INVESTIGATION OF FOODBORNE *CAMPYLOBACTER* SPECIES IN ANIMAL PRODUCTS AND HUMANS FROM SELECTED DISTRICTS OF SIDAMA REGION, ETHIOPIA

FACULTY OF VETERINARY MEDICINE

BY:

TAJEBE JERJERO TEREFE (DVM)

OCTOBER, 2024
HAWASSA, ETHIOPIA

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A thesis submitted to the Faculty of Veterinary Medicine of Hawassa University for the partial fulfillment of the requirements for the degree of Master of Science in Veterinary Microbiology

OCTOBER, 2024
HAWASSA, ETHIOPIA

ADVISOR’S APPROVAL SHEET

SCHOOL OF GRADUATE STUDIES

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(Submission sheet-1)

This is to certify that the thesis entitled “**Investigation of foodborne *campylobacter* species in animal products and humans from selected districts of Sidama region, Ethiopia,**” submitted in partial fulfillment of the requirements for the degree of **Master’s** with specialization in Veterinary Microbiology, the graduate program of the Faculty of Veterinary Medicine, has been carried out by Tajebe Jerjero ID. No. GPVeMiR/0005/15, under my/our supervision. Therefore, I/we recommend that the student has fulfilled the requirements and hence hereby can submit the thesis to the Faculty of Veterinary Medicine.

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We, the under signed, members of the Board of Examiners of the final open defense by **Tajebe Jerjero** have read and evaluated his/her thesis entitled "**Investigation of foodborne *campylobacter* species in animal products and humans from selected districts of Sidama region, Ethiopia**" and examined the candidate. This is, therefore, to certify that the thesis has been accepted in partial fulfillment of the requirements of the Master's in **<u>Veterinary Microbiology</u>**.

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DECLARATION

I, the undersigned, declare and affirm that this MSc thesis is my original work. I followed all the ethical principles in the preparation, data collection, analysis, and completion of this thesis. All the citations and references used for the Thesis have been duly acknowledged, and every serious effort has been made to avoid any plagiarism in the preparation of this work.

The thesis is submitted for partial fulfillment for the requirements of the requirements of a Master of Science degree in Veterinary Microbiology at Hawassa University. To the best of my knowledge, I solemnly declare that the main results formulated here have not been previously presented either in whole or in part for the award of any academic degree, diploma, or certificate in other institution elsewhere.

Name: Tajebe Jerjero Signature: ______________________
Date of submission: _____________________
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LIST OF ABBREVIATIONS

16S rRNA	the 16S ribosomal Ribose Nucleic Acid
BHI	Brain Heart Infusion
BPW	Buffered Peptone Water
CDT	Cytolethal Distending Toxin
CFU	Colony Forming Unit
CI	Confidence Interval
DNA	Deoxyribonucleic Acid
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
H_2S	Hydrogen Sulfide
HIV	Human immunodeficiency virus
ISO	International Organization for Standardization
mCC	modified Campy-Cefex
mCCDA	modified Charcoal Cefoperazine Deoxycholate Agar
OR	Odds ratio
PCR	Polymerase Chain Reaction
Spp.	Species
SPSS	Statistical Package for the Social Sciences
TBE	Tris/Borate/EDTA buffer
TSI	Triple Sugar Iron
USA	United States of America
UV	Ultraviolet
WHO	World Health Organization

ABSTRACT

Campylobacter species, small, microaerophilic, spirally curved, Gram-negative rods with characteristic fast corkscrew motility, are pathogens of major public health concern of global importance and among the leading cause of zoonotic foodborne gastroenteritis and acute diarrhea in humans worldwide. Human infection typically occurs through ingestion of contaminated foods or water and direct contact with feces of infected animals or humans. A cross-sectional study was conducted from October 2023 to June 2024 with the aim of investigating the presence of *Campylobacter* species in selected animal-derived foods (beef, milk, and eggs) and stool samples of diarrheic patients in three districts of Sidama region. For this study, a total of 284 samples, comprising raw beef (n=46), chicken eggshell swabs (n=47), raw cow milk (n=91), and stool samples of diarrheic patients (n=100), were purposively collected. The individual samples were examined using standard microbiological culture techniques and biochemical tests for isolation and identification, followed by species-level confirmation of presumptive isolates by polymerase chain reaction using specific primers. Molecular analysis confirmed the detection of thermophilic *Campylobacter* species in 17 (6%) of the samples with species distribution of *C. lari* (35.3%), *C. coli* (17.6%), *C. jejuni* (17.6%) and other unidentified thermophilic *Campylobacter* species (29.4%). The majority of the isolates were detected in chicken eggshell swabs (12.8%, n=6), followed by stool samples of diarrheic patients (6%, n=6), raw beef samples (4.35%, n=2) and raw cow milk samples (3.3%, n=3), but there was no difference ($p > 0.05$) in all of these prevalence values. The presence of thermophilic *Campylobacter* species in raw beef, egg, and milk can suggest the products to be important reservoirs posing the risk of foodborne public health hazards. Therefore, to mitigate these risks, it is crucial to enforce adequate safety measures and good hygienic practices in egg, milk, and beef producers and retailers. Adoption of the One Health approach to promote medical and veterinary sectors' collaboration is recommended to prevent the spill-over transmission of the pathogen between animals and humans and improve public health outcomes.

Keywords: *Campylobacter species, Gastroenteritis, Sidama, Zoonotic importance*

1. INTRODUCTION

Infectious foodborne illnesses arise from the consumption of microbe contaminated foods, particularly those of animal origin. Food-producing animals serve as significant reservoirs for pathogens like *Campylobacter*, non-typhoidal *Salmonella*, Shiga toxin-producing *Escherichia coli* and *Listeria monocytogenes* (EFSA and ECDC, 2018). Among these pathogens, *Campylobacter*, especially thermophilic species, presents a serious global public health risk, by causing a leading foodborne illness called campylobacteriosis. Several of the so-called thermophilic *Campylobacter* species are zoonotic; and are foremost important causal agents of gastroenteritis in human beings (Silva *et al*., 2011; WHO, 2020).

Members of the genus *Campylobacter* are responsible for most bacterial gastrointestinal infections in human, making it one of the leading causes of global foodborne diarrheal diseases, with an estimated 400–500 million cases annually. The condition is currently recognized as a major public health concern and is the most frequently reported zoonotic bacterial gastroenteritis of human around the globe (WHO, 2013; Kaakoush *et al*., 2015; WHO, 2020). Beyond gastroenteritis, *Campylobacter* species can also lead to extra-intestinal infections such as bacteremia, abscesses, meningitis, Guillain-Barré syndrome, reactive arthritis, and irritable bowel syndrome (Fitzgerald, 2015). The incidence of campylobacteriosis has increased over the past decade in industrialized countries, surpassing other common foodborne pathogens. In developing countries, the incidence and disease burden is even high due to inadequate food safety, sanitation, and healthcare systems, but its significance is poorly understood and often underestimated (Kaakoush *et al*., 2015).

Campylobacter organisms are commensal organisms which reside in the intestinal tract of a variety of domestic and wild mammals and birds, and reptiles as well as in related environments (water and soil), and are associated with abortion, gastroenteritis and/or diarrhoea in livestock. These animals are the major sources of food and water contamination with these pathogens. Among the domestic animals, poultry are found to be the primary host and reservoir of these zoonotic organisms (EFSA and ECDC, 2018; Hlashwayo *et al*., 2020).

The widespread presence of *Campylobacter* and its various transmission routes contribute to its broad distribution in the ecosystem pathogen. Risk factors that are significant for contracting campylobacteriosis include the consumption of raw or undercooked contaminated foods and untreated water, contact with domestic animals, and poor food hygiene practices. Especially, in low and middle-income countries, such as in sub-Saharan Africa, poor hygiene and sanitation status; and close contact with animals are the major contributors to the transmission of this pathogen of zoonotic importance to humans (EFSA and ECDC, 2018; Hlashwayo *et al*., 2020).

Most of the cases of *Campylobacter*-associated gastroenteritis are mild and self-limiting and require no antibiotic therapy. However, the infection can sometimes lead to severe and sometimes fatal complications, particularly in young children, the elderly, and immunocompromised individuals. Nonetheless, *Campylobacters* species are becoming important targets for both animal and human health research as a result of their zoonotic and public health importance, wide range of reservoir hosts, environmental perseverance (survival in water), and their emerging resistance to some of the commonly used antimicrobial agents. Furthermore, the pathogens are of paramount importance in food safety, in addition to being of both human and veterinary medical importance (Fitzgerald, 2015; WHO, 2020).

Studies conducted in Ethiopia on thermophilic *Campylobacter* species from human, domestic animals and their food products illustrated the zoonotic importance of the bacteria. Raw animal products, especially meat and milk, are commonly consumed in traditional Ethiopian diets, a dangerous practice for human campylobacteriosis, but the problem is rarely studied compared to other countries (Woldemariam *et al*., 2009). Moreover, updated information is warranted on the sources of infection and disease prevalence in different localities of the country. Despite the public health risks posed by this pathogen and the poor sanitation and hygiene practices in the region, no citable work has been done so far regarding the prevalence of thermophilic *Campylobacter* species in animal-derived foods in the Sidama region. Identifying sources of contamination and understanding the dissemination means of *Campylobacter* species in the food chain are the main keys to develop effective mitigation strategies.

Given the above gaps in knowledge and the potential link between thermophilic *Campylobacter* species in foods of animal origin and human illness, research is needed to guide interventions aimed at reducing contamination of animal products and preventing subsequent human infections in the study area. From a public health perspective, understanding the sources, risk factors and transmission modes is crucial for protecting both human and animal health and for designing appropriate control and prevention measures. Therefore, assessing the occurrence of *Campylobacter* species from foods of animal origin in the vibrant Sidama region is crucial. Hence, this research was conducted with the following objectives into consideration:

1.1. Objectives of the study

1.1.1. General objective

- To investigate the prevalence, distribution, and potential public health impact of foodborne *Campylobacter* species in animal products and human stool samples from selected districts of the Sidama Region, Ethiopia.

1.1.2. Specific objectives

- ✓ To estimate the prevalence of thermophilic *Campylobacter* in different animal products in selected districts of the Sidama region using culture and molecular method.
- ✓ To estimate the prevalence of thermophilic *Campylobacter* in human in selected districts of the Sidama region using culture and molecular method.

1.2. Statement of the problem

Campylobacteriosis currently is regarded as the most common zoonotic bacterial case of gastroenteritis and diarrhea both in developed and developing countries of the world. Campylobacters majorly cause gastrointestinal infections; even it is a number one cause of bacterial gastrointestinal infections. Particularly, in many developing countries the problem is conceived to be a hyper endemic condition owing to poor food, lack of environmental sanitation and close contact with animals (WHO, 2018).

In Ethiopia, numerous research works have been conducted over the past years that investigate the presence of *Campylobacter* species in cow milk, beef and feces as well as the emergence of patterns of resistance to various antimicrobials both in human and veterinary medicine (Nigatu *et al*., 2015; Kebede *et al*., 2017). In addition no information is available concerning the occurrence of *Campylobacter* species in the study area, particularly in Wondogenet and Yirgalem districts. Thus in this regard, there is a need for research based information on the occurrence of food borne campylobacter species to help undertake necessary interventions to minimize the contamination of animal products and the subsequent human infections in the study area.

1.3. Significance of the study

The genus *Campylobacter* is of paramount importance both in human and veterinary medicine as well as food safety worldwide. These organisms are known to occur in the intestinal tracts of a wide variety of domestic and wild animals. Their ubiquitousness as well as the variety of ways by which they can disseminate contributes to the broad distribution of the organism in the ecosystem, resulting in human and animal infections and food contaminations (Sahin *et al*., 2017). Thermophilic *Campylobacter* species from farm and wild animals and environmental sources have been associated with human gastroenteritis (Huang *et al*., 2015; WHO, 2018).

Livestock including chicken and cattle play a significant role in campylobacter infections in humans. Foods of animal origin such as egg, meat and milk when contaminated are food vehicles associated with sporadic cases of campylobacteriosis in humans. Of the foods intended for human consumption, those of animal origin tend to be most hazardous unless the principles of food hygiene are employed (Savita *et al*., 2018; Hlashwayo *et al*., 2020). Therefore, reducing the chance of contamination can reduce the burden of campylobacter infections in humans. Comprehending the occurrence of thermophilic campylobacter will assist researchers and livestock owners establish when and where risks for acquisition of campylobacter remain high. Additionally, findings on predisposing factors of campylobacteriosis will provide potential areas to guide public health players to formulate control strategies for minimizing and preventing zoonotic transmission of *Campylobacter* species. The study can provide information on the bacterial load and will form the basis for future quality control of animal products in order to control food borne diseases including campylobacteriosis in the country. Moreover, it may also fill the literature gaps and may serve as a baseline data for further studies on this pathogen of immense medical and veterinary importance.

