureus* | 11 (10.28%) | 0 (0%) | 7 (6.54%) | 13 (12.5%) | 5 (4.67%) | 18 (16.82%) |
| *C. freundii* | 4 (3.74%) | 5 (4.67%) | 30 (28.04%) | 25 (23.36%) | 14 (13.08%) | 39 (36.45%) |
| *M. morganii* | 4 (3.74%) | 4 (3.74%) | 0 (0%) | 7 (6.54%) | 1 (0.93%) | 8 (7.48%) |
| *P. mirabilis* | 4 (3.74%) | 3 (2.80%) | 1 (0.93%) | 6 (5.62%) | 2 (1.87%) | 8 (7.48%) |
| Total | 52 (48.60%) | 17 (15.89%) | 38 (35.5%) | 85 (75.7%) | 21 (24.30%) | 107 (100%) |

Pearson-Chi-Square comparison indicated a statistically significant difference between the isolation rates of *E. coli, S. aureus, C. freundii, M. morganii*, and *P. mirabilis* and study districts ($p < 0.05$). ($\chi$ 2 = 68.1336 P = 0.000) and absence of significant difference

between the isolation rates of *E. coli, S. aureus, C. freundii, M. morganii*, and *P. mirabilis* and sex of ticks ($p<0.05$). ($\chi 2 = 6.4821$  $P = 0.166$).

In the present study, a total of 107 bacterial isolates were isolated from eight species of ticks collected from cattle. The 107 different bacterial strains isolated were differentiated into different genera including, (N=34; 31. 8%) were *E. coli* isolated from seven species of ticks. From a total of 34 *E. coli* bacterial isolates, 13(38.2%) were from *Hy. truncatum*, 3(8.8%) were from *Rh. decoloratus*, 10(29.4%) were from *Hy. rufipes*, 4(11.7%) were from *Am. variegatum*, 1(2.94%) were from *Am. gemma*, 2(5.9%) were from *Rh. evertsi*, and 1(2.94%) were from *Rh. pulchellus and no E. coli* growth was observed from *Am. cohaerens*. Out of the total of 34 *E. coli* bacterial isolates, 29 (85.3 %) were from Ada'a districts whereas 5(14.7%) were from Lome districts. 30(88.2%) *E. coli* bacterial isolates were identified from male ticks while 4(11.7%) were from female ticks. Whereas, 39(36.5%) were *C. freundii* isolated from six species of ticks. From the total of 39 *C. freundii* bacterial isolates, 1(2.6%) were from *Hy. truncatum*, 5(12.8%) were from *Rh. decoloratus*, 5(12.8%) were from *Hy. rufipes*, 14(35.9%) were from *Am. variegatum*, 11(28.2%) were from *Am. cohaerens*, 3(7.7%) were from *Rh. evertsi* and no bacterial growth was obtained from *Am. gemma* and *Rh. pulchellus.* Out of the total 39 *C. freundii* bacterial isolates 4(10.3%) were from Ada'a districts, 5(12.8%) were from Lome districts and 30(76.9%) were from Ezha districts. 25(64.1%) *C. freundii* bacterial isolates were identified from male ticks while 14(35.9%) were from female ticks.

In the present study an overall of 18(16.8 %) *S. aureus* was isolated from five species of ticks collected from cattle. From the total of 18 *S. aureus* bacterial isolates 2(11.1%) were from *Hy. truncatum*, 4(22.2%) were from *Rh. decoloratus*, 4(22.2%) were from *Hy.rufipes*, 6(33.3%) were from *Am. variegatum*, 2(11.1%) were from *Am. cohaerens* and no bacterial growth was obtained from *Am. gemma*, *Rh. pulchellus*, and *Rh. evertsi.* Out of the total 18 *S. aureus* bacterial isolates 11(61.1%) were from Ada'a districts and 7(38.9%) were from Ezha districts and no bacterial growth was obtained from Lome district. 13(72.2%) *S. aureus* was isolated from male ticks while 5(27.8%) were from female ticks. Whereas, (N=8; 7.5%) were *Morganella morganii* and (N=8; 7.5%) were *Proteus mirabilis* isolated from three and five species of ticks respectively. From the total of 8(7.5%) *Morganella*

*morganii* isolates 6(75%) were from *Hy. truncatum*, 1(12.5%) were from *Rh. decoloratus*, 1(12.5%) were from *Hy. rufipes*, and no bacterial growth was obtained from *Am. variegatum, Am. gemma, Am. cohaerens, Rh. pulchellus*, and *Rh. evertsi.* Out of the total 8 *M. morganii* bacterial isolates 4(50%) were from Ada'a districts and 4(50%) were from Lome districts and no bacterial growth was obtained from Ezha district. 7(87.5%) *M. morganii* were isolated from male ticks while 1(12.5%) were from female ticks. Out of the total 8 (7.48%) *Proteus mirabilis* isolates 1(12.5%), were from *Hy. truncatum*, 1(12.5%), were from *Rh. decoloratus*, 2(25%), were from *Hy. rufipes*, 3(37.5%), were from *Am. variegatum*, 1(12.5%) were from *Am. cohaerens* and no bacterial growth were obtained from *Am. gemma, Rh. pulchellus* and *Rh. evertsi.* Out of the total 8 *P. mirabilis* bacterial isolates 4(50%) were from Ada'a districts, 3(37.5%) Lome district, and 1(12.5%) were from Ezha district. 6(75%) *P. mirabilis* were isolated from male ticks while 2(25%) were from female ticks.

**Table 4:** Aerobic pathogenic bacteria species isolated from study ticks

| Bacteria isolated | *Hy. truncatum* | *Rh. decoloratus* | *Hy. Rufipes* | *Am. variegatum* | *Am. gemma* | *Am. cohearens* | *Rh. evertsi* | *Rh. pulchellus* | Total |
|---|---|---|---|---|---|---|---|---|---|
| *E. coli* | 13 | 3 | 10 | 4 | 1 | 0 | 2 | 1 | 34 |
| | (12.15%) | (2.8%) | (9.35%) | (3.74%) | (0.93%) | (0%) | (1.87%) | (0.93%) | (31.78%) |
| *S. aureus* | 2 | 4 | 4 | 6 | 0 | 2 | 0 | 0 | 18 |
| | (1.87%) | (3.74%) | (3.74%) | (5.61%) | (0%) | (1.87%) | (0%) | (0%) | (16.82%) |
| *C. freundii* | 1 | 5 | 5 | 14 | 0 | 11 | 3 | 0 | 39 |
| | (0.93%) | (4.67%) | (4.67%) | (13.08%) | (0%) | (10.28%) | (2.88%) | (0%) | (36.45%) |
| *M. morganii* | 6 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 8 |
| | (5.61%) | (0.93%) | (0.93%) | (0%) | (0%) | (0%) | (0%) | (0%) | (7.48%) |
| *P. mirabilis* | 1 | 1 | 2 | 3 | 0 | 1 | 0 | 0 | 8 |
| | (0.93%) | (0.93%) | (1.87%) | (2.87%) | (0%) | (0.93%) | (0%) | (0%) | (7.48%) |
| Total | 23 | 14 | 22 | 27 | 1 | 14 | 5 | 1 | 107 |
| | (21.5%) | (13.08%) | (20.56%) | (25.23%) | (0.93%) | (13.08%) | (14.67%) | (0.93%) | (100%) |

Pearson-Chi-Square comparison indicted a statistically significant difference between the isolation rates of *E. coli, S. aureus, C. freundii, M. morganii*, and *P. mirabilis* and species of ticks ($p < 0.05$). ($\chi 2 = 53.4878$, $P = 0.003$)

