Thesis Ref. No. _____

A STUDY ON PREVALENCE, PUBLIC HEALTH SIGNIFICANCE AND ASSSOCIATED RISK FACTORS OF *BACILLUS CEREUS* ON BOVINE RAW MILK IN AND AROUND ASSOSA DISTRICT, IN HOUSEHOLD DAIRY FARMS OF THE BENISHANGUL GUMUZ REGIONAL STATE,WESTREN ETHIOPIA

MSC THESIS

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ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

DEPARTMENT OF MICROBIOLOGY, IMMIUNOLOGY AND PUBLIC HEALTH

MSc PROGRAM IN VETERINARY PUBLIC HEALTH

JUNE, 2014

BISHOFTU, ETHIOPIA

A STUDY ON PREVALENCE, PUBLIC HEALTH SIGNIFICANCE AND THE ASSSOCIATED RISK FACTORS OF *BACCILUS CEREUS* ON BOVINE RAW MILK IN AND AROUND ASSOSA DISTRICT, IN SMALL HOUSEHOLD DAIRY FARMS OF THE BENISHANGUL GUMUZ REGIONAL STATE, WESTERN ETHIOPIA

A Thesis submitted to School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Public Health

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Approval Sheet of Thesis

As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by **Shibabaw Bejano** entitled **A study on prevalence, public health significance and the asssociated risk factors of *Bacillus cereus* on bovine raw milk in and around Assosa district, of small household dairy farms of the Benishangul Gumuz regional state,Western Ethiopia.** And recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Veterinary public health.

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DEDICATION

This thesis/dissertation manuscript is dedicated to my wife, W/o Mesert Mamo, and to all my family and friends those who are behind my success.

STATEMENT OF AUTHOR

First, I declare that this thesis is my *original* work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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ACKNOWLEDGEMENTS

I am glad to thank my advisors, Dr. Aklilu Feleke and Dr. Tesfaye Sisay for their time devotion in correcting this manuscript, forwarding constructive comments and suggestion on the editorial and scientific aspect of this manuscript paper and supportive encouragement throughout the research.

I would like to acknowledge my host institution Assosa Agricultural, Technical and Vocational Training College and thematic research on Mastitis, Diarrhea and Pneumonia project of Addis Ababa University for sponsoring me to attend this postgraduate study program and providing me Chemical reagents ,fuel and vehicle.

I would like to thank Assosa Regional Veterinary Laboratory, Agriculture and Animal Health Departments of the district and the Peasant Associations for their valuable support on sample collection and the whole research activities conducted at Assosa.

My special appreciation also goes to Dr. Teshale Sori and Dr.Ashebafi Fiysa who had advised me on statistical analysis to the thesis.

My special heartfelt gratitude goes for all my family members, my close friends Dr.Samuel Aytenfsu, Dr.Yared Dendana and Mr.Teshome Kifle for their moral and technical support.

Lastly, I offer my sincere thanks for all who are on behind of my success.

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LIST OF ABBREIVATIONS

ATP	Adenosine Triphosphate
ATVET	Agricultural Technical and Vocational Education
BACARA	Bacillus cereus Rapid Agar
CFU	Colony Forming Units
CMT	California Mastitis Test
DPA	Dipicolinic Acid
DNA	Deoxyribonucleic Acid
HACCP	Hazard Analysis Critical Control Point
HBL	Haemolysin BL
IU	International *Unit*
MYP	Mannitol-Eggyolk- polymyxine
MPN	most probable number
NAD+	Nicotinamide Adenine Dinucleotide
NHE	Non-Hemolytic Enterotoxin
PAS	Peasant Associations
RNA	Ribonucleic Acid
Spp	Species
SPC	Standard Plate Count
UK	United Kingdom
VP	Voges Prorkauer

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A STUDY ON PREVALENCE, PUBLIC HEALTH SIGNIFICANCE AND THE ASSSOCIATED RISK FACTORS OF *BACCILUS CEREUS* ON BOVINE RAW MILK IN AND AROUND ASSOSA DISTRICT, IN SMALL HOUSEHOLD DAIRY FARMS OF THE BENISHANGUL GUMUZ REGIONAL STATE,ETHIOPIA