2. LITERATURE REVIEW

2.1. Historical overview and taxonomy of *Campylobacter*

Campylobacter species were first described in 1886 by Theodore Escherich (Butzler *et al*., 2004), who observed non-culturable spiral-shaped bacteria in the large-intestinal mucus of infants who had died of cholera infantum (Vandamme *et al*., 2010). The first isolation of bacterial pathogens, currently known as campylobacters, was made by McFadyean and Stockman in1906 from the uterine exudate of aborting sheep (Butzler, 2004). Later, in 1963, Sebald and Veron suggested a new genus, Campylobacter (Greek word for curved rod), for these Vibrio-like organisms, leading to the change of *Vibrio fetus* to *Campylobacter fetus* and *Vibrio bubulus* to *Campylobacter bubulus*. Afterward, the isolation of Campylobacter became routine in the field of clinical microbiology since many selective media were developed, and *Campylobacter* species rapidly became recognized as a common cause of bacterial gastroenteritis (Fitzgerald *et al*., 2008).

The genus *Campylobacter* belongs to the family Campylobacteraceae, order Campylobacterales, in the class Epsilonproteobacteria. The family Campylobacteraceae comprises other closely related genera, including *Arcobacter*, *Dehalospirillum,* and *Sulfurospirillum* (Vandamme *et al*., 2015). Genus *Campylobacter* comprises 32 officially described species, nine subspecies, and four biovars, with *C. jejuni* and *C. coli* serving as the species of most significant concern causing most of the zoonotic campylobacteriosis cases worldwide, followed by *C. lari* (Man, 2011; ITIS, 2020). The species in this genus are grouped into five distinct groups, namely: the *C. fetus* group, *C. jejuni* group, *C. lari* group, *C. concisus* group, and *C. ureolyticus* group. Among these, *C. jejuni*, *C. upsaliensis*, *C. coli*, *C. lari*, *C. helveticus*, and *C. hepaticus*, among others, form a genetically homogenous grouping and are identified as the thermophilic campylobacters due to their requirement of higher incubating temperature of 42^{o}C, although they don't exhibit true thermophily (growth ≥ 55°C). Therefore, it was suggested that those which could grow at 41–43°C should be referred to as "thermotolerant" *Campylobacter* species (Fitzgerald, 2015; Costa and Iraola, 2019).

Among them, *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* are the most implicated pathogens in both human and veterinary medicine and are considered the principal species of public health and clinical significance. While *Campylobacter* organisms do not typically cause disease in animals, *C. fetus* subspecies *fetus* and *C. fetus* subspecies *venerealis* are important causes of reproductive system disorders and abortions in ruminants, particularly *C. fetus* subsp. *venerealis* is restricted to cattle and causes bovine genital campylobacteriosis (Fitzgerald, 2015; Costa and Iraola, 2019).

2.2. Morphological, biochemical, and survival characteristics

The genus name *Campylobacter* originated from an ancient Greek word meaning "crooked stick or curved rod, "which describes the typical S-shaped bacterium's morphology (Kreling *et al*., 2020). Members of the bacterial species in the genus *Campylobacter* are slender, S-shaped or spirally curved, non-spore-forming, Gram-negative rods, having small sizes ranging from 0.2 to 0.9µm in width and 0.5 to 5µm in length. However, some species, such as *C. hominis* and *C. gracilis,* exhibit straight rod morphology. The typical shape of *Campylobacter* looks more like a spiral or helical one rather than a curved rod shape, which can change its shape into filamentous or coccoid to adapt to stressful conditions. When two or more bacterial cells are grouped, an "S" or a "V" gull-wing shape is formed. The majority of the species are motile by means of a single polar unsheathed flagellum inserted at one (monotrichate) or both (amphitrichate) poles of the cells, which confer them a characteristic rapid darting or corkscrew-like motility, with the bacteria spinning around their long axes in a corkscrew fashion. The only exceptions are *C. showae*, by possessing up to five flagella at one pole, and *C. gracilis*, which has no flagella at all and is therefore nonmotile (Vandenberg *et al*., 2005; Fitzgerald *et al*., 2008; Silva *et al*., 2011). On account of their small size and motility, campylobacter *is* able to pass through membrane filters of 0.45 to 0.65µm pore size with relative ease, and this property is used for isolating *Campylobacter* species from different samples (Fitzgerald *et al*., 2008).

The bacteria, when grown on selective charcoal cefoperazone deoxycholate agar, colony morphological characteristics appear grey/white or creamy grey in color and moist in appearance, flat, irregular, and thinly spreading, but round, convex, or glistening colonies may also be formed. Furthermore, their colonies are usually non-pigmented (Vandenberg *et al*., 2005). Gram staining or wet mount on phase contrast microscopy is usually used to assess the morphology and motility. If the characteristic curved rods with darting motility appearance are noticed, then the presumption of *Campylobacter* species is made. If colonies have been obtained from selective media incubated at microaerobic conditions, this characteristic morphological finding combined with an oxidase-positive biochemical result can be reliably used to report the bacteria as being *Campylobacter* species. On the other hand, when using a filtration technique and a non-selective agar media, the presumptive isolation of *Campylobacter* species is made by confirmation of the isolate as Gram-negative, oxidase-positive, and l-alanine aminopeptidase negative (Jokinen *et al*., 2012; Vandamme *et al*., 2015).

Physiologically, *Campylobacter* species require uniquely fastidious growth conditions and are usually more sensitive to environmental stress as they lack many of the well-characterized adaptive responses that support the resistance to stress in other bacteria. The organisms are fastidious and slow-growing bacteria with a general requirement of specific microaerophilic incubation conditions of low oxygen tension (5% O_2, 10% CO_2, and 85% N_2) by most species for optimal growth. Nevertheless, certain species, such as *C. concisus* can grow under or prefer anaerobic conditions for growth. In addition, several *Campylobacter* species, including *C. concisus, C. curvus, C. gracilis, C. mucosalis, C. rectus, C. showae* , and some strains of C. hyointestinalis, require extra hydrogen (3–7% H_2,), or formate to act as electron donor for microaerobic growth and successful isolation. These factors may lead to the difficulty of recovering campylobacters from food and food-related environmental samples (Lastovica, 2006; Vandamme *et al*., 2015; Jokinen *et al*., 2012).

The typical temperature requirement for optimal growth is 37–42°C. Thermotolerant *Campylobacter* species (*C. jejuni, C. coli, C. lari, C. upsaliensis, C. helveticus,* and *C. insulaenigrae*) optimally grow at a higher incubating temperature of 42°C. In comparison, nonthermotolerant ones grow at an incubation temperature of 37°C (Vandamme *et al*., 2015).

For *in vitro* growth, 37°C and 42°C are mostly used, which reflects the temperature of their mammalian and avian hosts, respectively. A primary incubation temperature of 42°C, the internal temperature of the chicken body, allows for the growth of usually just *C. jejuni* and *C. coli*. However, an incubation temperature of 37°C is more appropriate for the isolation and maintenance of *Campylobacter* species that are able to infect mammals as other *Campylobacter* species grow poorly or not at all at 42°C. Furthermore, campylobacters grow at a pH between 6.5 and 7.5 (Goni *et al*., 2017). Because of the evidence that *Campylobacter* species lack cold shock protein genes, which play a role in low-temperature adaptation, they are incapable of growth at temperatures below 30 °C. These physiological characteristics collectively reduce the ability of campylobacters to multiply outside of animal hosts and in foods during their processing and long-term storage (Silva *et al*., 2011).

Campylobacter species also possess metabolic peculiarity. These non-spore-forming and fastidious bacteria are generally considered asaccharolytic bacteria, as they neither ferment nor oxidize carbohydrates for energy generation. Instead of metabolizing sugars, they obtain their energy from the degradation of amino acids or tricarboxylic acid cycle intermediates. Due to this property, they lack many discerning biochemical tests for their laboratory identification. However, all species except *C. gracilis* synthesize the enzyme oxidase. When exposed to the urease test, thermophilic campylobacters are negative except for *C. lari*. In addition, thermophilic campylobacters are catalase-positive except for *C. upsaliensis* (Silva *et al*., 2011; Goni *et al*., 2017).

Regarding their survival characteristics, these bacteria are very fragile and more susceptible than most bacteria to many environmental conditions, including sunlight, extremes of temperature, pH changes, low humidity, the presence of oxygen, UV irradiation, and many chemical agents, including disinfectants. When exposed to adverse environmental conditions, such as temperature and atmospheric stress, *Campylobacter* species change their morphology into the so-called “viable but non-culturable” physiological state. Despite their non-culturable state, they are not regarded as dead cells because their cell membrane is still intact, and their genetic information is not damaged. Campylobacters at this state may affect the sensitivity of culture-dependent detection procedures (Vandenberg *et al*., 2005; Silva *et al*., 2011).

2.3. *Campylobacter* virulence factors and pathogenesis

Pathogenic microorganisms, including *Campylobacter* species, use various factors known as virulence factors to evade the host immune system or to survive and flourish within a new environment. It is well established that *Campylobacter* species have complex and not completely known mechanisms for survival to conquer the host barriers and to cause sicknesses in their host organisms. It is documented that *Campylobacter* infections can occur with infectious doses of as low as 500-800 colony-forming units (CFU). Their pathogenicity is driven by multi-factorial virulence mechanisms, including secretion systems, capsules, flagellum, toxin production, adhesions, and lipopolysaccharides (García-Sánchez *et al*. 2018; Lopes *et al*., 2021). The severity of diseases due to *Campylobacter* species is dependent on the multitude of virulence factors and pathogenesis mechanisms used, including adhesion to the intestinal wall, colonization of the digestive tract, invasion of targeted cells, and toxin production. The infection process involves colonizing the intestine, adhering to gut cells, and producing toxins that damage the intestinal lining, leading to severe, bloody diarrhea (García-Sánchez *et al*. 2018; Kreling *et al*., 2020).

The key virulence factors of these bacteria include the flagellum for motility, chemotaxis, colonization, adhesion, invasion, and transport of effector protein by acting as a type three secretion system (Hamer *et al*., 2010; Kreling *et al*., 2020), surface structures such as capsular polysaccharide and lipooligosaccharide for adherence, colonization and resistance to host immune system (García-Sánchez *et al*. 2018; Lopes *et al*., 2021), adhesins such as *Campylobacter* adhesion to fibronectin (CadF) protein, capsular polysaccharide and flagella for adhesion to the epithelial cells (García-Sánchez *et al*. 2018; Kreling *et al*., 2020; Lopes *et al*., 2021), invasion factors including *Campylobacter* invasion antigen B (CiaB) for invasion of host cells (García-Sánchez *et al*. 2018; Lopes *et al*., 2021) and toxin production such as cytolethal distending toxin (CDT), the main toxin for inducing cell death by damaging DNA (García-Sánchez *et al*. 2018; Lopes *et al*., 2021). Despite these insights, the exact mechanisms of campylobacter pathogenesis responsible for their disease are multifaceted and are still not well comprehended (Kreling *et al*., 2020; Lopes *et al*., 2021).

2.4. Sources of infection and transmission routes of *Campylobacter* species

Campylobacter organisms are extensively ubiquitous. They live as commensals on the oral cavity mucosa, in the gut of a wide variety of animal reservoirs, including both wild and farmed animals, as well as domestic and wild birds and in related environments (including soil and water contaminated with animal feces). Warm-blooded farm animals such as poultry, pigs, cattle, and sheep are major reservoirs and sources of infection for human beings. While most farm animals are the potential reservoirs for the organisms, poultry are the major reservoirs of thermotolerant *Campylobacter* species and carry the most risk burden of campylobacteriosis (Huang *et al*., 2015; WHO, 2018). The reason that poultry remains the most important reservoir is due to the high body temperature of chickens at 42°C, which makes their gut the ideal niche for optimum growth of thermophilic campylobacters. Furthermore, the free-roaming nature of chickens, especially under a free-range system, increases the spread of the pathogen through fecal contamination of the environment, pasture, and surface water. They often carry large amounts of the organism without showing clinical signs and shed the bacteria in feces, contaminating water and the environment and act as common sources of human and animal exposure. Consumptions of raw or undercooked contaminated animal products and direct contact with animals play a significant role in the epidemiology of campylobacteriosis (Silva *et al.,* 2011; Huang *et al*., 2015; WHO, 2018; Hlashwayo *et al*., 2020).

The primary route of transmission for these bacteria is fecal-oral, mainly through the consumption of contaminated raw or undercooked foods (especially poultry products) or untreated water. In addition, infections due to non-food exposure can occur because of contacting the feces of carrier animals or humans, close contact with infected animals, or even from environments contaminated with feces from infected animals shedding bacteria. In developing countries, poor hygienic and sanitation conditions and close proximity to animals contribute to the easy and frequent acquisition of these pathogens. Although it is well-known fastidious nature, campylobacters are also isolated from environmental sources, such as lakes, rivers, soil, sea, and sewage, suggesting that environmental water is a possible vehicle of transmission (Silva *et al*., 2011; Huang *et al*., 2015; WHO, 2018).