### 4.4. Antibiotic sensitivity pattern of aerobic pathogenic bacteria

An antimicrobial susceptibility test was performed against the bacterial isolates obtained from ticks of cattle. Nine antimicrobial impregnated discs (OXOID) were used to evaluate the susceptibility of *C. freundii, E. coli, S. aureus, P. mirabilis,* and *M. morganii* against the commonly used antimicrobial drugs. The antibiogram profile of *C. freundii* strains indicated a marked susceptibility to streptomycin, chloramphenicol, trimethoprim-sulfamethazole and gentamicin and resistant to clindamycin, Bacitracin, penicillin, oxytetracycline, and tetracycline (Table 5). Most of the strains of *E. coli* have shown a high level of sensitivity to gentamicin, streptomycin, chloramphenicol and trimethoprim-sulfamethazolem however, they were resistant to clindamycin, bacitracin, penicillin, tetracycline, and oxytetracycline (Table 5). Streptomycin, tetracycline, gentamicin, and oxytetracycline were the drugs that revealed high level of efficacy against strains of *Staph. aureus,* however, on the other side trimethoprim-sulfamethazole, clindamycin, penicillin, and bacitracin showed various degrees of efficacy (Table 5). The antibiogram profile of *P. mirabilis* bacterial strains indicated a marked susceptibility to gentamycin, streptomycin, and chloramphenicol, however resistance against penicillin, bacitracin, and trimethoprim-sulfamethazole, with various degrees of efficacy was recorded. On the other hand, tetracycline, oxytetracycline, clindamycin, and streptomycin demonstrated intermediate levels of efficacy (Table 5). *M. morganii* was susceptible to the action of chloramphenicol, streptomycin, gentamycin, and trimethoprim-sulfamethazole however, it showed 100% resistance against clindamycin, Bacitracin, penicillin, and tetracycline and on the other hand, oxytetracycline showed intermediate efficacy against the tested bacteria.

**Table 5:** Antibiotic susceptibility tests of the aerobic pathogenic bacteria isolates (disk diffusion method) from ticks.

| | % Of strains sensitive to the antibacterial agents | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Bacterial species | N | CN | P | TE | OT | C | DA | SXT | B | S |
| *C. freundii* | 10 | 100 | 0 | 30 | 30 | 100 | 0 | 100 | 0 | 70 |
| *E. coli* | 10 | 100 | 0 | 60 | 60 | 80 | 0 | 100 | 0 | 40 |
| *S. aureus* | 10 | 100 | 30 | 90 | 90 | 30 | 10 | 0 | 0 | 80 |
| *P. mirabilis* | 8 | 100 | 0 | 25 | 37.5 | 87.5 | 37.5 | 0 | 0 | 75 |
| *M. morganii* | 8 | 100 | 0 | 12.5 | 25 | 100 | 0 | 75 | 0 | 87.5 |

N= number of isolates tested, CN= Gentamicin, P= Penicillin, TE= Tetracycline, OT= Oxytetracycline, C= Chloramphenicol, DA= Clindamycin, SXT= sulfamethazole-Trimethoprim, B= Bacitracin, S= Streptomycin.

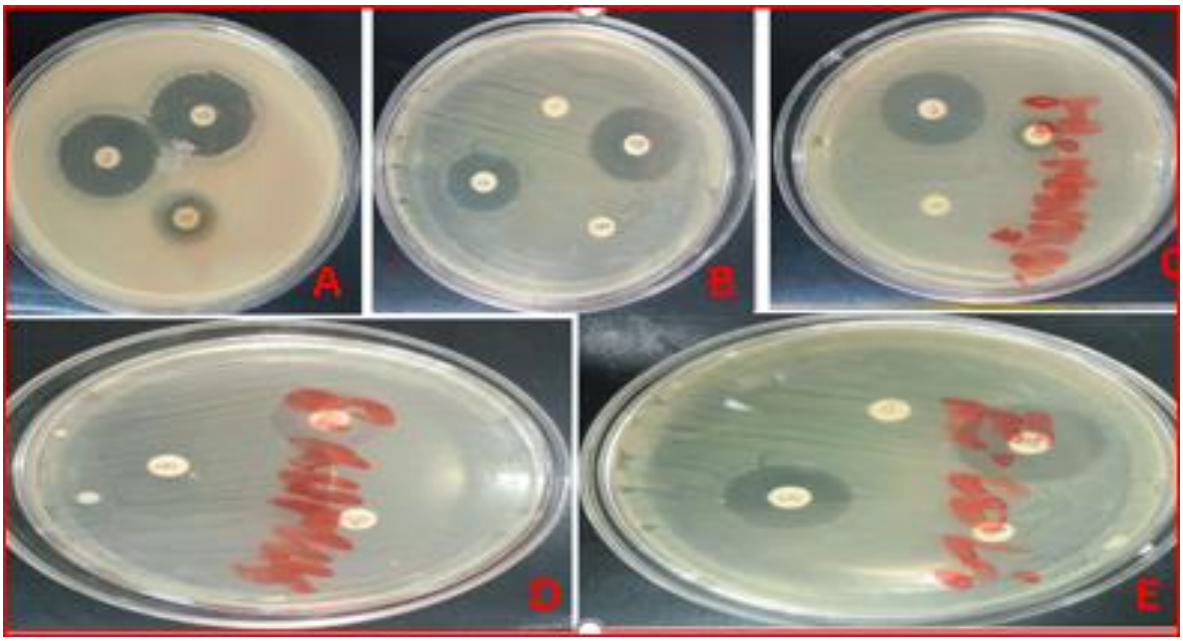


**Figure 9:** Antimicrobial sensitivity test showing the different degrees of zone of inhibition. (A) *P. mirabilis* (B) *C. freundii* (C) *M. morganii* (D) *S. aureus* (E) *E. coli*

## 5. DISCUSSION

Ticks are one of the most important arthropods responsible for major obstacles to cattle production and are known to have great medical and veterinary importance worldwide (De la Fuente *et al.,* 2015) including Ethiopia (Kumsa *et al.,* 2015a). In addition, they play a serious role in the transmission of many pathogens to various host species and some are life-threatening (Blom *et al.,* 2015). Information isn't available on the presence of cultivable pathogenic bacteria isolated from ticks of cattle in Ethiopia. Therefore, the present study was carried out with the objectives to isolate and identify cultivable aerobic pathogenic bacteria from ticks collected from cattle in central Ethiopia, namely, Ada'a, Lome, and Ezha districts.

Results of the present study demonstrated the presence of cultivable aerobic pathogenic bacteria from ticks collected from cattle in central Ethiopia. The bacteria detected in different tick species most probably were endogenous to the tick itself or may have originated from the environment or the animal skin microbiota or the host blood meal (Moreno *et al.,* 2006; Van Treuren *et al.,* 2015; Clow *et al.,* 2018; Estrada-Peña *et al.,* 2018). Out of the total of 205 ticks, 107 (52.2%) ticks were positive and 98 (47.8%) ticks were found negative for bacterial isolation and a total of 107 bacterial isolates of different species were identified based on the bacterial identification and biochemical tests from all the study areas, namely, Ada'a, Lome, and Ezha districts. More than one type of bacteria species was isolated from all tick species revealing that mixed bacterial species isolates were a common scenario. Similar previous findings were reported across many ticks (Murrell *et al.,* 2003; Rahuma *et al.,* 2005; Egyed and Makrai, 2014; Li, C. H *et al.,* 2014; Anbalagan *et al.,* 2014; Kirecci *et al.,* 2015; Jalil and Zenad, 2016; Khoo *et al.,* 2016; Khalaf *et al.,* 2018; Myahi *et al.,* 2019; Loong *et al.,* 2020), suggesting that ticks ingest and transport bacteria from their surroundings; these bacteria can survive and, in some cases, replicate in ticks. These bacteria may have a role in the biology of the tick, the transmission of tick pathogens or protect themselves from external harmful influences of the environment inside their body and perpetuate their life in nature (Rahman MH and Rahman MM, 1980; Amoo *et al.,* 1987). In the same instance, the ticks play a role in an

evolutionary process of bacteria and might lead to create new bacterial strains or change their virulence (Duron *et al.*, 2015; Kang *et al.*, 2014).