ABSTRACT

A cross-sectional study was conducted on 384 lactating dairy cow to assess the prevalence of Bacillus cereus, with pathogenic bacteria load in raw milk. The associated risk factors, public health significance and source of contamination were also assessed at Assosa district, small household dairy farms from September 2013 to June 2014. The overall prevalence of B. cereus in raw milk sample was 22.4% (86). The B. cereus count ranges from $1.09x10^3$- $1.10x10^7$CFU/ml, 65/86 (75.6%) of total positive samples have significant counts (> 10^5 CFU/ml) which were above legal limit in raw milk intended for human consumption. The positivity of bacillus was significantly associated with hygienic practices, CMT test and lactation length ($p < 0.05$). As the result indicated that poor hygienic conditions and mid lactation stage and mid parity were highly contaminated with B. cereus. An attempt was made to assess public health implication and source of raw milk contamination by using semi structured questionnaire survey. Furthermore, antibiotic susceptibility testing of the isolates showed that infections with Bacillus cereus may not be cured by treatment regimes polymixin B, tetracycline,ampicilin ,penicillin G and kanamycin; the isolates were found to be susceptible to clindamycin, chloramphenicol and vancomycin. In conclusion, our study results indicated that raw milk samples were highly contaminated with Bacillus cereus, exceeding the legal limit set for raw milk (>10^5 $_{CFU/ml}$), suggesting the need for effective hygienic measures to be introduced in milk value chains during milk production, distribution and processing and food service establishments to avoid public health hazards and economic losses from milk spoilage.

Key words: *Antibiotic susceptibility, Assosa, Bacillus cereus, Bacterial count, CMT test, District,Prevalence, Public health, Raw milk, Small house hold*

1. INTRODUCTION

In many parts of the world in general and Ethiopia in particular milk contributes significant nutritive value which ensures benefits from its consumption, especially the rural areas where milk represent a good source of protein, calcium and vitamin D that stimulate the growth and body functions (Teka, 1997; Hoppe *et al.,* 2006) and the wholesomeness of human diets especially during childhood. Milk is considered an attractive source of energy, proteins and calcium for infants and young children who have few alternative sources for these nutrients. Besides its beneficial effects on nutrition, milk is an excellent culture and protective medium for certain microorganisms, particularly bacterial pathogens, whose multiplication depends mainly on temperature (Grassman and Barries, 2010).

Pathogenic organisms in milk can be derived from the cow itself, human handlers and from the environment such as soil, litter, feed, water, feces and other items in a farm environment commonly contaminate the surface of the udder, teats, hair and skins of cows. From these sources they can get into the milk during unhygienic milking procedures. Milk residues left on equipment and utensil surfaces provide nutrients to support the growth of many microorganisms including pathogenic bacteria (Grassman and Barries, 2010). Use of non potable water may also cause entry of pathogens into milk. It is known that tropical conditions which have a hot, humid climate for much of the year are ideal for quick milk deterioration so pose particular problems because the temperature is ideal for growth and multiplication of many bacteria (Godefay and Molla, 2000).

The distribution of *Bacillus cereus* is worldwide (Logan *et al*., 2006). *B. cereus food poisoning* are commonly found in places where there is improper food handling and it is known for causing 25 % of food-borne intoxications due to its secretion of emetic toxins and enterotoxins (FDA, 2007). *Bacillus cereus* is one of the bacterial spp causing milk-borne zoonosis and food poisoning in humans. The Genus *Bacillus* was established in 1872 with *B. subtilis* as type species. *B. cereus* was added fifteen years later by Frankland (Maarten, 2009). Several accounts of food poisoning attributed to members of the genus *Bacillus* appeared in the European literature before 1950. During this period, a number of papers also described the isolation of *Bacillus*

species other than *B. anthracis* from a variety of non-gastrointestinal infestations. Since the early 1950's, there have been an increasing number of well-documented reports substantiating the role of *B. cereus* as a food poisoning organism (Kramer *et al*., 1982).