2.5. Diagnosis and detection methods

Laboratory isolation and identification of *Campylobacter* species is laborious as they are fastidious and necessitate special atmospheric requirements to grow. For the identification of *Campylobacter* species, both phenotypic (based on the detection of an organism's production of certain proteins) and genotypic tests can be employed. Speciation of *Campylobacter* species is difficult because of the complex and rapidly evolving taxonomy along with the biochemical inertness of the pathogen, and these problems have resulted in a proliferation of both phenotypic and genotypic methods for identifying members of these bacterial genera (Fitzgerald, 2015; Ricke *et al*., 2019).

2.5.1. Bacteriological culturing of Campylobacter species

Campylobacter species exhibit dynamic and malleable physiological and metabolic characteristics that have impacts on the sensitivity and specificity of culture-dependent methods. So far, there is no generally accepted single or "standard" method of isolating and detecting all *Campylobacter* species from all kinds of sample types due to different requirements of temperature, microaerobic conditions, nutrients, and susceptibility to selective antibiotics and, therefore, culture techniques used vary between different research facilities. However, protocols have been published by recognized authorities, including the International Standards Organisation (ISO) and the US Food and Drug Administration (FDA) for isolating and detecting common types of *Campylobacter* species from some samples (Ricke *et al*., 2019; Silva *et al.,* 2020).

The detection and isolation of *Campylobacter* species traditionally rely on culture-based methods, which remain the gold standard for confirming the presence of live bacteria in various samples. Three common procedures for culture-based methods include direct plating, selective broth enrichment, and membrane filtration. Selective enrichment improves the recovery of low or stressed bacteria, while membrane filtration separates campylobacters from larger bacteria and also reduces the need for antimicrobial supplements into culture media (Jokinen *et al*., 2012; Silva *et al.,* 2020).

The membrane filtration method is carried out by taking advantage of the small size and unique motility of campylobacters to allow these organisms in samples to penetrate cellulose filters of 0.45-mm or 0.65-mm pore sizes to antibiotic-free blood or other agar media. Suspected colonies are then identified through Gram staining, motility tests, oxidase and catalase tests, and growth conditions. Further identification involves the use of tests such as susceptibility to cefalotin and nalidixic acid, hydrolysis of indoxyl acetate, hippurate hydrolysis, and H_2S production in media such as triple sugar iron (Jokinen *et al*., 2012; Silva *et al.,* 2020).

2.5.2. Molecular detection and identification methods

Phenotypic identification can be challenging because of the fastidious growth requirements, the asaccharolytic nature and possession of few distinguishing biochemical characteristics by *Campylobacter* species. Because of the difficulties and the unreliability of the phenotypic identification, several molecular methods may be used as supplementary to biochemical tests or even to replace them. Nowadays, several molecular techniques have been developed into powerful tools to characterize, solve the inconveniences of traditional methods and make it faster. Molecular identification methods, such as PCR and sequencing, has resolved some problems in pathogen detection research by offering fast and accurate tools for detecting *Campylobacter* species especially when conventional growth methods fail to recover stressed cells as they have high sensitivity, specificity and speed. These methods can be employed either to directly identify *Campylobacter* species from test samples or to confirm the species identity of isolates obtained from culture methods. The techniques identify unique DNA or RNA segments, with multiplex PCR enabling detection of multiple species in a single sample. Sequencing further refines identification to the species or subspecies level (Yamazaki-Matsune *et al*., 2007; Kreling *et al*., 2020; Baaboua *et al*., 2021).

2.6. Public health significance of *Campylobacter* species

Campylobacteriosis, a bacterial zoonosis caused by *Campylobacter* species, has been documented as the major cause of foodborne infections, including gastroenteritis and other extra-intestinal infections, in humans globally. Campylobacters majorly cause gastrointestinal infections; even it is the number one cause of bacterial gastrointestinal infections. However, post-infection complications, such as reactive arthritis, can also occur outside of the gut (WHO, 2018).

Campylobacter gastroenteritis in humans is of incredible public health importance, which is caused mainly by *C. jejuni* and *C. coli*. Poultry are distinguished as asymptomatic carriers and the principal reservoirs of these agents, and thus, poultry and their products have been incriminated as being responsible for an expected 80% of *Campylobacter* infections in humans. Ruminants (particularly cattle) have also been documented to be the second leading source of *C. jejuni* infections in humans through the consumption of contaminated unpasteurized milk and dairy products from these animals. Infected chickens and cattle may shed the bacteria in their feces, thus contaminating the environment in which humans share. Other predisposing factors include close contact with domestic animals and unhygienic living and/or poor sanitary standards. Most human campylobacteriosis cases are usually sporadic, but there were many reported outbreaks. Despite its severity rate is low (0.03%), the quantity of human *Campylobacter* infection cases is elevating. All age groups are affected but, infections are recognized with increasing frequency in infants, children, and aged individuals suffering from deliberating disorders (Goni *et al*. 2017; García-Sánchez *et al*. 2018).

Globally, 166 million *Campylobacter* cases per year have been reported, but there is a great difference by region. In regions where surveillance programs for foodborne illness are well settled, the campylobacteriosis yearly rate is high. Interestingly, there is a significant difference between nations. In developing countries, *Campylobacter* is not the most common cause of bacterial foodborne illness because in the developing nations, there aren't national programs for surveillance of Campylobacter infection. Thus, the state of campylobacter infection is difficult to evaluate in these countries (WHO, 2018; García-Sánchez *et al*., 2018).

2.7. Treatment and prevention of *Campylobacter* infections

Campylobacter infections are usually self-limiting resolve on their own, and require no therapeutic intervention other than supportive therapy, such as fluid and electrolyte replacement. Therefore, antibiotics are reserved for prolonged, severe cases or for immunocompromised patients. In such cases,, macrolides (such as erythromycin) are the primary treatment choice, with fluoroquinolones, tetracyclines, beta-lactams, and aminoglycosides as alternatives. However, resistance to these drugs, which affects the effectiveness of clinical therapy, is rising, largely due to the inappropriate use of these drugs. For this reason, thorough attention should be paid when choosing the most appropriate antimicrobials for effective treatment (WHO, 2013; Kaakoush *et al*., 2015).

Prevention of human transmission is of paramount importance in reducing the incidence and burden of campylobacteriosis in humans. Prevention can be directly applied to humans in different ways, including sewage sanitary conditions, provision of portable water, public awareness concerning the significance of pasteurization of milk, proper cooking of food from animal origins, and the use of therapeutics in case of infections. In addition, every measure needs to be taken by farmers, processors, and consumers to improve the hygiene and safety of animal products along the various stages of the food chain in preventing the spread of foodborne pathogens, including *Campylobacter*. However, the ubiquity of campylobacters in the environment, animals, and their products presents significant difficulties in investigating the complex pathways for infection with no single specific point for effective control and prevention. Furthermore, as *Campylobacter* resistance to drugs of choice may limit the treatment options, controlling the indiscriminate use of antimicrobials in animal husbandry for non-therapeutic reasons such as promoting weight gains of birds or improving feed efficiency is essential to reduce the burden of Campylobacter infections (Moyane *et al*., 2013; WHO, 2013; García-Sánchez *et al*., 2018).

2.8. Status of *Campylobacter* in Ethiopian perspective

In Ethiopia, a number of published studies have reported the occurrence and antimicrobial susceptibility profile of *Campylobacter* species from animal feces (Kassa *et al*., 2007; Chanyalew *et al*., 2013; Abamecha *et al*., 2015; Nigatu *et al*., 2015; Chala *et al*., 2021), meat/carcass (Dadi and Asrat, 2008; Woldemariam *et al*., 2009; Chanyalew *et al*., 2013; Faris, 2015; Hagos *et al*., 2021), human stool (Beyene *et al*., 2004; Ewnetu and Mihret, 2010; Lengerh *et al*., 2013; Getamesay *et al*., 2014; Tafa *et al*., 2014; Kebede *et al*., 2017; Chala *et al*., 2021; Behailu *et al*., 2022) and water (Chala *et al*., 2021) across the different parts of the country. In the country, several studies that have been conducted from 2004 to 2022 reported *Campylobacter* species' prevalence and antimicrobial susceptibility from various sources: animal feces, meat, human stool, and water. The reported prevalence ranged from 6.1% in HIV-positive stool samples from Hawassa town (Kebede *et al*., 2017) to 86.6% in chicken feces from the Lare district of Gambella (Abamecha *et al*., 2015). In these studies, chickens and cattle were identified as the key reservoirs for human infection. Regarding the species identified by these studies, *C. jejuni* and *C. coli* were the main species reported, with *C. jejuni* being the predominant one. The studies also highlighted significant antibiotic resistance of the pathogens, particularly to erythromycin, tetracycline, ampicillin, nalidixic acid, and ciprofloxacin, indicating serious public health concerns. Table 1 below shows a summary of *Campylobacter* status across Ethiopia from 2004 to 2022.

Table 1: Summary of occurrence of *Campylobacter* species in different parts of Ethiopia spanning from 2004 to 2022

Author and year of publication	Study area	Study subject	Sample type	Characteristics	Detection methods	Prevalence (%)
Beyene *et al*., 2004	Jimma	Human	Stool	Children	Culture	11.6
Kassa *et al*., 2007	Jimma	Animals	Feces	Chicken	Culture	68.1
				Pig		50
				Sheep		38
				Cattle		12.6
Dadi and Asrat, 2008	Debre Zeit/ Addis Ababa	Food of animal origin	Raw meat	Raw meat	Culture	9.3

Woldemariam *et al*., 2009	Debre Zeit	Animals	Carcass	Sheep	Culture	10.6
				Goats		9.4
Ewnetu and Mihret, 2010	Bahir Dar	Human	Stool	Patients	Culture	8
		Animal	Cloacal swabs	Chickens		72.7
Chanyalew *et al*., 2013	Debre Berhan	Animal	Carcass and Fecal	Sheep	Culture	12.6
Lengerh *et al*., 2013	Gondar	Human	Stool	Under-five Children	Culture	15.4
Getamesay *et al*., 2014	Hawassa	Human	Stool	Under-five Children	Culture	12.7
Tafa *et al*., 2014	Jimma	Human	Stool	Under-five children	Culture	16.7
Abamecha *et al*., 2015	Lare (Gambella)	Animals	Feces	Chicken	Culture	86.6
				Cattle		48
				Sheep		39
				Goat		33.3
Faris, 2015	Addis Ababa	Animal	Raw meat	Cattle	Culture	9.4
Nigatu *et al*., 2015	Gonder	Animals	Faeces	Poultry	Culture	28.9
				Cattle		21.5
Kebede *et al*., 2017	Hawassa	Human	Stool	HIV-patients	Culture	6.04
Hagos *et al*., 2021	Mekele	Animals	Raw meat	chicken	Culture and PCR	43.93
				Cattle		11.9
				Goat		9.25
Chala *et al*., 2021	Addis Ababa	Animals	Faeces	Cattle	Culture and PCR	18.5
				Sheep		13.3
				Goat		7.1
				Poultry		13.0
		Human	Stool	Livestock owners		10.1
		Water	Water	Different water samples		10.5
Behailu *et al*., 2022	Hawassa	Human	Stool	Children	Culture	6.8

3. MATERIALS AND METHODS

3.1. Study area

The study was carried out in three districts of Sidama regional State: Hawassa town administration, Dale woreda and Wondogenet woreda. These areas were purposively chosen for their significant dairy cattle and poultry populations, milk and beef production, accessibility, and growing milk demand due to urbanization besides the logistical feasibility. Hawassa, the regional capital, is located 275 km south of Addis Ababa, at an altitude of 1750 meters above sea level with annual rainfall of 800-1000 mm and temperatures ranging from 20.1°C to 25°C. Dale, situated 313 km south of Addis Ababa and 40 km south of Hawassa, has an elevation ranging from 1200 to 3200 meters, with temperatures between 5.1°C and 27.4°C and 247 mm of average annual rainfall. Wondogenet, located 270 km south of Addis Ababa and 34 km east of Hawassa, spans elevations of 1720 to 2620 meters, with temperatures from 12°C to 26°C and annual rainfall ranging from 709 mm to 2062 mm (Argaye, 2022; Behailu *et al*., 2022).