The high isolation rate of bacteria from positive ticks referred to the contribution of these ticks in contamination of the environment, rather than transmission of these bacteria to their hosts. Even though no bacterial growth was observed for some tick specimens, we suggest that it was not due to the limitation of the agar culture media but the method employed for bacterial isolation (Stewart, 2012). Tick cell lines (Bell-Sakyi *et al.*, 2018) may be useful for cultivating non-culturable bacterial pathogens. In support of this, next-generation sequencing-based microbiome studies have identified hundreds of bacterial genera associated with single or pooled tick specimens (Khoo *et al.*, 2016; Trout Fryxell *et al.*, 2016; Estrada-Pea *et al.*, 2018; Lado *et al.*, 2018). Bacterial compositions of ticks are variable and likely depend on the tick species, environmental contamination, seasons, study system, and also type of animal feed led to making diversity in the distribution of livestock having bacteria (Shahzad *et al.*, 2013) or due to the environmental factors that ticks have manners which facilitate the touch with microorganisms in the surroundings (Andreotti *et al.*, 2011). Sampling location may account for much of the microbiome variation, especially over large spatial scales (Carpi *et al.*, 2011). Furthermore, the environment in which the eggs are deposited influences the form of bacterial types (Hendry and Rechav, 1981). Another study found that blood-feeding had a strong influence on microbial community structure, as well as richness and diversity (Swei and Kwan, 2017).

The observation of 39 (36.5%) *C. freundii* from six species of ticks where the most encountered bacterial isolates in the present study is in agreement with previous report of 28.3% prevalence of *C. freundii* from the viscera and salivary glands of *Rh. annulatus* and *Hy. turanicum* in Iraq (Jalil and Zenad, 2016), *Citrobacter* spp. were also identified from tick, *Boophilus microplus* in Australia (Murrell *et al.*, 2003). It was also reported from the digestive tract of the ground beetle, *Poecilus chalcites* (Lehman *et al.*, 2008), on the body surface of cockroaches (Czajka *et al.*, 2003; Moges *et al.*, 2016), and the surface of *Ctenocephalides felis* of cat from Indonesia (Rombot and Semuel, 2021). *Citrobacter freundii*, a member of the genus Citrobacter in the family Enterobacteriaceae, is generally described as a commensal resident of both human and animal intestinal tracts (Guerrant *et*

*al.*, 1986). It has been responsible for sporadic infections and outbreaks (Guerrant *et al.*, 1986; Tschape *et al.*, 1995).

The observation of an overall occurrence of *E. coli* was (N=34; 31.8%) isolated from seven different species of ticks in the current study is in line with several previous reports including, 13.41% of *E. coli* from *Rhipicephalus* spp. and 59.1% of *E. coli* from *Hyalomma* spp. have been isolated in Turkey and Iraq (Kirecci *et al.*, 2015), up to 28.3% of *E. coli* were isolated from the viscera and salivary glands of *Rh. annulatus* and *Hy. turanicum* in Iraq (Jalil and Zenad, 2016), 13% of *E. coli* were isolated from *Hayalomma* spp. of sheep in Iraq (Al-amura and Almyahii, 2012). The isolation of *E. coli* in several tick spp. might be due to the wide prevalence of this organism in nature and the extensive fecal contamination among cattle might probably assist in increasing tick's infection (or harboring such pathogens) and also this most probably reflects unhygienic sanitation as well as poor management, which ultimately increased *E. coli* pollution of the environment.

The finding of an overall 18(16.8%) of *S. aureus* isolates from five different species of ticks in the present study is in line with several previous reports including the prevalence of 18.1% of *staphylococcus* spp. isolated from ticks of three species (*Ixodes ricinus, Dermacentor reticulatus, and Haemaphysalis concinna*) in Hungary (Egyed and Makrai, 2014), up to 13.6% of *S. aureus* isolated from the viscera and salivary glands of *Rh. annulatus* and *Hy. turanicum* in Iraq (Jalil and Zenad, 2016), and 23.8% *S. aureus* isolated from hard ticks *Rhipicephalus* spp. collected from cattle and sheep randomly from diverse regions of Baghdad city, Iraq (Al-rubaie *et al.*, 2019). Previous investigators argued that *Staphylococcus* spp. isolates reflect sources and depend on regions and times of collection of tick species (Murrell *et al.*, 2003; Rahuma *et al.*, 2005; Li, C. H *et al.*, 2014; *Anbalagan et al.*, 2014; Kirecci *et al.*, 2015; Jalil and Zenad, 2016; Khoo *et al.*, 2016; Myahi *et al.*, 2019; Loong *et al.*, 2020).

The present study identified an overall of (N=8; 7.5%) *Morganella morganii* from three species of ticks 6(75%) *Hy. truncatum*, 1(12.5%) *Rh. decoloratus* and 1(12.5%) *Hy. rufipes*. *M. morganii* belongs to the tribe Proteae of the Enterobacteriaceae family which is a motile, non-lactose fermenting bacterium and shares with the Proteus members on the

capacity for urease production and presence of phenylalanine deaminase. This bacterium is commonly found in the environment and intestinal tracts of humans, mammals, and reptiles as part of the normal flora have been isolated along with *Proteus mirabilis* frequently in patients with diarrhea than in healthy controls and found in the environment, intestinal tracts of humans, mammals, and reptiles as part of the normal flora (Mobley *et al.*, 1988; Falagas *et al.,* 2006). *M. morganii* bacteremia is not common and only accounts for 0.7% of bacteremia reported by Lee *et al.* (2006). Custovic *et al.* (2008) found 3.6% among nosocomial infections in newborns. Li *et al.* (2018) identified in infected cattle milk for the first time resulted in a high mortality rate (57%) and severe pathological lesions.

The present study identified an overall of (N=8; 7.5%) *P. mirabilis* from five species of ticks 1(12.5%) *Hy. truncatum*, 1(12.5%) *Rh. decoloratus*, 2(25%) *Hy. rufipes*, 3(37.5%) *Am. variegatum* and 1(12.5%) *Am. cohaerens*. Proteus is a motile, Gram-negative bacterium that can live in soil, water, and the intestines of animals (Drzewiecka, 2016). *Proteus mirabilis* is becoming more well-known as an opportunistic pathogen that causes nosocomial infections in humans and animals (Jacobsen and Shirtliff, 2011; Adams-Sapper *et al.,* 2012). In support of the findings of the present study *P. mirabilis* were also identified from *Rh. decoloratus* in Nigeria (Omoya *et al.,* 2013). Previous researchers argue that *P. mirabilis* isolates are affected by various factors including regions and times of collections of many tick species.

Antimicrobial drugs are important resources that must be conserved for proper use. Choosing the most effective antimicrobial agent is often difficult. A judgment should be made based on in vitro sensitivity results (Caprioli *et al.,* 2000). Hence, in the present study antibiotic susceptibility tests were done for *C. freundii, E. coli, S. aureus, P. mirabilis,* and *M. morganii* using diverse oxoid antibiotic disks.

The present study showed a high level of resistance among the bacterial pathogens against the commonly used antimicrobials. The majority of isolates of bacteria obtained from ticks infesting cattle in the present study, *C. freundii, E. coli, S. aureus, P. mirabilis,* and *M. morganii* pathogens were susceptible to streptomycin, gentamicin, chloramphenicol, and trimethoprim-sulfamethazole but resistant to the efficacy of clindamycin, Bacitracin,

penicillin, oxytetracycline and tetracycline. The findings of 100% susceptibility to chloramphenicol, trimethoprim-sulfamethazole, gentamicin, and 70% susceptibility to streptomycin and resistant to clindamycin, Bacitracin, penicillin, oxytetracycline, and tetracycline in *C. freundii* strains isolated from ticks in the current study is in agreement with previous studies conducted in Iraq by Al-Haider *et al.* (2019).