Contamination of dairy products with *B. cereus* occurs initially at farm level where the incidence of *B. cereus* in raw milk is almost inevitable due to the organism's abundance in soil and feed (Griffiths and Phillips, 1990; Saran, 1995; Slaghuis *et al*., 1997). Incidence, count in milk and transmissions of *B. cereus* are dependent on housing, daily management and hygienic practices and climatic conditions (Connell, 2013). When cows were housed, the incidence of *B. cereus* is lower in raw milk as compared with cows outside (Slaghuis *et al.*,1997;Stenfors *et al.,* 2008).

Bacillus cereus is a gram-positive, an endemic, soil-dwelling, rod-shaped, beta-hemolytic, spore-forming, motile bacterium that is widely distributed in the environment and undoubtedly the most important aerobic pathogen found in milk (Christiansson, 2011).

The presence of spores in raw milk, their high resistance to pasteurization temperature (Janstova and Lukasova, 2001), and also the adherence of spores and vegetative cells to metal surfaces and dairy equipment used in dairy plants (Ronner and Husmark, 1992; Faille *et al.,* 2001) make *B. cereus* an unwelcome but very frequent contaminant of dairy products (Huang *et al.*, 1999).

Another important trait of *Bacillus cereus* is their ability to grow at the storage temperature of milk (4 – 7 ^{o}c), which mainly determines the shelf life of pasteurized milk and derived milk products (Te Giffel *et al.,* 1995).The unique nature of *Bacillus cereus* like heat resistance, endospore forming abilities, toxin production and psychrotrophic feature (ability to growing below 7 ^{0}C) gives ample scope for this organism to be a prime cause of public health hazard (Griffiths and Schraft, 2002).

Another issue regarding *Bacillus cereus* in milk is the possible production of toxin. It is a well known food pathogen that can cause two types of food poisoning syndromes: an emetic (vomiting) intoxication due to the ingestion of a toxin (cereulide) pre-formed in the food and a diarrheal infection due to the ingestion of bacterial cells or spores (enterotoxins) in the small

intestine (Dierick *et al.*, 2005, e.g., post-traumatic wound infections, ophthalmitis, septicaemia, bovine mastitis and bovine abortion (Tumbuii and Krarner,1991; Melling ***et*** *al*., 1976).

In addition to its familiar role in food-borne infections and intoxications *Bacillus cereus* is an emerging human food-borne pathogen and has long been known to be capable of causing opportunistic infections in individuals recovering from surgery and in immunecompromised individuals (Turnbull *et al.*, 1979; Kamar *et al*., 2013). It has, however, not been regarded as a true infective pathogen of healthy individuals and the identification of *B. cereus* in the clinical laboratory is usually dismissed as either insignificant or as contamination (Miller *et al.*, 1997). Nevertheless, several later reports have documented fatal and life-threatening *B. cereus* infections in apparently healthy individuals (Miller *et al*., 1997; Hoffmaster *et al*., 2004; Avashia *et al.*, 2007) and sudden deaths of eight healthy wild chimpanzees in Cote d'Ivoire, and three chimpanzees and one gorilla in Cameroon were reported (Leendertz *et al*., 2004; Leendertz *et al*., 2010).

Different researches have suggested Gram-positive spore-forming microorganisms such as *B. cereus* are the predominant microorganisms in raw and pasteurized milk during refrigerated storage. Even though the dairy industry is often confronted with severe implications caused by *Bacillus cereus*, there is no previous research has been done in Ethiopia on the prevalence of *B. cereus* in raw milk at small household farmer level, except the works done in Debre Zeit and Alage Agricultural Technical and Vocational Education dairy farm, Ethiopia, by Alemneh (2012) and Seblewengel (2013), respectively.

Therefore, this study was conducted with the following objectives:

- To determine prevalence and bacterial load of *Bacillus cereus* in raw bovine milk samples in and around Assosa, Benishangul Gumuz Regional State.
- To identify the associated risk factors that favor *Bacillus cereus* occurrence in raw bovine milk under small household production system in the study area.
- To assess the public health implication and source of raw milk contamination of *Bacillus cereus* through questionnaire survey in the study area.