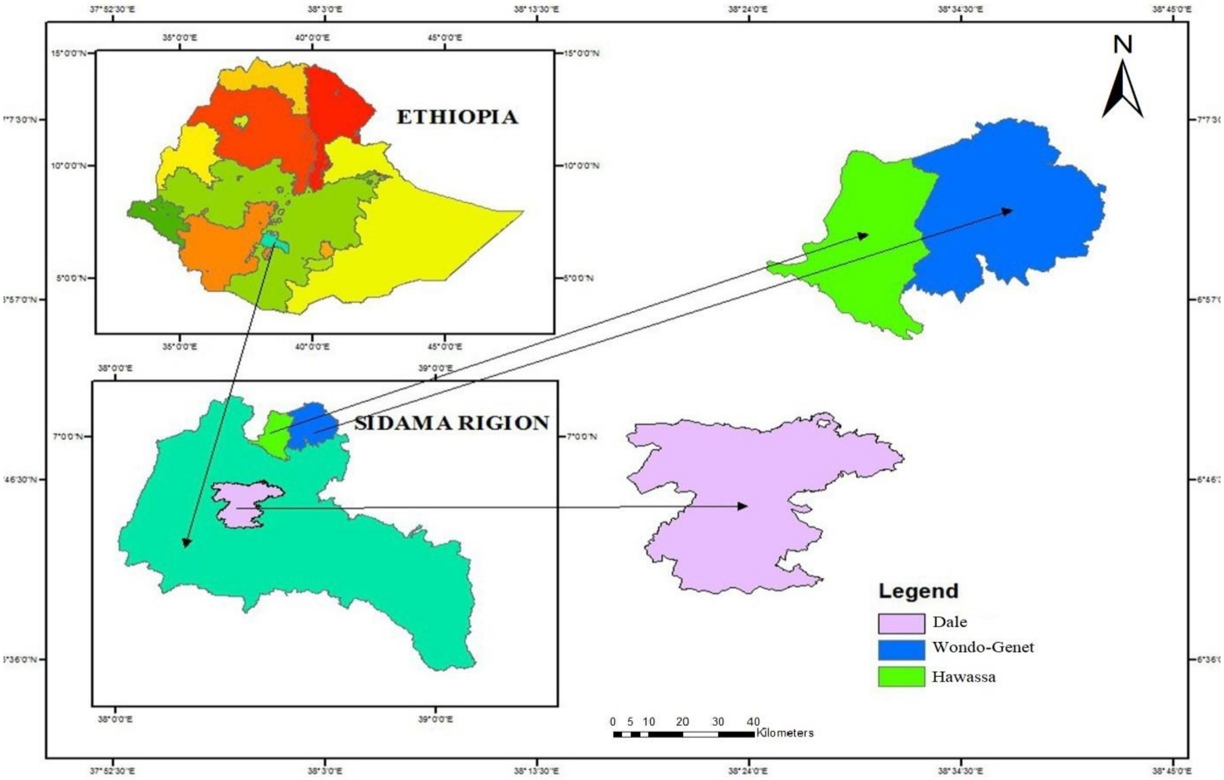


Figure 1: Map of the study area

3.2. Source of samples

The samples required for the study were collected from various sources: raw beef from butcher shops, hotels, and restaurants; raw cow milk from dairy farms and milk retailer shops; chicken eggshell swabs from poultry production farms and egg retailer shops; and stool samples from diarrheic patients at hospitals in the study areas. For milk, pooled samples were taken from milking equipment and containers of individual dairy farms, but pooling was not undertaken in milk retailer shops since their milk itself is a pool from different sources. Similarly, pooled chicken eggshell swab samples were collected from each poultry production farm and retail point. Raw beef samples that were in contact with a cutting table and/or weighing balance were collected from butcher shops, hotels, and restaurants. Human stool samples from diarrheic patients were collected with consideration for the patient's age, sex, residence, and stool consistency.

Prior to the start of collecting all these samples, support letters were sought and obtained from Sidama regional state agricultural and health bureaus and delivered to their respective offices of each district to inform and seek their facilitation.

3.3. Study design

A cross-sectional study was conducted from October 2023 to June 2024 to determine the occurrence of *Campylobacter* species and identify the species involved from different animal products and human stool samples in the three selected districts to determine the occurrence of *Campylobacter* species and identify the species involved from different animal products and human stool samples in the three selected districts of the Sidama region.

3.4. Sample size determination and sampling strategy

For this study, a purposive sampling method was employed, focusing on areas with relatively high dairy and poultry production potential. The required minimum sample size was determined using the standard formula for a single population proportion, as described by Thrusfield, (2018),

$$n = \frac{1.96^2 \; X \; Pexp(1-Pexp)}{d^2}$$

Where 1.96 is the value of Z at a 95 % confidence interval
n is the required sample size,
Pexp is the expected *Campylobacter* prevalence and
d is the desired absolute precision (0.05).

Accordingly, the required sample size for stool samples from diarrheic patients visiting the selected governmental hospitals in the study districts was calculated using an expected prevalence of 6.8% (Behailu *et al*., 2022), resulting in a final sample size of 100. This total number is proportionally allocated to three participant hospitals according to their patient's flow rate.

Likewise, the required sample size for different animal products was determined using an expected prevalence of 9.3% from a previous study by Dadi and Asrat (2008), giving the final calculated sample size of 184.

This sample size was also proportionally allocated for the three participant districts according to their potential for dairy cattle and chicken populations. Proportionally stratified allocation was also conducted for each sample type of animal product (raw beef vs eggshell swab vs raw milk). In this respect, raw beef-derived samples were further stratified based on sources in butcher shops, hotels, or restaurants, whereas eggshell swab and raw milk-derived samples were stratified into either farm or retailer shop sources. Accordingly, the representative sampling plan entailed the sampling of 46 raw beef, 47 eggshell swabs, and 91 raw milk samples from the three study districts.

3.5. Sample collection, handling and transport

Prior to the start of sample collection, support letters written by the respective district veterinary departments were obtained and presented to the selected poultry and dairy farms, requesting them to collaborate in the study after briefing them on the study objective. Consequently, samples were collected from those farms whose owners showed a willingness to participate in the study. In a similar way, samples from retail shops, butcher shops, hotels, and restaurants were collected after obtaining willingness from their respective owners after briefing the objective of the study.

In the process of collecting food samples from each of the three districts, the participating study sub-cities or kebeles (the smallest administrative units) were purposively selected on the basis of relatively high potential and accessibility for dairy and poultry farms and the willingness of farm owners to participate in the study after briefing them the study objective.

For this study, about 20-25 ml of pooled milk samples were aseptically collected from milking equipment's and milk containers of each dairy farms and milk retailer shops using sterilized universal sampling bottles as described elsewhere (Salihu *et al.,* (2010).

In the process, raw milk samples from different milking equipment/containers or different cattle pens in a study dairy farm were pooled and taken as one sample. In the case of beef sample taking, approximately 10 grams of raw beef samples in contact with weighing balances, cutting tables, or that were hung in retail environments were taken and placed in zip-locked sterile plastic bags as described by Baaboua *et al*. (2021).

Swab samples from intact shells of selected chicken eggs were also collected as a pool from each participant poultry farm and egg retailer shop. While sampling, 3 to 5 intact chicken eggs were randomly selected from different egg containers or different poultry sheds in a study poultry farm and their shell swabs were pooled and taken as one sample. In the process of swabbing, the entire eggshell surface of selected intact chicken eggs first rubbed using sterile cotton-tipped swabs soaked in sterile buffered peptone water. On completion of the rubbing process, the shaft was broken by pressing it against the inner wall of a test tube containing 9 ml of sterile buffered peptone water and disposed of leaving the cotton swab in the test tube, following the method described by Savita *et al*. (2018).

Hospital-based cross-sectional study was also conducted on human campylobacteriosis by collecting stool samples from diarrheic patients visiting the outpatient department (OPD) of hospitals on sampling days. Study subjects of the study were volunteer outpatients who attended the selected governmental hospitals for cases of gastrointestinal symptoms (diarrhea) and who had not been recently treated with antibiotics. To achieve this purpose, permission letters were obtained from the Sidama Regional State Health Bureau and were presented to the medical directors of the respective hospitals to seek their willingness and facilitation. The stool samples were collected purposively from diarrheic patients whom physicians ordered for stool examination in collaboration with laboratory professionals of the hospitals. To do this, the purpose of the study was first explained to the patients or caretakers of children through the facilitation of the assigned laboratory professionals. Subsequently, freshly passed diarrheal stool specimens were collected from each volunteer participant using sterile cotton-tipped swabs, placed immediately in screw-capped test tubes containing 9 ml of sterile buffered peptone water as a transport medium, labeled, and promptly transported to the laboratory.

Verbal consent was also sought from the patients or caretakers, and information on sex, age, place of residence, and stool consistency was obtained from each volunteer individual while sampling. The consistency of collected stool samples was determined in the hospital's laboratory while collecting the samples.

Finally, all the collected animal products and stool swab samples were properly labeled accordingly with the necessary information and unique codes, placed in a cooler ice box containing ice packs, and immediately transported to the microbiology laboratory of the faculty of veterinary medicine at Hawassa University. Then, the samples were processed for microbiological analysis in the university laboratory within 4-6 hours of collection using aseptic techniques to ensure the viability and culturability of the organisms.

3.6. Sample processing and isolation of *Campylobacter* species

For the isolation and identification of thermophilic *Campylobacter* species, all the collected and laboratory-transported samples were processed using conventional methods optimized for the detection of thermophilic *Campylobacter* species according to the techniques and guidelines recommended by the International Organization for Standardization (ISO, 2017), with slight modifications. As per the method described by Bolton (2000), a resuscitation step was performed for all sample types by incubating them aerobically at 37 °C for 4 hours. This step was crucial for initiating the growth of culturable *Campylobacter* cells or for the recovery of sub-lethally injured cells that may have been compromised due to exposure to drying, heat, starvation, freezing, or oxidative stress (Williams *et al*., 2009; Zhou *et al*., 2011).

Briefly, the collected raw beef samples were homogenized in sterile buffered peptone water at a ratio of 1:9 (beef: broth) within a zip lock sampling plastic bag by manually massaging for about 3 minutes at room temperature. The rinse mixture was then subjected to an enrichment procedure, starting with a resuscitation step of incubating at 37 °C for 4 hours. Likewise, pooled milk samples were vortexed and mixed with sterile buffered peptone water at a 1:9 ratio (milk: broth) and incubated at 37 °C for 4 hours for resuscitation.

Regarding the samples collected as swabs, the swab samples collected from chicken eggshells and human stools were directly incubated for resuscitation at 37 °C for 4 hours, as above.

Following the completion of the resuscitation period, the samples were thoroughly homogenized. Subsequently, 1 ml of each sample was transferred to 9 ml of sterile modified Rappaport Vassiliadis enrichment broth (Microxpress, India) and incubated at 41.5 °C for 48 hours to achieve a successful enrichment purpose, as described elsewhere (Williams *et al.*, 2009).

Subsequent to the 48 hours of incubation for enrichment, a loopful of growth from the enrichment broth was streak plated onto modified charcoal cefoperazone deoxycholate agar (mCCDA) (CM0739, Oxoid) plates with *Campylobacter* selective supplement (SR0155E, Oxoid). The plates were then incubated at 41.5˚C in a microaerophilic environment (5% O_2, 10% CO_2, and 85 % N_2) created by CampyGen™ sachets (CN0025A, Oxoid) in a 2.5-liter anaerobic jar (Oxoid) and for 72 hours period.

3.7. Identification of *Campylobacter* species

After 72 hours of incubation, the plates were examined for the presence of *Campylobacter* typical colonies (greyish, flat, and moistened, with a tendency to spread, and may have a metal sheen). The colonies were selected for further identification, through biochemical tests (motility test, Gram staining, catalase, and oxidase tests, and H_2S production). The presumptively identified colonies were then preserved in a deep freezer at −20 °C in brain heart infusion broth (Difco, Detroit, Mich) supplemented with 35% (vol/vol) sterile glycerol until sent for further PCR analysis for species confirmation.

Annex iv shows the flow chart representing the sequence of laboratory processing, isolation, and identification of thermophilic *Campylobacter* species from this study's samples.

3.8. Identification and speciation of *Campylobacter* by PCR

Forty six presumptively detected *Campylobacter* isolates were obtained using culture and biochemical methods. All the presumptively detected isolates were then subjected to PCR analysis for final confirmation. The PCR analysis was carried out in two steps: first, all presumptive isolates were subjected to level PCR identification of *Campylobacter* using primers targeting the 16S rRNA gene sequence as described by Yamazaki-Matsune *et al.* (2007). Next, all isolates positive for the first PCR reaction were subjected to a multiplex PCR analysis for *Campylobacter* species identification. Multiplex PCR was conducted using primers targeting the 16S rRNA gene for genus-level identification and cj0414, glyA, cstA and ask genes for identification of *C. jejuni*, *C. lari*, *C. fetus*, and *C. coli,* respectively, as previously described (Yamazaki-Matsune*et al*., 2007). All the forward and reverse primer sequences, target genes and amplicon size used in this study are shown in table 2.