The findings of 100% sensitivity to gentamicin and trimethoprim-sulfamethazole and 80% to chloramphenicol in most the strains of *E. coli* and varying degrees of resistance to clindamycin, bacitracin, penicillin, tetracycline, and oxytetracycline in the present study is in line with the previous report in India (ICAR) Shakuntala *et al.* (2003).

The observation of a very high level of sensitivity to most isolates of the *S. aureus* towards Streptomycin, tetracycline, gentamicin, and oxytetracycline and on the contrary, the presence of resistance against trimethoprim-sulfamethazole, clindamycin, penicillin, and bacitracin recorded in the current study revealed corroborates the previous report by Edward *et al.* (2002) who reported Bacitracin highly resistant for *S. aureus* 94%, According to San martin *et al.* (2007) report *S. aureus* resistant to penicillin (68%). (Gentilini, 2002) reported in Argentina *S. aureus* is highly susceptible to Gentamycin (90%).

The observation of marked susceptibility to gentamycin, streptomycin and chloramphenicol and resistant to penicillin, Bacitracin and trimethoprim-sulfamethazole in *P. mirabilis* bacterial strains and the detection handoff resistance against, tetracycline, oxytetracycline, clindamycin, and streptomycin. The present study is in line with the previous observation of studies conducted in India by Trivedi and Branton, (2015).

The finding of susceptibility to chloramphenicol, streptomycin, gentamycin, and trimethoprim-sulfamethazole, and on the contrary, the detection of 100% resistance against clindamycin, bacitracin, penicillin, and tetracycline in *M. morganii* isolates obtained from ticks in the present study agrees with the previous study by Jensen *et al.* (1996) and Stock and Wiedeman, (1998) who reported Biovar C *M. morganii* and *M. morganii* ssp. *sibonii* strain is tetracycline-resistant and sensitive to trimethoprim-sulfamethazole and chloramphenicol.

## 6. CONCLUSION AND RECOMMENDATIONS

In conclusion, findings of the current study showed that in addition to common intracellular tick-borne pathogens such as the rickettsial agents, other extracellular bacterial pathogens associated with human or animal infections could also be recovered and this provides additional baseline information of the cultivable aerobic pathogenic bacteria in ticks collected from cattle in central parts of Ethiopia, namely, Ada'a, Lome, and Ezha districts. Result of the present study demonstrated that out of the total 205 tick examined for bacteria 107 (52.2%) ticks were positive for different species of bacteria. Out of the total of 107 different pathogenic bacteria isolates obtained from ticks of cattle *C. freundii* and *E. coli* were the predominant bacteria isolated mainly from *Am. variegatum* and *Hy. truncatum*, respectively. Findings of the presented study suggested that more than one type of bacteria species were isolated from positive tick species revealing that mixed bacterial species isolates were a common scenario. Out of the total of 107 bacterial isolates obtained from positive ticks a total of 5 species of bacteria were identified including *Citrobacter freundii, Escherichia coli, Staphylococcus aureus, Proteus mirabilis,* and *Morganella morganii*. In vitro study on antibiotic efficacy study showed different degrees of resistance against the commonly used antibiotic drugs. The majority of *C. freundii, E. coli, S. aureus, P. mirabilis,* and *M. morganii* showed a multidrug resistance pattern to penicillin, bacitracin, and clindamycin. However, these bacteria were sensitive to gentamycin, chloramphenicol, and streptomycin. Altogether, the present study attempts to culture aerobic pathogenic bacteria from ticks infesting cattle in central Ethiopia and highlights information on their susceptibility to the most commonly used antibiotics in Ethiopia.

Therefore, based on these conclusions, the following recommendations were accorded:

- Appropriate practically applicable control of ticks to rescue tick-borne bacterial diseases in cattle of the study area is recommended.
- Improve general knowledge and awareness about the aerobic cultivable pathogenic bacteria in regards to biology, ecosystem niches, and microbial community remains to be investigated.

- Further advanced molecular studies should be conducted on aerobic cultivable pathogenic bacteria in ticks of cattle to characterize the specific pathogenic bacteria, determine their clinical significance, pathogenicity, transmission pattern and detect the presence of antibiotic resistance determinants which may provide a full figure of genotypic antimicrobial resistance pattern in addition to phenotypic one.

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## 8. ANNEXES

**Annex 1:** Sample (ticks) collection layout.

| No | Sample code | Species of ticks | Sex | Sample collection districts |
|---|---|---|---|---|
| 1 | | | | |
| 2 | | | | |
| 3 | | | | |

**Annex 2:** Results of cultural colony characteristics of isolated pathogenic bacteria.

| | Nutrient agar | Blood agar | MacConkey agar | XLD agar | EMB agar | Mannitol Salt agar |
|---|---|---|---|---|---|---|
| *E. coli* | | Greyish to white-colored | Pink colored Circular | yellow to yellow-red colonies. | metallic green sheen | - |
| *C. freundii* | | Circular, flat, red colored | pale-colored | yellow to yellow-red | Brown colored | - |
| *S. aureus* | | Golden yellow | Golden yellow (Beta hemolysis) | - | - | appeared as yellow |
| *M. morganii* | | Circular, dome-shaped, | cream-colored | Flat, colorless (NLF) | Yellow colonies | Flat, colorless - |

| | | | | | | |
|---|---|---|---|---|---|---|
| *P. mirabilis* | Pale white | Pale white swarming growth | Flat colorless (NLF) | Translucent, black center | Yellow, translucent | - |

**Annex 3:** Results of primary identification of aerobic pathogenic bacteria.

| Isolates | Gram stain | Catalase tests | Oxidase tests | Motility tests | Coagulase Tests |
|---|---|---|---|---|---|
| *E. coli* | Gram -ve rod | +ve | -ve | Motile | - |
| *C. freundii* | Gram -ve rod | +ve | -ve | Motile | - |
| *S. aureus* | Gram +ve cocci | +ve | -ve | Non-Motile | +ve |
| *M. morganii* | Gram -ve rod | +ve | -ve | Motile | - |
| *P. mirabilis* | Gram -ve rod | +ve | -ve | Motile | - |

**Annex 4:** Results of secondary biochemical tests of isolated aerobic pathogenic bacteria.

| Isolates | Coagulase Tests | Citrate | TSI | | | | Indole test | MR test | VP test | Lysin |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Slant | Butt | Gas | H2S | | | | |
| *E. coli* | -ve | -ve | A | A | +ve | -ve | +ve | +ve | -ve | +*ve* |
| *C. freundii* | -ve | +ve | A | A | +ve | +ve | -ve | +ve | -ve | -ve |
| *S. aureus* | +ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve |
| *M. morganii* | -ve | -ve | K | A | -ve | -ve | +ve | +ve | -ve | -*ve* |
| *P. mirabilis* | -ve | +ve | K | A | +ve | -ve | -ve | +ve | -ve | -*ve* |

**Annex 5:** Antibiotic susceptibility test registration set-up using Oxoid antibiotic disks

| No | Bacterial isolate | Antibiotic Disk used | Disc code | Potency | Inhibited growth zone in mm | Interpretation |
|---|---|---|---|---|---|---|
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |

**Annex 6:** Antibiotic discs, disc content and interpretive criteria for Enterobacteriaceae

| Antimicrobial agent | Symbol | Disc content | Susceptible | Intermediate | Resistant |
|---|---|---|---|---|---|
| Pencillin G | P | 10IU | ≥ 15 | - | ≤ 14 |
| Bacitracin | B | 10μg | ≥ 13 | 9-12 | ≤ 13 |
| Gentamycin | CN | 10 μg | ≥ 15 | 13-14 | ≤ 12 |
| Clindamycin | DA | 2 μg | ≥ 21 | 15-20 | ≤ 14 |
| Tetracycline | T | 30 μg | ≥ 15 | 12-14 | ≤ 11 |
| Streptomycin | S | 10 μg | ≥ 15 | 12-14 | ≤ 15 |
| Sulphametoxazol-Trimetoprime | SXT | 23.75/1.5 μg | ≥ 16 | 11-15 | ≤ 10 |
| Chloramphenicol | C | 30 μg | ≥ 18 | 13-17 | ≤ 12 |