2. LITERATURE REVIEW

2.1. Bacillus Cereus

2.1.1. Taxonomy and classification of Bacillus cereus

The genus Bacillus is Gram positive bacteria Classified under Kingdom Bacteria, Phylum Firmicutes, Class Bacilli, Order Bacillales, Family Bacillaceae, Genus Bacillus, and Species Group: *Bacillus cereus* group. The word bacillus means large rod, and *cereus* can be translated from Latin to mean wax-like. The name reflects the easily recognizable morphology of *Bacillus cereus* when viewed in the microscope or on blood agar plates (Helgason *et al*., 2004; Ciccarelli *et al*., 2006; Wu *et al*., 2009).

The *Bacillus cereus* group is a very homogenous cluster within the genus *Bacillus* and comprises six recognized species: *B. cereus, B. thuringiensis, B. anthracis, B. mycoides, B pseudomycoides and B. weihenstephanensis*. and among these the first four species are closely related based on their morphology (Jensen *et al*., 200; Priest *et al*., 2004). Of these, *B. cereus* and *B. anthracis* have received considerable attention due to their clinical importance, but the former is implicated with food borne disease (Guinebretière and Sanchis, 2003).

Table 1: Criteria to differentiate among four closely related *Bacillus* species

Species	**Colony**	**motility**	**haemolysis**	**Susceptibility Penicillin**	**Parasporal Body**	**Virulent to Mice**
Bacillus cereus	turquoise to peacock blue	Yes	Yes	No	No	No
B. anthracis	White	No	No	Yes	No	Yes
B. mycoides	Rhizoid	No	No	No	No	No
B. thuringiensis	White/ Grey	Yes	Yes	No	Yes	No

Source: (Guinebretière and Sanchis, 2003)

These species can be distinguished by phenotypic and genotypic features, *Bacillus cereus* is currently considered as a food borne disease agent, *Bacillus thuringiensis* produces a parasporal crystal toxic against insects and is used as a bio-pesticide, *Bacillus anthracis* is the agent of anthrax, and *Bacillus weihenstephanensis* is able to grow at 4-5 C° (Helgason *et al.*, 2004). *Bacillus* species can also be broadly divided into three groups based on the morphology of the spores and their sporangium (HPA, 2007).

According to (HPA, 2007) those clustered as group 1 consists Gram positive, possessing licithinase system, producing central/terminal ellipsoidal/cylindrical spores without distend sporangium are (*Bacillus cereus, Bacillus anthracis, Bacillus megaterium, Bacillus mycoides* and *Bacillus thuringiensis*); and clusters in group 2 consists Gram variable, possess ellipsoidal spores with swollen sporangia and cluster group 3 consists Gram-variable, with bulged sporangia having terminal/sub-terminal spherical spores (HPA, 2007).

2.1.2 .Characteristics of Bacillus cereus

Bacillus cereus is a large, Gram-positive, rod-shaped, endospore forming, facultative aerobic bacterium (Vilain *et al*.,2006) with growth temperature ranging from 10 - 48 ^{0}C with optimal growth between 28 ^{0}C and 35 ^{0}C, pH values of 4.9 to 9.3 and water activities of 0.92 to 1.0 (OSPBH, 2005). The organism does not ferment mannitol and has a very active phospholipase and lecithinase enzymes (Nieminen *et al.,* 2007). *Bacillus cereus* forms ellipsoidal spores in a central or terminal position without swelling the sporangium (Stenfors *et al.,* 2008)

Bacillus cereus can grows on common blood agar media to large colonies (2–5mm diameter) with a rather flat, greyish and 'ground-glass' appearance, often with irregular borders. Most strains will form endospores within a few days on commonly used agar media (helgason *et al.,* 2004).

Bacillus cereus is motile by means of flagella and exhibits two types of motility including swimming and swarming, depending on the environment. Single cells exhibit swimming motility by means of short flagellated rods (Senesi *et al.,* 2002). On the other hand, swarming is a collective movement of swarm cells with flagellum that is observed to be three to four times longer, and also forty times more flagellated than single swimming cells (Senesi *et al.,* 2002).