Table 2: Primer pairs used for the detection and speciation of *Campylobacter* species (Yamazaki-Matsune *et al.,* 2007)

Species	Expected amplicon Size (bp)	Target gene	Primer	Sequence (5’ to 3’)
Genus	816	16S	C412F	5’-GGATGACACTTTTCGGAGC-3’
Campylobacter		rRNA	C1228R	5’-CATTGTAGCACGTGTGTC-3’
C. coli	502	*Ask*[¥]	CC18F	5’-GGTATGATTTCTACAAAGCGAG-3’
			CC519R	5’-ATAAAAGACTATCGTCGCGTG-3’
C. jejuni	161	*cj0414*[*f*]	C-1	5’-CAAATAAAGTTAGAGGTAGAATGT-3’
			C-3	5’-CCATAAGCACTAGCTAGCTGAT-3’
C. lari	251	*glyA*[β]	CLF	5’-TAGAGAGATAGCAAAAGAGA-3’
			CLR	5’-TACACATAATAATCCCACCC-3’
C. fetus	359	*cstA*[⸸]	MG3F	5’-GGTAGCCGCAGCTGCTAAGAT-3’
			MG3F	5’-AGCCAGTAACGCATATTATAGTAG-3’

[¥] presumed to encode for *aspartokinase* gene.

[⸸] presumed to encode for *carbon starvation protein A* gene.

[*f*] presumed to encode an *oxidoreductase.*

[β] presumed to encode for *serine hydroxy methyltransferase.*

All PCR reactions and cycling parameters were according to Yamazaki-Matsune *et al*. (2007) with slight modification (see Annex viii). The PCR products were analyzed by gel electrophoresis using a 1.8% (w/v) agarose gel in 1xTBE buffer, stained with ethidium bromide. DNA bands were visualized under UV transillumination (BTS-20) and photographed. Isolates that were positive for the genus-specific PCR but negative for *C. lari*, *C. fetus*, *C. coli,* or *C. jejuni*-specific PCR fragments were classified as unidentified thermophilic *Campylobacter* species. In the process, a 1000 bp DNA ladder was used as a molecular size marker.

3.9. Data management and statistical analysis

The raw data from the study were initially recorded in Microsoft Excel 2010, coded appropriately, and then exported to IBM SPSS Statistics for Windows, version 25 (SPSS Inc., Chicago, IL, USA) for analysis. Descriptive statistics were used to determine the prevalence of *Campylobacter* and to analyze the frequencies of other variables. The prevalence of *Campylobacter* was calculated as the ratio of *Campylobacter*-positive samples to the total number of samples examined. The association between the dependent and independent variables was evaluated using Fisher's exact test. A p-value of less than 0.05 was considered statistically significant.

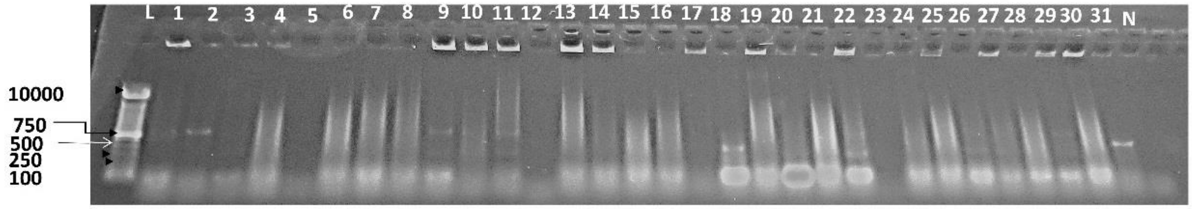
3.10. Ethical considerations

Prior to the conduct of this study, ethical approval was obtained from the Research Ethics Review Committee of College of Natural and Computational Sciences of Hawassa University (Ref. No. RERC/009/22). Support letters were also sought and secured from the Sidama Regional State Health Bureau for the respective hospitals, and permission was granted by the medical directors of each hospital to carry out the research. In addition to ethical clearance, verbal consent was sought from all diarrheic patients/care givers visiting the study hospitals on sample taking days prior to the sampling process and only volunteers were sampled.

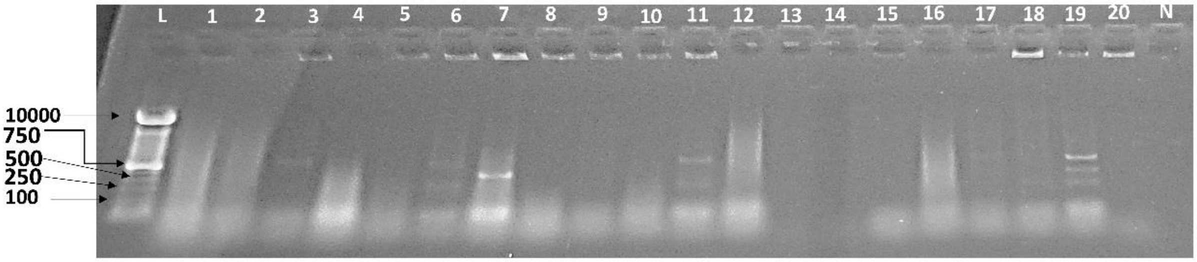
4. RESULTS

4.1 Occurrence of *Campylobacter* species in different samples

In this study, a total of 284 samples, including 184 animal products and 100 stool samples, were collected and analyzed for the presence of thermophilic *Campylobacter* species. Out of these, the PCR analysis result confirmed the presence of *Campylobacter* species in 17 of the samples, giving an overall sample-level prevalence of 6% (Figure 2). Among these 17 positive samples, 11 (64.7%) were from the tested animal products, and 6 (35.3%) were from human stool samples (Table 3). In this study, all types of the sampled animal products revealed contamination by thermophilic *Campylobacter* species. The PCR analysis of the positive isolates revealed that *Campylobacter lari* was isolated more frequently (35.3%), followed by unidentified thermophilic *Campylobacter* species (29.4%) and equally isolated *Campylobacter jejuni* (17.6) and *Campylobacter coli* (17.6). Among the animal-based food samples, chicken eggs had the highest contamination rate of 12.8% (n=6), followed by raw cow milk at 3.3% (n=3) and raw beef at 4.35% (n=2). However, the differences in the occurrence of *Campylobacter* among these various animal-based food samples were not statistically significant (P>0.05). District-wise analysis revealed that the highest contamination rate of 11.5% (n=6) was found in samples from Wondogenet, followed by Hawassa with a rate of 4.5% (n=4) and Dale with a rate of 2.3% (n=1). Nevertheless, these differences were also not statistically significant (P > 0.05).



L: DNA ladder (1kb); Lane 1,2 and 29 are positive only for genus level; Lane 9,11 and 18 (C. lari); Lane 22,26 and 27 (C. jejuni); Lane 31 (C. coli); N: Negative control



L: DNA ladder (1kb); L3 and 17 are positive for genus level only; L6,11 and 18 (*C. lari*); L7and19 (*C. coli*); N: Negative control

Figure 2: Representative gel image of multiplex PCR for *Campylobacter* identification and speciation. In the first raw: Lanes 1, 2, and 29 are positive only for genus level; Lanes 9, 11, and 18 are positive for *C. lari*; Lanes 22, 26, and 27 are positive for *C. jejuni*; Lane 31 is positive for *C. coli*. In the second raw, Lanes 3 and 17 are positive only for genus level; Lanes 6, 11, and 18 are positive for *C. lari*; Lanes 7 and 19 are positive for *C. coli*. In both rows, L = DNA ladder and N = negative control (sterile DNAse/RNAse free distilled water).

Table 3: Overall occurrence rate and species distribution of *Campylobacter* across the different sample types and districts

	Hawassa		Wondogenet		Dale		*Campylobacter* species			
Sample type	n	No (%) positive	n	No (%) positive	n	No (%) positive	*C. jejuni* (%)	*C. coli* (%)	*C. lari* (%)	Others (%)
Raw beef	20	1(5)	12	1(8.3)	14	0(0)	0(0)	1(50)	1(50)	0(0)
Eggshell swab	25	3(12)	13	2(15.4)	9	1(11)	1(16.7)	0(0)	2(33.3)	3(50)
Raw milk	43	0(0)	27	3(11)	21	0(0)	2(66.7)	0(0)	0(0)	1(33.3)
Human stool	40	2(5)	28	1(3.6)	32	3(9.4)	0(0)	2(33.3)	3(50)	1(16.7)
Total	128	6(4.7)	80	7(8.8)	76	4(5.3)	3(17.6)	3(17.6)	6(35.3)	5(29.4)

n = number of samples examined

In terms of *Campylobacter* contamination rates across different animal-derived food samples, the highest contamination rate (17.24%) was observed in chicken egg samples from poultry farms, followed by milk samples (3%) from dairy farms. In comparison, the lowest (0.0%) was recorded in beef samples from restaurants. However, no statistically significant differences ($p > 0.05$) were observed in the proportion of *Campylobacter* species positivity among the various food sample types and their sources (Table 4).

Table 4: Comparison of occurrence of *Campylobacter* among the different sample types/sources and districts

Sample type	Sample source	Districts						P value
		Hawassa		Wondogenet		Dale		
		No (%) examined	No (%) positive	No (%) examined	No (%) positive	No (%) examined	No (%) positive	
Raw beef	Butcher shop	3	0(0)	6	1(16.7)	3	0(0)	0.215
	Hotel	8	1(12.5)	3	0(0)	3	0(0)	
	Restaurant	9	0(0)	3	0(0)	8	0(0)	
Raw milk	Dairy Farm	36	0(0)	19	2(10.5)	12	0(0)	0.063
	Retailer shop	7	0(0)	8	1(12.5)	9	0(0)	
Eggshell swab	Poultry farm	16	3(18.75)	8	1(12.5)	5	1(20)	0.923
	Retailer shop	9	0(0)	5	1(20)	4	0(0)	

District based analysis of contamination rate in the foods of animal origin indicated that beef and chicken egg samples from Wondogenet district were more contaminated (8.3%, and 15.4%) than from Hawassa (5% and 12%) or Dale (0% and 11%) districts, respectively. On the other hand, *Campylobacter* species detection from raw cow milk samples was revealed only from samples of Wondogenet district (11%) and not from Hawassa (0%) or Dale (0%) districts, as illustrated in Table 5.

Table 5: Distribution of thermophilic *Campylobacter* in different food samples in three districts

Sample type	Sample district	Sample tested	Positive (%)	P-value
Raw beef	Hawassa	20	1(5)	0.729
	Dale	14	0(0)	
	Wondogenet	12	1(8.3)	
	Total	46	2(4.35)	
Eggshell swab	Hawassa	25	3(12)	1.000
	Dale	9	1(11)	
	Wondogenet	13	2(15.4)	
	Total	47	6(12.8)	
Raw milk	Hawassa	43	0(0)	0.035
	Dale	21	0(0)	
	Wondogenet	27	3(11)	
	Total	91	3(3.3)	
Total		184	11(6)	

In the hospital-based survey conducted in this study, there was no statistically significant difference (P>0.05) in the occurrence of *Campylobacter* among the hospitals. However, relatively the highest detection rate (50%) was recorded at Yirgalem General Hospital and the lowest (16.7%) at Wondogenet Primary Hospital. The isolation rate of *Campylobacter* was higher in female patients (66.7%) compared to males (33.3%), though this difference was not statistically significant (P>0.05). Similarly, a slightly higher occurrence rate was observed in patients from rural areas (40.0%) compared to those from urban areas (20.0%), but this difference was also not statistically significant (P>0.05). Additionally, no statistically significant differences (P>0.05) were found among different age groups or stool types concerning the occurrence of *Campylobacter*.

Table 6: Occurrence rate of *Campylobacter* species from different diarrheic patients visiting different hospitals

Characteristics	No examined	No (%) positive	P-value
District (Hospital)			
Adare general hospital	40	2(5)	
Yirgalem general hospital	32	3(9.4)	0.662
Wondogen primary hospital	28	1(3.6)	
Total	100	6(6)	
Sex of patient			
Female	53	4(7.5)	
Male	47	2(4.25)	0.681
Total	100	6(6)	
Place of residence			
Urban	44	2(4.55)	
Rural	56	4(7.14)	0.692
Total	100	6(6)	
Stool consistency			
Bloody	4	1(25)	
Mucoid	17	2(11.8)	
Semi fluid	65	3(4.4)	0.146
Watery	14	0(0)	
Total	100	6(6)	
Age group			
Early childhood (0-6yrs)	33	2(6.06)	
Late childhood (7-13 yrs)	13	1(7.7)	
Adolescent (14-20)	9	0(0)	1.000
Young adult (21-55 yrs)	38	3(7.9)	
Old adult (≥56yrs)	7	0(0)	
Total	100	6(6)	

5. DISCUSSION

Campylobacter species, among the leading zoonotic pathogens, are responsible for a significant number of gastroenteritis cases and associated diarrhea worldwide. The occurrence of *Campylobacter*-associated gastroenteritis has largely been attributed to the consumption of contaminated foods, particularly those of animal origin, and water. In the food industry, contamination by pathogenic *Campylobacter* species primarily occurs during production, processing, distribution, and/or preparation stages (WHO, 2018; Hlashwayo *et al*., 2020).