Source: (CLSI, 2020)

**Annex 7:** Tick species identification key

| *Amblyomma variegatum* | |
|---|---|
| **Male** | **Female** |
| Eyes are distinctly convex.<br>Primary punctation sizes on conscutumare small to medium.<br>Primary punctuation distribution on conscutum is sparse.<br>Mesial area of enamel ornamentationon conscutum is elongate.<br>Lateral median areas of enamel ornamentation on conscutum are absent (small areas may occur in some populations of this tick).<br>Festoon enamelling is absent. Posteromedian stripe is narrow. Enamel color is pink to orange. Leg coloration is with pale rings. Coxae 1 external and internal spur lengths are: external medium and internal short. | Eyes are distinctly convex.<br>Primary punctation sizes on scutum aresmall to medium.<br>Primary punctation distribution onscutum is regular.<br>Enamel color pink to orange. Genital aperture posterior lips have a broad U shape.<br>Coxae 1 external and internal s purlengths are: external medium and internal short.<br>Mesial area of enamel ornamentat ionon scutum is elongate (it may be indistinct in its anterior part).<br>Lateral areas of enamel ornamentati onon scutum are small.<br>Scutum sides are straight. Scutum posterior ang le is bird.<br>Leg coloration is with pale rings. |
| ***Amblyomma gemma*** | |
| **Male** | **Female** |

| | |
|---|---|
| Eyes are slightly convex (as shown forthe female)<br>.<br>Primary punctation sizes on conscutumare small to medium.<br>Primary punctation distribution<br>on conscutum is localized (between theeyes).<br>Mesial area of enamel ornamentationon conscut um is elongate.<br>Lateral median areas of<br>enamel ornamentation on conscutumare largean d complex.<br>Festoon enamelling is partial (6 of 11festoons w ith enamel).<br>Posteromedian stripe is broad Enamel colour is pink to orange. Leg colouration is with pale rings.Coxae 1 external and internal spurlengths are: external medium and internal short. | Eyes are slightly convex.<br>Primary punctation sizes on scutum aresmall to medium.<br>Primary punctation distribution<br>on scutum is localized (between the eyes).Enamel colour is pink<br>to orange.<br>Genital aperture posterior lips have a narrow V shape.<br>Coxae 1 external and internal sp urlengths are: external medium<br>and internal short.<br>Mesial area of enamel ornamentat ionon scutum is large and elongat e.<br>Lateral areas of enamel ornamentati onon scutum are large and complex<br>.<br>Scutum sides are straight. Scut um posterior angle is<br>broad. Leg colouration is with<br>pale rings. |
| ***Rhipicephalus (Boophilus) decoloratus*** | |
| **Male** | **Female** |

| | |
|---|---|
| Cornua are distinct.<br>Coxae 1 spurs length is short. Ventral plate spurs are distinct (3 =accessory adanal plate). Ventral plate spurs are distinct (4 =adanal plate).<br>Caudal appendage is narrow in males.Ventral plate spurs are visible dorsally. | Hypostomal teeth are in 3 + 3 columns.Palp articles 1 internal margin has a protuberance with pectinate setae. Coxae 1 spurs are distinct. Coxae 2 and 3 spurs are present.<br>Genital aperture posterior lips have a narrow U shape.<br>Porose areas shape is a narrow oval. |
| ***Hyalomma truncatum*** | |
| **Male** | **Female** |
| Cervical field‟s depression is notapparent.<br>Conscutum is dark colored (appearssmooth and shiny).<br>Lateral grooves are long (distinct grooves in posterior part, extending asli nes of punctations towards eyes).<br>Posterior ridges number two. Caudaldepression is present (conspicuously deep and with large punctations).<br>Central festoon is dark colored. Paracentral festoons are separateanteriorly.<br>Central festoon of *Hyalomma albiparmatum* is pale white and forms adistinct Parma.)<br>Posteromedian groove is absent. Paramedian grooves are absent. Leg coloration is with pale rings.<br>Punctuation size is small.<br>Punctuationdistribution is localized (restricted | Scapular grooves profile is steep (grooves reach the posterior marginof scutum).<br>Scutum is dark colored. Scutum posterior margin isdistinctly sinuous<br>Leg coloration is with pale rings. Punctation size is small.<br>Punctation distribution is localized (on cervicalfields and scapulae).<br>Genital aperture anterior groove is shallow.<br>Genital aperture pretrial fold isconcave.<br>Genital aperture posterior lips have a broad U shape (distinctly wider than long). |

| | |
|---|---|
| to marginal areas and caudal depression,central area is smooth and shiny). | |
| ***Hyalomma rufipes*** | |
| **Male** | **Female** |
| Cervical fields depression is not apparent.Con scutum is dark colored.<br>Lateral grooves are short.<br>Posterior ridges are absent. Caudal depressionis ab sent.<br>Central festoon is dark colored (form of festoons is indistinct). Paracentral festoons ares eparate anteriorly.<br>Posteromedian groove is absent. Paramediangroo ves are absent.<br>Leg coloration is with pale rings.Punctation size is small.<br>Punctationdistribution is dense. | Scapular grooves profile isstee p.<br>Scutum is dark colored. Scutum posterior mar gin isdistinctly sinuous<br>Spiracle areas have dense setae.Leg coloration is with pale rings.<br>Punctation size is small. Punctation distribution is dense.Genital aperture anterior groo veis deep.<br>Genital aperture preatrial fold iscon vex. |

| | |
|---|---|
| Subanal plate alignment is with the adanalplate s.<br>Subanal plates are distinct.<br>Adanal plates shape has square ends.Spiracle areas have dense setae. | Genital aperture posterior lipshave a broad V shape. |
| ***Rhipicephalus evertsi*** | |
| **Male** | **Female** |
| Interstitial punctation size is small tomedium.<br>Interstitial punctation distribution isdense.<br>Setiferous punctations are indistinct.Coxae 1 anterior spurs are visible. Cervical fields depression is not apparent. Cervical fields texture has wrinkled areas.<br>Eyes are very<br>convex. Conscutum colour is dark.<br>Accessory adanal plates are small<br>(theymay be absent).<br>Adanal plates shape is broad and curved Caudal appendage is absent in fed<br>males.Spiracle plate areas have dense setae.<br>Posterior grooves are<br>indistinct (represented by three flat posterior area sof wrinkled texture).<br>Lateral grooves type is a distinct groove.Lateral gr ooves texture is wrinkled.<br>Legs are colored a distinctive paleorange, | Interstitial punctation size is small tomedium. Interstitial punctation di stribution is dense.<br>Setiferous punctations are indistinct.Basis capituli lateral angles are blunt.<br>Porose areas separation is narrow .Palp pedicels are short.<br>Cervical fields shape is<br>not apparent. Cervical fields textur e haswrinkled areas.<br>Scapular grooves profile is shallow.Eyes are very convex.<br>Spiracle plate areas have dense setae.<br>Scutum posterior margin is slightlysinuous.<br>Scutum color is dark.<br>Legs are colored a distinctive paleo range, uniformly over<br>each segment) |
| ***Amblyomma coherens*** | |
| **Male** | **Female** |
| Eyes flat to slightly convex | Eyes flat to slightly convex. |