In the microbiological laboratory, Species differentiation from other closely related Bacillus species, such as *Bacillus anthracis*, *Bacillus thuringiensis*, and *Bacillus mycoides*, is based on phenotypic differences and genetic sequencing results (Sankararaman and Velayuthan, 2013).

Members of the *B. cereus* group exclusive of *B. anthracis* display a range of morphological forms depending upon the milieu in which they are observed. In Gram-stained smears prepared from agar growth will show more uniform bacillary morphology with, centrally situated spores, which do not distort the bacillary form (senesi *et al.*,2002)

When grown under aerobic conditions on sheep blood agar at 37°C, *B. cereus* colonies are dull gray and opaque with a rough matted surface. Colony perimeters are irregular and represent the configuration of swarming from the site of initial inoculation, perhaps due to *B. cereus* swarming motility (senesi *et al*.,2002; Edward,2010). Zones of beta-hemolysis surround and conform to the colony morphology (Turbull *et al*., 1990).

2.1.3. Sporulation and germination of B. cereus

B. cereus is a spore-forming bacteriurn. Spores are formed intra-cellularly in round, oval and occasionally cylindrical shapes at the end of exponential cell growth or when vegetative cells are transferred from a rich to a poor medium. The spores are designated as endospores. Sporulation is a multi-phasic, ordered, sequential process of spore formation, which results in the changes of cellular components such as DNA, membranes, peptidoglycan and dipicolinic acid (DPA). Therefore, spore structure is the important part for the survival of this bacterium. The spore's coat is made of proteins, small amounts of lipids and carbohydrates which contribute great resistance to oxidizing agents and chemicals by blocking toxic molecules and its outer structure helps for heat and γ-radiation (Pol *et al.,* 2001). The highly resistant *Bacillus cereus* spores can survive heating, drying, radiation, freezing, and pasteurization (Kotiranta *et al.,* 2000)

Under suitable conditions, germination of *B. cereus* spores may take place within-minutes. Germination is a process by which spores are activated into growing vegetative cells. The process usually involves several phases, i.e., activation, triggering, initiation and outgrowth (Bergère, 1992). Activation means a treatrnent responsible for breaking dormancy and heat treatment at 70 - 80°C for 10 - 30 min is normally employed. The triggering of germination involves the interaction of a specific compound, called germinant, within the spore that irreversibly comrnits the spore to lose its dormant properties. The initiation stage of germination involves metabolic

reactions that occur as a result of the triggering reaction. Outgrowth is defined as the development of the new vegetative ce11 from the germinated spores and comprises synthesis of new macromolecules (RNA, proteins and DNA). The structural, physiological and biochemical changes of spores during germination mainly include the release of DPA and calcium, loss of refractivity or phase darkening, onset of stain ability, increase in permeability and loss of heat resistance (Claus and Berkeley, 1986; Bergère and Cerf, 1992).(Fig.1).

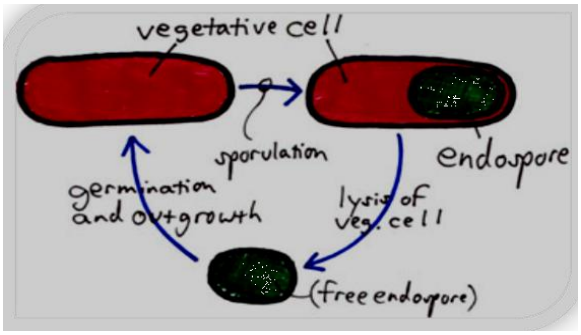

Figure 1: *Bacillus cereus* spore cycle

Source*: (*Kotiranta *et al.,* 2000)

2.1.4. Metabolism of Bacillus cereus

Bacillus cereus is a facultative aerobe so it can utilize oxygen as a terminal electron accepter, but also has methods of anaerobic respiration as a mechanism of energy release (Duport *et al.,* 2006). In aerobic respiration, reducing equivalents produced from glycolysis and the Krebs cycle is reoxidized by the electron transport chain, creating a proton motive force and ATP by ATP synthase (Duport *et al.,* 2006). In anaerobic respiration, *Bacillus cereus* utilizes fermentation to generate energy. Fermentation recycles NAD+ by reducing pyruvate and produces lactate and ethanol (Duport *et al.,* 2006).