This study aimed at providing insight into the prevalence of thermophilic *Campylobacter* species in selected animal products and stool samples across three districts of Sidama region. The overall sample-level prevalence of thermophilic *Campylobacter* species was found to be 6%. Based on this study, it is evident that different *Campylobacter* species could be isolated in variant percentages from the examined samples. The observed recovery rate of *C. lari* isolates in more frequency over *C. jejuni* and *C. coli* isolates in this study agrees with previous study findings of El-Kholy *et al*. (2016) and Waldenstrom *et al.* (2002) from El-Minia and Beni-Suef sites of Egypt and South Eastern Sweden, respectively. This might be due to the selection of samples, specific isolation procedures, and type of culture media, as these factors can influence the diversity of *Campylobacter* species recovered from contaminated samples (Williams *et al*., 2012). It can also be explained by the differences in the mechanism of pathogenesis and elimination between the different thermophilic *Campylobacter* species within the host cells (Szczepanska*et al*., 2017). Moreover, the observed differences in the proportions of these isolates might also be related to the actual variations in the composition of common *Campylobacter* species in the study area and, hence, in the collected samples (Kashoma *et al*., 2016).

In this study, the unidentified other thermophilic *Campylobacter* species may comprise an infrequently isolated species (such as *C. concisus*, *C. upsaliensis*, and *C. ureolyticus)*, which have been reported mainly by studies involving cattle and chickens, and hence their products, which should be scrutinized to investigate their role of attribution and zoonotic potential (Williams *et al*., 2012; Vandamme *et al*., 2015).

Regarding the presence of *Campylobacter* species in different foods of animal origin, the study demonstrated a prevalence of 4.35% in raw beef. This result is roughly consistent with previous findings, such as those reported by Debelo *et al*. (2022), Baaboua *et al*. (2021), Dadi and Asrat (2008), and Berhanu *et al*. (2021), which indicated prevalences of 4.1%, 6.25%, 6.27%, and 7.9% from Jimma town, Northern of Morocco, Central Ethiopia, and Jimma, respectively. However, the prevalence found in the current study is lower than other reports, such as 9.4% in Addis Ababa (Faris, 2015), 9.5% in Tanzania (Kashoma *et al*., 2016), 11% in Northern Iran (Raeisi *et al*., 2017), 11.8% in Arba Minch (Tonjo *et al*., 2022), 11.9% in Mekelle (Hagos *et al*., 2021), 12.7% in Northern Poland (Andrzejewska *et al*., 2019) and 14% in Ahvaz, Iran (Maktabi *et al*., 2019). These discrepancies may be due to differences in study methodologies, sample sizes, culturing conditions, sampling protocols, and sample sources (Jonaidi-Jafari *et al*., 2016; Szczepanska, *et al*., 2017; Hlashwayo *et al*., 2020). Most of the mentioned research employed swabbing of carcass surfaces as a sampling methodology, which can increase the chance of taking the bacteria along with the swabs. In addition, in most of the studies, the samples were pre-enriched in Bolton broth, which has been shown to affect both the number and species of thermophilic campylobacters isolated from naturally contaminated samples (Williams *et al*., 2012).

In this study, the isolation frequency of *Campylobacter* from the tested raw beef samples varies insignificantly across the study districts and source of samples. These might be due to several variables, including sanitary conditions and slaughter practices, among others. Raw meat, particularly beef, is widely consumed in Ethiopia, and this habit can, therefore, increase the likelihood of pathogen transmission to humans (Woldemariam *et al*., 2009).

Contamination of raw meat, including beef, with *Campylobacter* species can occur during slaughtering and/or processing stages, often originating from animal feces and hides/skins that come into contact with the carcass. Furthermore, processed meat can also be contaminated by the environment, including contaminated handling areas and unhygienic practices during various processing stages (Datta *et al*., 2012; EFSA and ECDC, 2018).

Consumption of contaminated chicken' meat and other poultry products, such as eggs are evidenced to be responsible for an important percentage of human campylobacteriosis cases (Savita *et al*., 2018; Hlashwayo *et al*., 2020). Therefore, a better understanding of the role of chickens' eggs in the spread of *Campylobacter* has become necessary. Thus, one of the objectives of this study was to investigate the occurrence rate of thermophilic *Campylobacter* on eggshells to help with risk assessment of human infections caused by the consumption of undercooked eggs, food produced with raw eggs, or by handling of contaminated eggs. In this current study, the occurrence rate of *Campylobacter* species from chicken eggshell swab samples of different sources was 12.8%. This finding is higher than the previously documented reports from Northern Morocco (0% by Baaboua *et al*. (2021)), Central Kerala (6.67% by Savita *et al*. (2018)), Iran (7% by Jonaidi-Jafari *et al*. (2016)), and Northern Iran (8% by Sabzmeydani *et al*. (2020)). However, the current finding is lower than previous reports by Modirrousta *et al*. (2016) and Gharbi *et al*. (2022), who reported occurrence rates of 31.6% and 25.6% from Iran and North-East Tunisia, respectively. These variations may be attributed to differences in study methodologies, isolation methods, sample sources, and sample sizes (Jonaidi-Jafari *et al*., 2016; Baaboua *et al*., 2021). The relatively higher occurrence of the bacteria in egg samples than in other samples is consistent with the evidence that poultry are the documented main asymptomatic reservoirs for thermophilic Campylobacter species and, hence, the cause of eggshell contamination (WHO, 2018; Hlashwayo *et al*., 2020).

This study also revealed that *Campylobacter* species can be isolated from chicken eggshells regardless of study districts and sample source. This might probably be due to the comparable poor sanitary practices and management and housing conditions across the study districts, poultry farms (especially), and egg retailer shops.

Eggshell contamination with *Campylobacter* can occur either in the hen's reproductive tract or through contact with excreta, dust, litter materials, or unhygienic handling conditions after oviposition. Contaminated plastic trays and environmental contamination can also serve as potential sources of contamination. Contamination of egg contents during cracking and processing from these contaminated eggshells poses a risk for human *Campylobacter*iosis (Baron and Jan, 2010; Savita *et al*., 2018). Therefore, the present study highlights that eggs from chickens raised without proper sanitary measures can be a significant risk to consumer health.

Thermophilic *Campylobacter* species were also recovered from raw cow milk samples collected from different sources in the study area at a prevalence of 3.3%. This result aligns with previous estimates from India (2.91% by Modi *et al*. (2015)) and Sokoto, Nigeria (4.8% by Salihu *et al*. (2010)). On the contrary, this finding is slightly higher than the prevalence estimates of 1.64% and 2.5% from Northern Morocco and Isfahan, Iran, which were reported by Baaboua et al. (2021) and Kazemeini et al. (2011) in their respective order. However, the 3.3% prevalence observed in this study is lower than other reports, such as 8.7% in Northern Iran (Raeisi *et al*., 2017), 11.8% from Northern Poland (Andrzejewska *et al*., 2019), 13% from El-Minia and Beni-Suef of Egypt (El-Kholy *et al.*, 2016), 13.4% in Tanzania (Kashoma *et al*., 2016), 15.8% in Iraq (Almashhadany, 2021), and 18% and 22% in Egypt (Zeinhom *et al*., 2021; El-Zamkan *et al*., 2016). The observed variations in occurrence rates could be attributed to factors such as sampling methods, geographical locations, seasons, bacterial culture conditions, detection method sensitivity, hygiene levels, and the presence of natural reservoirs of *Campylobacter* (Kaakoush *et al*., 2015; Szczepanska, *et al*., 2017; Hlashwayo *et al*., 2020). Therefore, finding a direct correlation among various studies might be difficult.

In this study, the isolation rate of the bacteria is comparable among the districts (Hawassa, Wondogenet, and Dale) and sample sources (dairy farms and milk retailer shops). The possible explanation for the absence of significant variation among the districts and sources is supposed to be due to their comparable hygiene and management practices, housing conditions, milking, and milk handling practices.

Cow milk, which contains high-quality animal protein and fats as well as vitamins and minerals, is an important source of income and basic food in the human diet, either in its original form or in the various dairy products. This lacteal product can be contaminated with *Campylobacter* species due to several factors, including the health status of cows, production and milking environments, poor hygiene practices during milking and storage, insufficient cleaning of milking equipment, and lack of access to clean water (Andrzejewska *et al*., 2019; Thomas *et al*., 2020).

The consumption of contaminated milk is a potential source of human *Campylobacter*iosis, particularly in areas with limited awareness of the risks associated with consuming raw or contaminated milk. Owing to their natural health properties, organic and raw foods are becoming increasingly popular. However, there is also a lack of consumers' awareness regarding the risks associated with the consumption of these foods in their raw state, such as milk in unpasteurized form (Andrzejewska *et al*., 2019; Thomas *et al*., 2020).

In the current study, a hospital-based screening of human stool swab specimens for thermophilic *Campylobacter* species revealed a prevalence of 6%. This finding is consistent with previous studies conducted in Bahir Dar (Ewnetu and Mihret, 2010), Hawassa (Kebede *et al*., 2017), and Hawassa (Behailu *et al*., 2022), which reported prevalences of 8%, 6.04%, and 6.8%, respectively. However, the current prevalence estimate is lower than those reported in Addis Ababa (10.1% by Chala *et al*. (2021)), Jimma (11.6% by Beyene *et al*. (2004)), Hawassa (12.7% by Getamesay *et al*. (2014)), Gondar (15.4% by Lengerh *et al*. (2013)), Northern of Morocco (16.7% by Baaboua *et al*. (2021)), Jimma (16.7% by Tafa *et al*. (2014)), and Beni-Suef Governorate, Egypt (48% by Zeinhom *et al*. (2021)). These discrepancies might be due to factors such as the degree of contact with animals and animal products, socio-economic status, environmental sanitation, personal hygiene, food handling practices, sample collection and transportation, culture conditions, and laboratory methods employed (Tafa *et al*., 2014; Behailu *et al*., 2022).

Furthermore, while the precise cause of differences in prevalence is not clear, it can, however, arise as a result of several methodological variables, including differences in the number of samples and sampling type, sampling technique, sampling units, and laboratory methodologies employed (Szczepanska *et al*., 2017; Baaboua *et al*., 2021). Except for Chala *et al*. (2021) and Hagos *et al*. (2021), who used both culture and molecular techniques (PCR) for the isolation and identification of *Campylobacter* species, respectively like the current study, all of the previous reports in Ethiopia employed culture as the only isolation and identification method. Therefore, the employed methodological difference in different studies may result in variations in the prevalence. The varying prevalence observed in the selected government hospitals may reflect the levels of food, water, and environmental contamination in the communities since infection is usually transmitted by ingesting contaminated food and water or through contact with animal and human feces (Silva *et al*., 2011; Huang *et al*., 2015; WHO, 2018; Hlashwayo *et al*., 2020).

In this study, the distribution of *Campylobacter* infection between male and female patients was not statistically significant ($p > 0.05$), which is consistent with the findings of Beyene *et al*. (2004) in Jimma and Behailu *et al*. (2022) in Hawassa. The relatively lower infection rates among urban residents compared to rural residents contradict the findings of Behailu *et al*. (2022) in Hawassa. Regarding the age distribution of infection, children and older adults are more prone to *Campylobacter* infection. The relatively high prevalence observed in these age groups in this study could be attributed to their comparatively lower immunity (Fitzgerald, 2015; WHO, 2020).

5.1. Limitations of the study

The study was done only on limited foods of animal origin due to limited resources and hence the findings may not represent the true reflection of *Campylobacter* prevalence in the study area and in other food types. In addition, the research was initially proposed to include the antimicrobial susceptibility profile of isolated thermophilic *Campylobacter* species. However, because of resource and facility limitations, the organisms' antimicrobial susceptibility profile testing was not conducted.

6. CONCLUSIONS AND RECOMMENDATIONS

The present study showed the presence of *C. jejuni*, *C. coli*, *C. lari*, and other unidentified thermophilic *Campylobacter* species at different recovery rates in raw beef, egg, milk, and stool samples of diarrheic patients in the study area. In these findings, relatively higher prevalence was observed in chicken eggs. The detection of these species in food samples can suggest these animal products as important reservoirs and potential sources for foodborne risk of significant public health hazards. Furthermore, the prevalence of the bacteria in raw foods and human stool samples highlights the widespread nature of this pathogen across different sources in the study area. The findings emphasize the need for improved hygiene practices during the production, processing, and handling of animal products to minimize the risk of contamination. Additionally, the presence of *Campylobacter* in human stool samples underscores the importance of addressing food and water safety and personal hygiene to reduce the burden of campylobacteriosis. Generally, this study highlights the ongoing risk of infections with thermophilic *Campylobacter* species in the region, particularly among vulnerable populations such as children and the elderly. Furthermore, the prevalence of these bacteria in the study area has the implication of posing high economic burden due to loss of working hours and clinical testing costs in humans. In line with the above conclusions, the following recommendations can be forwarded:

- Public health interventions aimed at raising awareness, improving food safety, and enhancing hygiene practices are essential to mitigate pathogen's human health impact.
- Avoiding the consumption of raw or under cooked animal products, especially raw meat and milk, is warranted to prevent or at least minimize the risk of infections.
- Applying an integrated One Health approach is crucial to create multisectoral linkages to better understand and tackle the disease, and thus achieve optimal health outcomes.
- Further in-depth research is recommended to explore the specific factors contributing to *Campylobacter* prevalence and to devise targeted strategies to control its spread.