| | |
|---|---|
| Scutum very smooth, puntuations small, very few, though denser on anterior of the shoulders. Marginal line a grocve rumming forward to posterior to eye, becoming very shallow at anterior end.<br>coxae 1 with long, pointed external and short, stubby internal spures, coxae 11 and 111 with posterior, coxa 4 with long, stout, internal spur<br>A large tick | Punctuation small, grouped around eyes with remainder of scutum smooth. Ornamentation a pale median stripe, which may be broken and incomplete anteriorily. Lateral borders may have very small patches of ornamentation.<br>Coxal spurs similar to those of the male though not as long. |
| ***Rhipicephalus pulchellus*** | |
| ✓ Interstitial punctation size in both sexes is minute to small.<br>✓ Interstitial punctation distribution is dense in both sexes.<br>✓ Setiferous punctations are distinct in both sexes.<br>✓ Basis capituli lateral angles are blunt.<br>✓ Porose areas separation is narrow.<br>✓ Palp pedicels are short.<br>✓ Cervical fields shape is not apparent in either sex.<br>✓ Eyes are flat in both sexes.<br>✓ Coxae 1 anterior spurs are visible on males.<br>✓ Female scutum colour is with enamel ornamentation (ivory white all over).<br>✓ Male conscutum colour is with enamel orna mentation (an ivory white in a pattern again | |

| | |
|---|---|
| st a dark brown background).<br>✓ Posterior grooves are distinct.<br>✓ Caudal appendage is narrow in fed males.<br>✓ Lateral<br>grooves type is punctations only. Lateral groo<br>ve texture is distinctly punctate. | |

Source: (Pegram *et al*., 1987; Houseman, 2013; Walker *et al*., 2014).

**Annex 8:** Primary biochemical identification tests used

**Gram's Stain** (Quinn *et al*., 2002)

**Principle :** Gram staining is a bacteriological laboratory technique used to differentiate bacterial species into two large groups (Gram-positive and Gram-negative) based on the physical properties of their cell walls.

**Procedure:** A thin smear was made & allowed to dry on air. The smear was fixed by passing through the Bunsen flame 3-4 times. Then the film was flooded with crystal violet for 1 minute. The crystal violet was washed off with water & drained. The slide was flooded with iodine solution for 2 minutes. The iodine was washed off with water & the smear was let dry. The film was decolorized with 95% ethanol alcohol for about 15-20 seconds and washed with water. Then the smear was counter stain with safranin for 1 minute, washed with water, blot dried and finally observed via 100microscope using oil immersion.

**Catalase Test (**Quinn *et al*., 2002)

**Principle:** catalase is an enzyme that converts hydrogen peroxide into water and oxygen. The bacteria that contain this enzyme are usually aerobic or facultative anaerobes. A positive reaction is indicated by a continuous bubble formation when the hydrogen peroxide is introduced into bacterial colonies.

**Procedure:** A loop full of bacterial growth was taken from the nutrient agar and placed on a clean microscope slide. A drop of 3% $H_2O_2$ was added. Then an effervescence of oxygen gas within few seconds indicates a positive reaction.

**Oxidase Test (**Quinn *et al*., 2002)

**Principle:** Cytochromes, found principally in organisms that can utilize free oxygen, are heme- containing proteins involved with oxidative phosphorylation. The enzyme cytochrome oxidase catalyzes the oxidation of cytochrome C, transferring the electrons to the terminal electron acceptor, oxygen. The oxydase reagent tetramethyl-p-phenylenediamine reacts with the oxidized cytochrome C producing a dark purple color.

**Procedure:** A solution of 1% tetramethyl-p-phenylenediamine dihydrochloride was used to moisten a piece of filter paper placed in a clean petridish. The test bacteria were streaked across the reagents placed on the filter paper using a sterile stick. A development of dark purple color along the streak line with in 10 seconds indicates a positive reaction.

**Motility Test** (Quinn *et al.*, 2002)

**Procedure:** SIM medium was prepared to evaluate the motility nature of the bacteria. The medium was stab inoculated using a straight wire. The tubes were incubated at $37^0$c for 24-48 hrs & checked for motility. A diffused growth throughout the medium indicates a motile bacterium but the growth of non-motile bacteria was confined to a stab line. While reading the result, the tubes were hold against a good light & the inoculated tubes were compared with the uninoculated tube as a control.

**Annex 9:** Secondary biochemical identification test used.

**Methyl Red (MR) Test** (Quinn *et al*., 2002)

**Principle:** It is a quantitative test for acid production (mainly used in the identification of Enterobacteriaceae), requiring positive organisms to produce strong acids (lactic, acetic &formic) from glucose through the mixed acid fermentation pathway. Since many species of the Enterobacteriaceae may produce sufficient quantities of strong acid that can be

detected by methyl red indicator during the initial phases of incubation, only organisms that maintain this low PH after prolonged incubation (48-72 hrs) overcoming the PH buffer system of the medium, can be called methyl red positive.

**Procedure:** MR-VP broth was inoculated with the pure culture of test organism & then incubated at 37$^{o}$c for 48 hrs. Then about 5 drops of MR solution were added in to the media. A positive result was indicated by the production of red color and negative result was indicated by yellow color in the test.

**Voges-Proskauer (VP) Test** (Quinn *et al.,* 2002)

**Principle:** The Voges-Proskauer (VP) test is used to determine if an organism produces acetylmethyl carbinol from glucose fermentation. If present, acetylmethyl carbinol is converted to diacetyl in the presence of ∝- naphthol, strong alkali (40% KOH), and atmospheric oxygen. The ∝-naphthol was not part of the original procedure but was found to act as a color intensifier by Barritt and must be added first. The diacetyl and quanidine-containing compounds found in the peptones of the broth then condense to form a pinkish red polymer.

**Procedure:** MR-VP broth was inoculated with the pure culture of test organism & then incubated at 37$^{o}$c for 24 hrs. Then added 6 drops of 5% alpha-naphthol, and mix well to aerate and also added 2 drops of 40% potassium hydroxide, and mix well to aerate. A positive result was indicated by the production a pink-red color at the surface within 30 min by shaking the tube vigorously and negative result was indicated by yellow color in the test.

**Indole Test (**Quinn *et al*., 2002)

**Principle:** The Indole Test is based on the formation of a red color complex when indole reacts with aldehyde group of p-dimethyl aminobenzaldehyde. This is the active chemical in Kovac‟s reagent. Indole positive bacteria possess an enzyme tryptophanase which converts tryptophan to indole.

**Procedure:** SIM medium was stab inoculated with the test bacterium using straight wire & then incubated at 37$^{o}$c for 24 hrs. Then 0.3 ml of Kovac‟s reagent was added & let stood

for about 1 minute. The formation of dark red ring indicates a positive reaction but a negative reaction forms a yellow ring.

**Citrate Utilization Test** (Quinn *et al*., 2002)

**Principle:** Certain bacteria can obtain energy in a manner other than the fermentation of carbohydrate by utilizing citrate as a sole of Carbon source. The measurement of these characteristics is important in the identification of the Entrobactericeae.

**Procedure:** The slant surface of Simmons citrate agar was inoculated with a pure colony & then incubated at 37$^{o}$c for 24-48hrs. A positive test was indicated by the development of a deep blue color within 24-48 hrs, indicating that the test organism was able to utilize the citrate contained in the medium with the production of alkaline products. The color remained green in a negative reaction.

**Triple Sugar Iron Agar Test (TSI)** (Quinn *et al.,* 2002)

**Principle:** The triple sugar- iron agar test employing Triple Sugar Iron Agar is designed to differentiate among organisms based on the differences in carbohydrate fermentation patterns and hydrogen sulfide production. Carbohydrate fermentation is indicated by the production of gas and a change in the color of the pH indicator from red to yellow. To facilitate the observation of carbohydrate utilization patterns, TSI Agar contains three fermentative sugars, lactose and sucrose in 1% concentrations and glucose in 0.1% concentration. Due to the building of acid during fermentation, the pH falls. The acid base indicator Phenol red is incorporated for detecting carbohydrate fermentation that is indicated by the change in color of the carbohydrate medium from orange red to yellow in the presence of acids. In case of oxidative decarboxylation of peptone, alkaline products are built and the pH rises. This is indicated by the change in color of the medium from orange red to deep red. Sodium thiosulfate and ferrous ammonium sulfate present in the medium detects the production of hydrogen sulfide and is indicated by the black color in the butt of the tube.

**Procedure:** Inoculated TSI by first stabbing through the center of the medium to the bottom of the tube and then streaked the surface of the agar slant with a pure colony and

leaved the cap on loosely and incubated at 35°-37°C in ambient air for 18 to 24 hours. Examined the reaction of medium.

**Staphylase Test Kit for Coagulase Test**

**Principle:** The generally accepted identifying characteristic of *Staphylococcus aureus* is the ability to produce free and bound coagulase (or clumping factor). The presence of clumping factor may be detected in a number of ways. The Oxoid Staphylase Test detects the presence of clumping factor through clumping of fibrinogen- sensitized sheep red blood cells. The specificity of the reaction is ensured by a simultaneous test with a control reagent (unsensitized sheep red blood cells), when of course no clumping reaction should be observed.

**Procedure:** Shaked the Test and Control reagents vigorously to obtain a homogenous suspension and any reagent cells that may be trapped in the dropping pipette must be mixed into the suspension. Using a loop, smear 1 to 3 of the suspected pure colonies from Mannitol Salt Agar on a test circle and a control circle on the Reaction Card and added 1 drop of the Test Reagent to the test circle and 1 drop of Control Reagent to the control circle then mixed the contents of the test circle using a loop. Flame the loop, then mixed the contents of the control circle and observe for agglutination while mixing.

**API (Analytical Profile Index) 20E Test**

**Principle:** API 20E is a biochemical panel for identification and differentiation of members of the family Enterobacteriaceae. The range provides a standardized, miniaturized version of existing identification techniques, which up until now were complicated to perform and difficult to read. In the API 20E, the plastic strip holds twenty mini-test chambers containing dehydrated media having chemically-defined compositions for each test. They usually detect enzymatic activity, mostly related to fermentation of carbohydrate or catabolism of proteins or amino acids by the inoculated organisms. A bacterial suspension is used to rehydrate each of the wells and the strips are incubated. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. All positive and negative test results are compiled to obtain a profile number,

which is then compared with profile numbers in a commercial codebook (or online) to determine the identification of the bacterial species.

**Procedure:** Confirmed the culture was of an Enterobacteriaceae. To test this, a quick oxidase test for cytochrome c oxidase may be performed. Picked a single isolated colony (from a pure culture) and made a suspension of it in sterile distilled water. Took the API 20E Biochemical Test Strip which contains dehydrated bacterial media/bio-chemical reagents in 20 separate compartments. Used a Pasteur pipette, fill up (up to the brim) the compartments with the bacterial suspension. Added sterile oil into the ADH, LDC, ODC, H2S and URE compartments. Put some drops of water in the tray and put the API Test strip and close the tray. Marked the tray with identification number (Patient ID or Organism ID), date and your initials. Incubated the tray at 37$^{o}$C for 18 to 24 hours.

**Maintenance of stock culture**

The pure culture of isolated bacterial species was inoculated into the tubes containing Tryptone Soya Broth and incubated at 37$^{0}$C for 24 hours. After the growth of organisms, the tubes were sealed with liquid paraffin (light) and kept in the refrigerator at +4$^{0}$C for further studies.

**Annex 10:** Microbial Media used for isolation and identification of the bacteria isolated

**PEPTONE WATER** (Oxide, Hampshire, England)

**Formula in grams per liter:** Peptone---10.0; Sodium chloride---5.0; Andrade's indicator (acid-Fuchsin) ---0.1 & the final PH is 7.4± 0.2 at 25$^{o}$c.

**Preparation:** 15 Grams of the medium was suspended in to 1000 ml of distilled water. Mix well to dissolve, distribute into tubes or bottles, containing Durham tubes and sterilize by autoclaving at 121°C for 15 minutes.

**Blood Agar** (Oxide, Hampshire, England)

**Formula in grams per liter:** Lab-Lemco powder---10.0; Meat peptone---10.0; Sodium chloride---5.0; bacteriological agar---15.0 & the final PH is 7, 3± 0, 2 at 25°c.

**Preparation:** 40 grams of the medium was suspended in to 1000 ml of distilled water. The mixture was allowed to stand for about 5 minutes until a uniform suspension was obtained. The medium was heated with gentle agitation & then sterilized at 121°c for 15 minutes. Finally, the medium was cooled to 45-50°c, 5-10% sterile defibrinated sheep blood was added, homogenized & poured in to sterile petri-dishes.

**NUTRIENT AGAR** (Oxide, Hampshire, England)

**Formula in grams per liter:** Lab-Lemco powder---1.0; Yeast extract---2.0; Peptone---5.0; Sodium chloride---5.0; bacteriological agar---15.0 & the final PH is 7.4± 0.2 at 25°c.

**Preparation:** 28 Grams of the medium was suspended in to 1000 ml of distilled water. The mixture was mixed well by boiling until a uniform suspension was obtained. It was sterilized in the autoclave at 121°C for 15 minutes & was let cooled to 45°C in a water bath. Finally, it was poured in to sterile petri-dishes, allowed to solidify & placed upside down to avoid excessive moisture.

**MacConkey Agar** (Oxide, Hampshire, England)

**Formula in grams per liter:** peptone---20.0; Lactose---10.0; agar---12.0; Bile salts---5.0; Neutral red---0.075 and the final PH is 7, 1± at 25 °c.

**Preparation:** 50 Grams of the medium was suspended in to 1000 ml of distilled water. The mixture was mixed well by boiling until a uniform suspension was obtained. It was sterilized in the autoclave at 121°C for 15 minutes & was let cooled to 45°C in a water bath. Finally, it was poured in to sterile petri-dishes, allowed to solidify & placed upside down to avoid excessive moisture.

**Mannitol Salt Agar (MSA)** (Oxide, Hampshire, England)

**Formula in grams per liter:** Proteose peptone---10.0; Sodium chloride---75.0; D-mannitol---10.0; Beef extract---1.0; Phenol red---0.025; Agar---15 and the final pH 6.8 ± 0.2 at 25 $^{o}$c.

**Preparation:** Suspended 111 grams of Mannitol Salt Agar in 1000 ml of distilled water. The mixture was mixed well by boiling until a uniform suspension was obtained. It was sterilized in the autoclave at 121$^{o}$C for 15 minutes & was let cooled to 60$^{o}$C in a water bath. Finally, it was poured in to sterile petri-dishes, allowed to solidify & placed upside down to avoid excessive moisture.

**Eosin Methylene Blue (EMB) Agar** (Oxide, Hampshire, England)

**Formula in grams per liter:** peptone---10.0; Lactose---10.0; Dipotassium hydrogen phosphate---2.0; Eosin Y---0.4; Methylene blue---0.065; Agar---15 and the final pH 6.8 ± 0.2 at 25 $^{o}$c.

**Preparation:** 37.5 Grams of the medium was suspended in to 1000 ml of distilled water. The mixture was mixed well by boiling until a uniform suspension was obtained. It was sterilized in the autoclave at 121$^{o}$C for 15 minutes & was let cooled to 60$^{o}$C in a water bath and Shaked the medium in order to oxidise the methylene blue (i.e., Restore its blue color) and to suspended the precipitate which is an essential part of the medium. Finally, it was poured in to sterile petri-dishes, allowed to solidify & placed upside down to avoid excessive moisture.

**Xylose Lysine Decoxycholate (XLD) Agar (**Oxide, Hampshire, England**)**

**Formula in grams per liter:** Yeast extract---3.0; L-Lysine HCl---5.0; Xylose---3.75; Lactose---7.5; Sucrose---7.5; Sodium desoxycholate---1.0; Sodium chloride---5.0; Sodium thiosulphate---6.8; Ferric ammonium citrate---0.8; Phenol red---0.08; Agar---12.5 and the final pH 7.4 ± 0.2 at 25 $^{o}$c.