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8. ANNEXES

Annex i. Field sample data collection format for *Campylobacter* investigation in different food sample types

S. No	District	Sample type	Sample source	Sample code	Date of sampling
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					
19					
20					

Annex ii. Field sample data collection format for *Campylobacter* investigation in stool sample

Ser. No	Hospital	Sample code	Stool consistency	Sex	Age	Residence	Date of sampling
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
15							
16							
17							
18							
19							
20							
21							
22							
23							
24							
25							
26							
27							
28							
29							
30							

Annex iii. Laboratory test parameters format for identification of *Campylobacter* spp.

Serial No	Sample type	Sample source	Sample code	District	Greyish, shiny & mucoid colonies with a tendency to spread	Typical corkscrew or darting motility	Typical gram –ve rxn & shape	Catalase test	H_2S production on TSI agar
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
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16									
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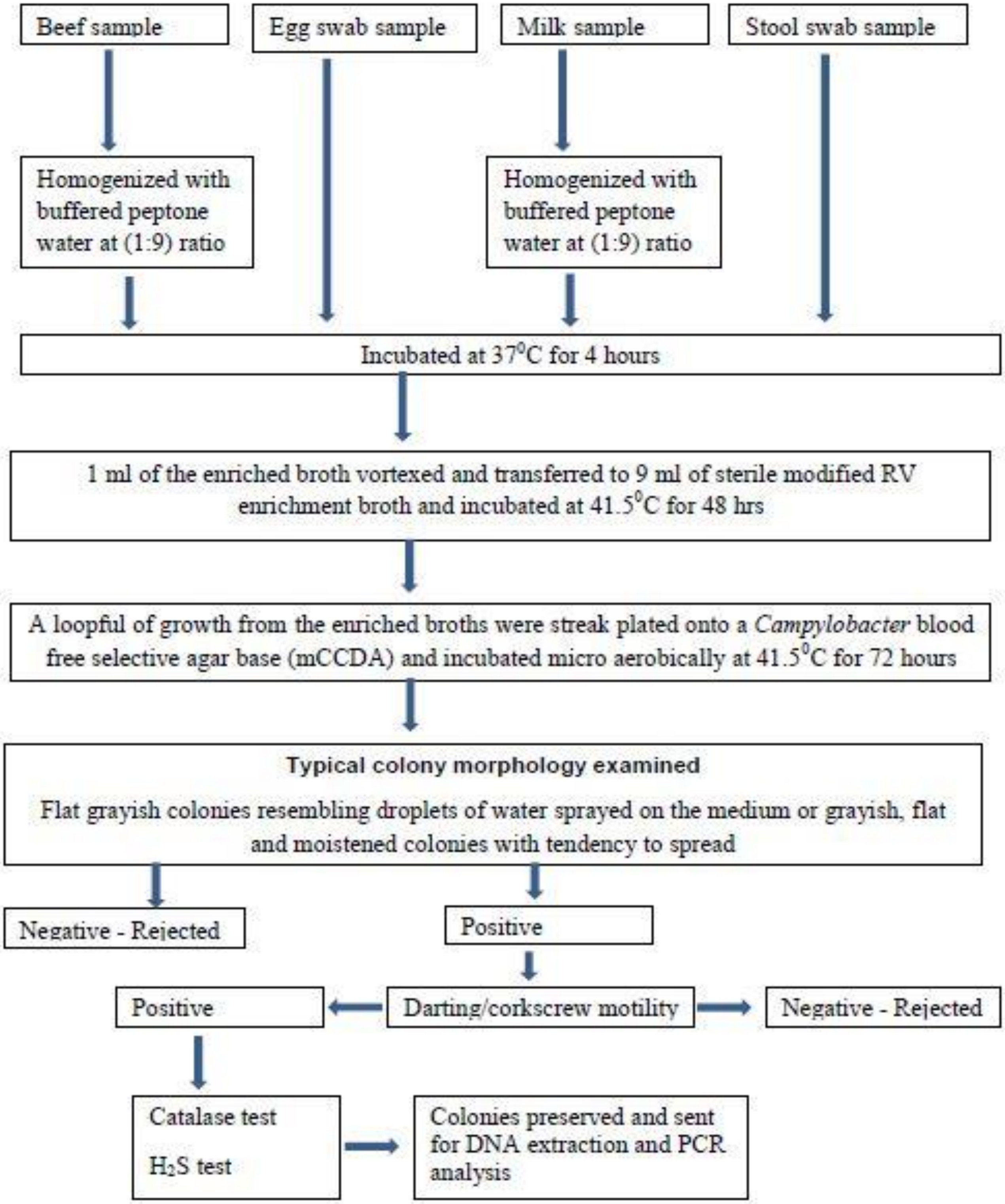
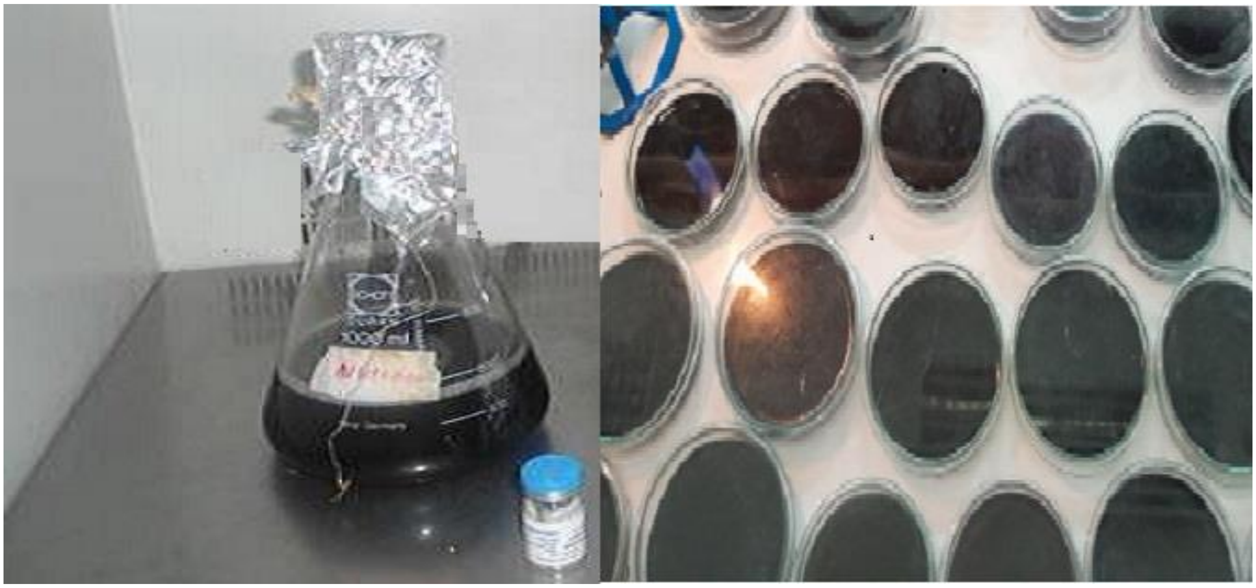
20									
21									
22									
23									
24									
25									
26									
27									
28									
29									
30									

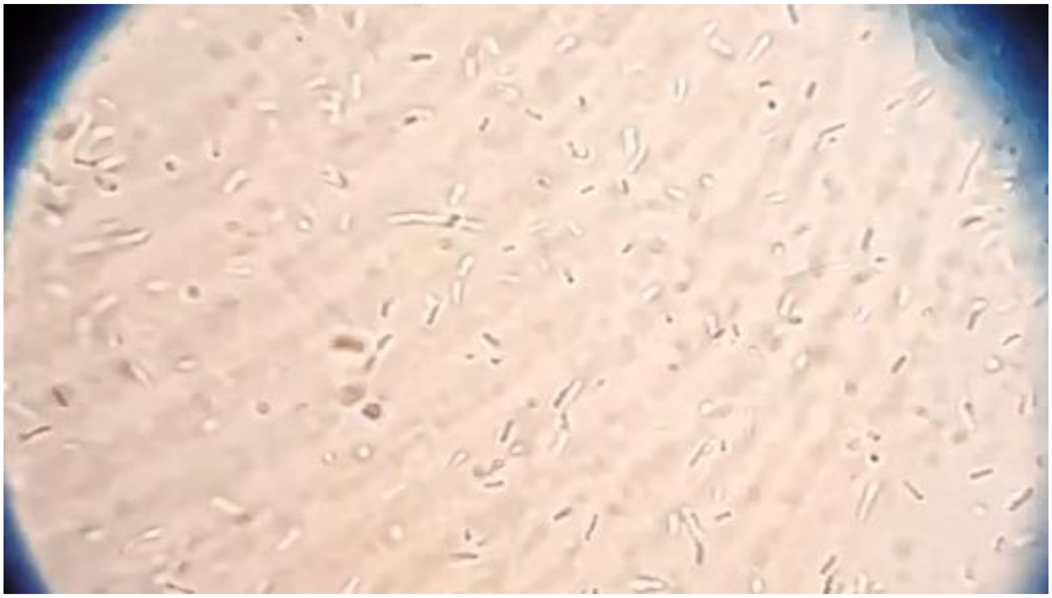
Annex iv. Flow chart showing the sequence of isolation, identification, and characterization of *Campylobacter* species



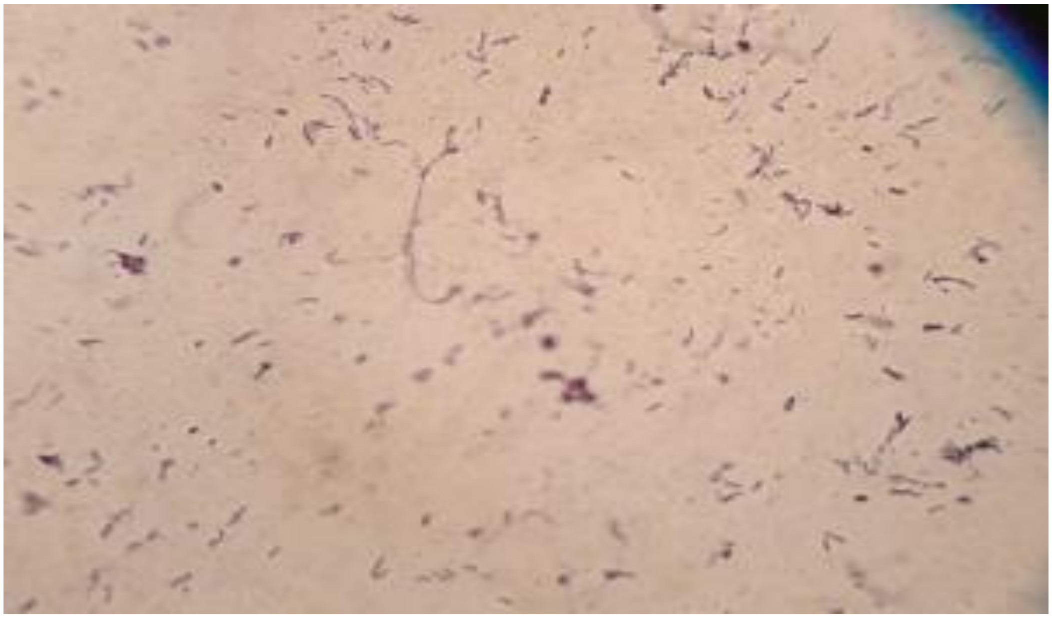
Annex v: Miscellaneous pictures taken during laboratory work of this study



1. mCCDA media preparation and dispensing for the isolation of *Campylobacter* species