**Preparation:** Suspended 53 g in 1000ml of distilled water. Heat with frequent agitation until the medium boiled. **DO NOT OVER HEAT**. Transferred immediately to a water bath

at 50°C. Poured into plates as soon as the medium has cooled and allowed to solidify & placed upside down to avoid excessive moisture.

**Sulfide Indole and Motility (SIM) media (**Oxide, Hampshire, England**)**

**Formula in grams per liter:** Casien peptone…20,00; meat peptone…6,10; Ferric ammonium sulfate…0,20; Sodium Thiosulfate …0,20; bacteriological agar…3,50 & the final PH is 7,3± 0,2 at 25$^{o}$c.

**Preparation:** 30 Gram of SIM basal Medium was measured & mixed in 1000 ml of distilled water in a flask & let dissolved. The solution was autoclaved at 121$^{o}$C for 15 minutes to sterilize. It was taken out from the autoclave & allowed to cool. Finally, the medium was poured in tosterile tubes, cooled & placed at +4$^{0}$ c for preservation.

**Simmons' Citrate Agar** (Oxide, Hampshire, England)

**Formula in grams per liter:** Ammonium Dihydrogen Phosphate…1.0; Dipotassium phosphate…1,00; Sodium chloride…5.0; Sodium citrate…2.0; Magnesium Sulfate…0.20; Bromthymol blue…0.08; Bacteriological agar…15.0 and the final PH is 6,8 ± 0.2 at 25$^{o}$c.

**Preparation:** 24.3gram of the powder was measured & suspended in 1000 ml of distilled water in a flask & let dissolved by boiling. The solution was autoclaved at 121$^{o}$C for 15 minutes to sterilize. The Medium was taken out from the autoclave & allowed to coll. Finally, the medium was poured in to sterile test tubes & cooled in a slanted position (The medium can also be sterilized by dispensing it in to tubes).

**Tryptone Soya Broth** (Oxide, Hampshire, England)

**Formula in grams per liter:** Pancreatic Digest of Casein ---17.0; Papaic digests of Soybean Meal ---3.0; Sodium Chloride --- 5.0; Dipotassium Phosphate ---2.5; Glucose---2.5; Final pH: 7.3 ± 0.2 at 25°C.

**Preparation:** 30 g of the medium was dissolved in 1 liter of distilled water. The solution was mixed thoroughly & sterilized at 121°c for 15 minutes. It was allowed to cool to 45-50°C and finally dispensed into sterile culture tubes.

**Triple Sugar Iron Agar** (Oxide, Hampshire, England)

**Formula in grams per liter:** Lab-Lemco powder---3.0; Yeast extract---3.0; Peptone---20.0; Sodium chloride---5.0; Lactose---10.0; Sucrose---10.0; Glucose---1.0; Ferric citrate---0.3; Sodium thiosulphate---0.3; Phenol red---0.024; Agar---15.0 and the final PH is 7.4 ± 0.2 at $25^{O}$C.

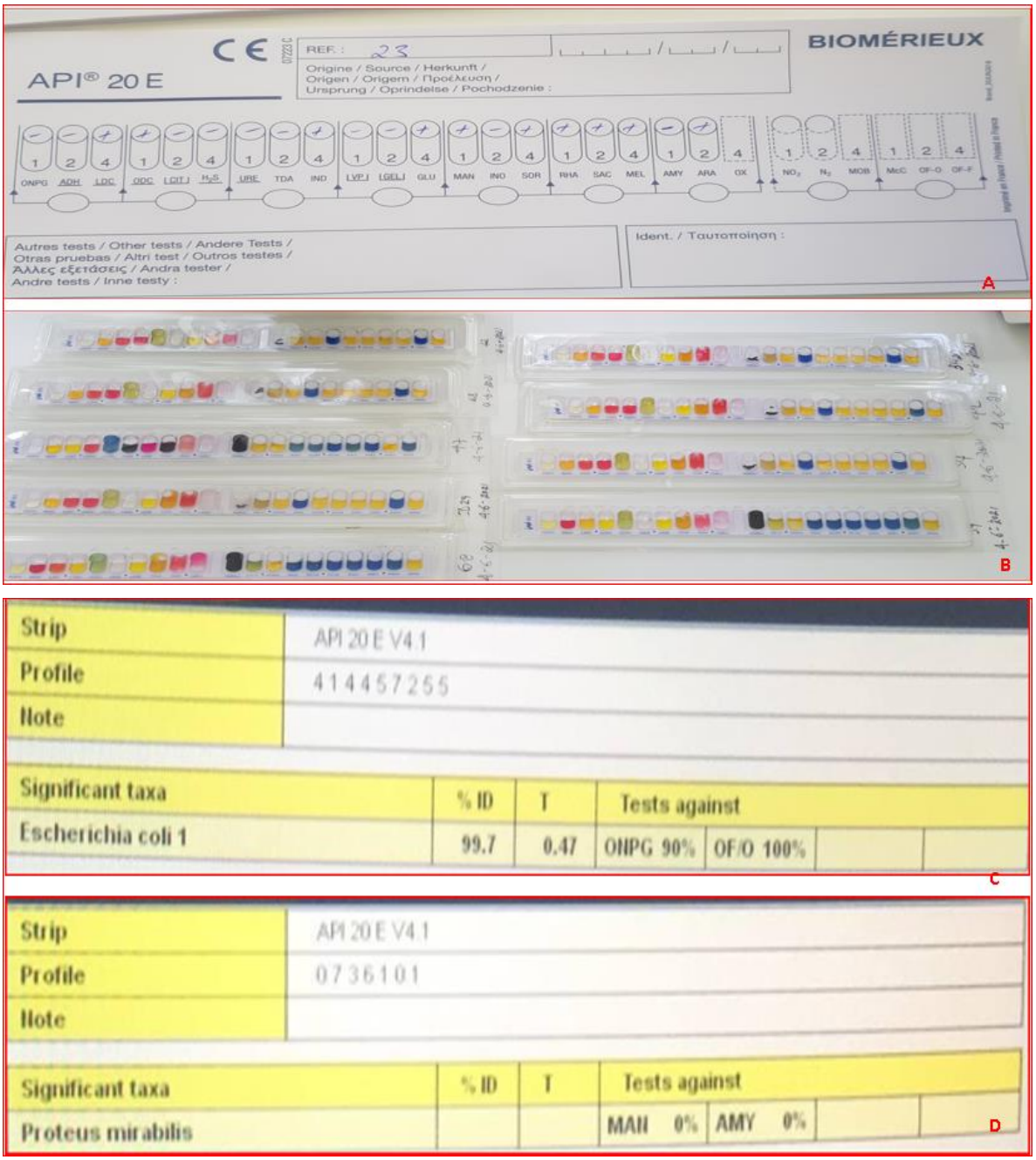
**Preparation:** Suspended 65 g in 1000ml of distilled water. Brought to the boil to dissolved completely. Mixed well and distributed. Sterilized by autoclaving at 121°C for 15 minutes. Allowed the medium to set in sloped form with a butt about 1 inch deep.

**MR-VP Medium** (Oxide, Hampshire, England)

**Formula in grams per liter:** Peptone---7.0; Glucose---5.0; Phosphate buffer---5 and the final pH 6.9 ± 0.2 at $25^{O}$C.

**Preparation:** Added 17 g to 1000ml of distilled water. Mixed well, distributed into final containers and sterilized by autoclaving at 121°C for 15 minutes.

**Annex 11:** Pictures taken during laboratory investigation of bacteria.




(A) API 20E test result register format. (B) Few API 20E test strip results after incubation of the bacteria for 24hr and addition of all necessary reagents. (C &D) The results of isolated test bacteria read from computer data base.

Pictures taken during laboratory investigation