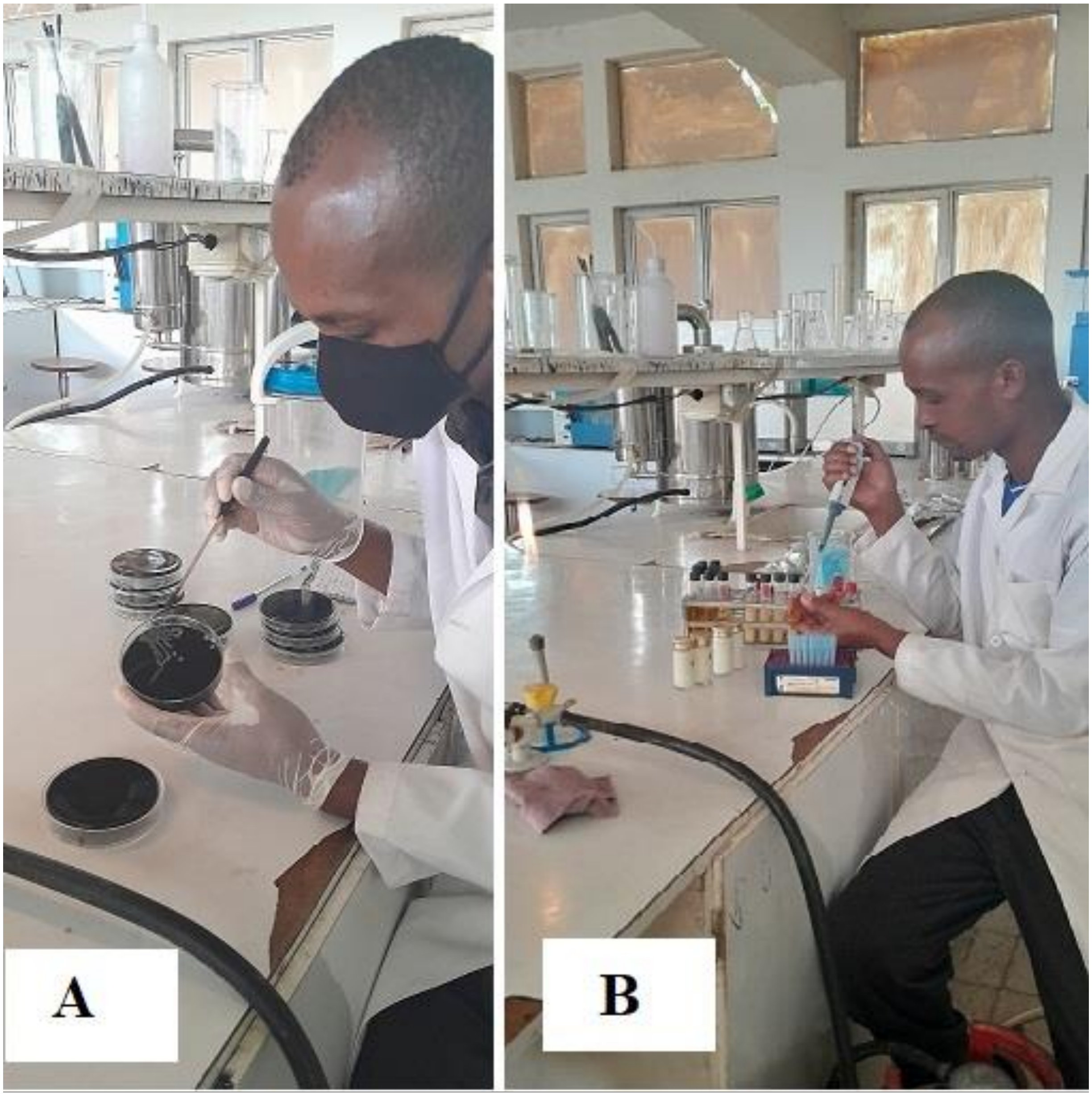


2. Microscopic view of wet mount showing motility of *Campylobacter* species



3. Gram stain of typical colony showing slender, curved or seagull wing appearance

4. Massaging of beef sample with buffered peptone water in a plastic bag



5. Colony characterization (A), milk sample inoculation (B)

Annex vi. Type, composition, and preparation of bacteriological media used for isolation and identification of *Campylobacter* species

Preparation of modified Charcoal Cefoperazone Deoxicolate Agar blood free medium (mCCDA)

1. Suspend 22.75g of *Campylobacter* blood-free selective agar base in 500 ml of distilled water and bring it to a boil
2. Sterilize the suspension by autoclaving at 121^0C for 15 minutes
3. Cool to 50^0C
4. Aseptically add 2ml sterile distilled water to one vial of mCCDA selective supplement (SR0155E, Oxoid) and mix gently to dissolve
5. Aseptically add the vial content to the 500ml *Campylobacter* blood-free selective agar base to it inhibit the growth of bacteria except *Campylobacter*.
6. Mix well and pour into sterile Petri-dishes

Composition(g/L): meat extract 10; enzymatic digest of animal tissues 10; enzymatic digest of casein 3.0; charcoal 4.0; sodium chloride 5; sodium deoxycholate 1; sodium pyruvate 0.25; iron (II) sulfate 0.25; agar 8-18; pH at 25 °C 7.4 ± 0.2.

Composition of mCCDA selective supplement (mg/L): cefoperazone 16mg/L; amphotericin B 5; PH at 25 °C 7.4±0.2.

Preparation of buffered peptone water

1. Suspend 20grams of the medium in 1000ml of distilled water
2. Bring to boil to dissolve the medium completely
3. Mix well and dispense as desired in to tubes
4. Sterilize by autoclaving at 121^0C for 15 minutes

Composition (g/L): enzymatic digest of casein 10; sodium chloride 5.0; disodium phosphate dodecahydrate 9.0; potassium dihydrogen phosphate 9.0; PH at 250C 7.0±02.

Preparation of Modified Rappaport Vassiliadis enrichment broth

1. Suspend 27.11grams of hydrated medium in 1000ml of distilled water
2. Heat to boil to dissolve the medium completely

3. Dispense as desired in to tubes
4. Sterilize by autoclaving at 121^0C for 15 minutes

Composition (g/L): soya peptone 4.5; sodium chloride 8.0; potassium dihydrogen phosphate 0.6; dipotassium phosphate 0.4; magnesium chloride hexahydrate 29; malachite green 0.036; PH at 250C 5.2±0.2.

Preparation of Triple sugar iron (TSI) agar

1. Suspend 65grams of the medium in 1000ml of distilled water
2. Bring to boil to dissolve the medium completely
3. Mix well and dispense as desired in to tubes
4. Sterilize by autoclaving at 121^0C for 15 minutes
5. Allow set as a slope with 2.5 cm butts

Composition (g/L): meat extract 3.0; yeast extract 3.0; peptone 20; sodium chloride 5.0; lactose 10; sucrose 10; glucose 1.0 ferric citrate 0.3; sodium thiosulfate; phenol red 0.024; agar 12; PH at 25^0C 7.4±0.2.

Preparation of Brain Heart Infusion (BHI) broth

1. Suspend 37gms of the medium in one liter of distilled water
2. Mix well and dissolve by heating with frequent agitation
3. Boil for one minute until complete dissolution
4. Dispense into screw-caped tubes and sterilize by autoclaving at 121 0c for 15 minutes

Composition (g/L): gelatin peptone 10; beef heart infusion 10; calf brain heart infusion 7.5; Sodium chloride 5.0; disodium phosphate 2.5; dextrose 2; PH at 25 ^{0}C 7.4±0.

Gram's staining

i. Crystal violet

Solution A

Crystal Violet 2.0gm

95% ethyl alcohol 20.0ml

Solution B

Ammonium oxalate 0.8gm

Distilled water 30.0ml

Crystal violet was dissolved in ethyl alcohol and ammonium oxalate in distilled water. Then solution A and B are mixed.

ii. Gram's Iodine

Iodine 1.0gm

Potassium iodide 2.0ml

Distilled water 300.0ml

Iodine and potassium iodide were dissolved in distilled water.

iii. Ethyl Alcohol (95%)

Absolute alcohol 95.0ml

Distilled water 5.0ml

iv. Safranin

Safranin 10.0ml

(2.5 % solution in 95% ethyl alcohol)

Distilled water 100.0ml

Procedure:

1. Heat fixed smear of bacterial culture was flooded with crystal violet for one minute and excess stain was washed out.
2. The slide was treated with Gram's Iodine for 1 minute and washed.
3. It was flooded with decolorize alcohol and immediately washed with water.
4. Then smear was treated with safranin for 1 minute and washed with water.
5. It was dried and observed under microscope.

Catalase Test

Catalase test is done to test the presence of enzyme catalase. The enzyme catalase splits hydrogen peroxide to water and oxygen.

Reagents: (3 % Hydrogen peroxides).

Composition: Concentration hydrogen peroxide 3ml**;** Distilled water 97ml

Procedure

1. Apply suspected colonies on glass slide
2. Add a drop of 3% H_2O_2 on to the suspected colonies on the glass slide
3. Look for the formation of gas bubbles as an indication of positive result

Annex vii. Biochemical tests for the detection of *Campylobacter* species

Characteristic	*C. jejuni*	*C. jejuni subsp. doylei*	*C. coli*	*C. lari*	C. fetus subsp. fetus	*C. hyo-intestinalis*	*"C. upsaliensis*"(b)
Growth at 25°C	-	+	-	-	+	D	-o
Growth at 35-37°C	+	+	+	+	+	+	+
Growth at 42°C	+	+	+	+	D	+	+
Nitrate reduction	+	-	+	+	-	+	+
3.5% NaCl	-	-	-	-	+	-	-
H2S, lead acetate strip	+	+	+	+	-	+	+
H2S, TSI	-	-	D	-	+	+(c)	-
Catalase	+	+	+	+	+	+	-
Oxidase	+	+	+	+	+	+	+
MacConkey's agar	+	+	+	+	+	+	-
Motility (wet mount)	+	+	+	+	+	+	+
Growth in 1% glycine	+	+	+	+	-	+	+
Glucose utilization	-	-	-	-	-	-	-
Hippurate hydrolysis	+	+	-	-	R	-	-
Resistance to nalidixic acid	S(d)	S	S	R	S(e)	R	S
Resistance to cephalothin	R	R	R	R		S	S

a Symbols: +, 90% or more of strains are positive; -, 90% or more of strains are negative; D, 11-89% of strains are positive; R, resistant; S, susceptible.

b Proposed species name.

c Small amount of H2S on fresh (<3 days) TSI slants.

d Nalidixic acid-resistant *C. jejuni* have been reported.

e Cephalothin-resistant C. fetus subsp. *fetu*s strains have been reported.

Source: Barret *et al.* (1988).

Annex viii. General procedures and protocols followed for PCR analysis DNA extraction

DNA Extraction

Genomic DNA from presumptive isolates was extracted using the boiling method from fresh cultures grown on modified Charcoal Cefoperazine Deoxycholate agar for 72 hours, as previously described (Jokinen *et al*., 2012; Kashoma *et al*., 2016). In brief, a loopful of suspected *Campylobacter* species isolated from a test culture was suspended in 100 µl of sterilized DNase and RNase-free water.

DNA Amplification

To obtain a final volume of 25 µl of PCR product for gel electrophoresis, the following steps were undertaken:

I) Preparation of the Premix (Primer Mixture): A volume of 0.6 µl of each forward and reverse primer specific to *C. coli, C. jejuni, C. fetus, C. lari*, and the genus-specific primers was used for a single DNA lysate (one sample). The primer mixture was prepared based on the number of samples to be amplified, ensuring the final 25 µl volume per sample. All forward and reverse primers used in this study are listed in Table 1 above.

II) Preparation of the PCR Reaction Mixture: Each PCR tube received the master mix (containing bases, cofactors, and polymerase), the prepared premix, 1 µl of template DNA, and DNase/RNase-free water to reach a final volume of 25 µl per tube.

The DNA amplification was conducted using a CCC thermocycler with cycling conditions as outlined by Yamazaki-Matsune et al. (2007), with slight modifications:

Initial denaturation at 95°C for 15 minutes

30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 90 seconds, and extension at 72°C for 1 minute

Final extension at 72°C for 7 minutes

Samples were then held at 4°C until analysis.

Gel Preparation and Electrophoresis

Each reaction mixture was analyzed by gel electrophoresis using a 1.8% (w/v) agarose gel in 1x TBE buffer for 1 hour and 30 minutes and visualized by UV transillumination after staining with ethidium bromide (0.5 µg/ml). DNA bands were photographed using a UV transilluminator (BTS-20), with a 1 kb DNA ladder serving as a molecular size marker.

1x TBE Buffer Preparation

Dissolve 10.8 g of Tris-base and 5.5 g of boric acid in 805 ml of distilled water.

Add 4 ml of 0.5M EDTA (pH 8.0) to the above mixture.

Adjust the volume to 1L with distilled water.

Preparation and Loading of a 1.8% Agarose Gel

Add 4.5 g of agarose to a 500 ml Pyrex flask containing 250 ml of 1x TBE buffer.

Heat in a microwave until the agarose is fully dissolved.

Add 10 µl of ethidium bromide to the dissolved agarose at 45°C, gently swirling to avoid air bubbles.

Pour the solution onto a 25 cm gel casting plate and allow it to solidify for about 40 minutes.

Place the solidified gel cast into the gel box after removing the combs and overlay with 1x TBE buffer until fully submerged.

Load the gel with the amplified PCR product, along with loading dye and a 1 kb DNA ladder and run the electrophoresis for 1.5 hours.