Bacillus cereus can metabolize a variety of compounds including carbohydrates, proteins, peptides and amino acids for growth and energy. Some of the major products produced from carbon sources such as sucrose or glucose during anaerobic respiration include L-lactate, acetate, formate, succinate, ethanol, and carbon dioxide (Mols *et al.*, 2007). During nitrate respiration, nitrate reductase converts nitrate into nitrite which is converted to ammonium by nitrite reductase (Mols *et al.*, 2007)

2.1.5. Pathogenesis and Virulence

Clinical isolates of *Bacillus cereus* have a glycoprotein S-layer over its peptidoglycan which consists of proteinaceous paracrystalline arrays and covers the cell surface. The S-layer is involved in the virulence of *Bacillus cereus* and functions to promote interactions with human polymorphonuclear; leucocytes (Mignot *et al.*, 2001).

Bacillus cereus produces many types of toxins, two of which are most frequently associated with food poisonings: 1) the thermo-labile enterotoxins that are destroyed when food is heated and usually associated with foods that were insufficiently cooked or contaminated after cooking, and 2) the emetic toxin, which is not inactivated by heating of food and usually associated with highly cooked and fried or boiled and rapidly cooled foods like rice (Quinn *et al.*, 1999; Granum, 2007).The diarrheal syndrome is associated with meat and dairy products while the emetic toxin is linked to starchy products such as cereals and rice (Jay *et al.*, 2005).

In addition to its familiar role in food-borne infections and intoxications *B.cereus* has long been known to be capable of causing opportunistic infections in individuals recovering from surgery and in immune-compromised individuals (Turnbull *et al*., 1979). It has, however, not been regarded as a true infective pathogen of healthy individuals and the identification of *B. cereus* in the clinical laboratory is usually dismissed as either insignificant or as contamination (Miller *et al.*, 1997). Nevertheless, several later reports have documented fatal and life-threatening *B. cereus* infections in apparently healthy individuals (Miller *et al.*, 1997; Hoffmaster *et al.*, 2004; Avashia *et al.*, 2007) .The first case of a life-threatening *B. cereus* infection was reported in 1994 in Louisiana and involved a welder by trade. The patient survived, but only after 44 days of mechanical ventilation, aggressive antimicrobial chemotherapy and a partial lobectomy. More

recently the sudden deaths of eight healthy wild chimpanzees in Cote d'Ivoire, and three chimpanzees and one gorilla in Cameroon were reported (Leendertz *et al*., 2004; Leendertz *et al*., 2010).

Virulence factors associated with diarrhoeal syndrome involve three enterotoxins including hemolysin BL (HBL), non-hemolytic enterotoxin (NHE), and cytotoxin K (Kotiranta *et al.*, 2000). HBL is a haemolysin consisting of three proteinaceous subunits: B, L1 and L2, a binding factor and two "lytic" factors, respectively (Kotiranta *et al.*, 2000). Cytotoxin-K is the "youngest" of the enterotoxins, since it has first been described in 2000 (Lund *et al.,* 2000). In structure as well as in mode of action cytotoxin-K resembles that of *Staphylococcus aureus* –toxin or *Clostidium perfringens* –toxin (Lund *et al.*, 2000).

The emetic syndrome is caused by cereulide peptide toxin which is secreted during stationary phase (Granum and Lund, 1997). This toxin has a ring structure, dodecadepsipeptide, which consists of four amino acids, repeating three times, and oxy acids (Granum and Lund, 1997). Cereulide is very stable; it may persist in heat treated foods after death of the *Bacillus cereus* cells (Ehling-Schulz *et al.*, 2004a and b). Because cereulide is resistant towards acid conditions, proteolysis and heat, it will not be destroyed by gastric acid, the proteolytic enzymes of the intestinal tract or by reheating foods that have been stored at room temperature after a first heating (Ehling-Schulz *et al.*, 2004a). In egg and meat products as well as in liquid foods such as milk and soy milk, only low cereulide levels were detected. In contrast, boiled rice and farinaceous foods could sustain production of high levels of cereulide (Agata *et al.*, 2002).

The manifestations of systemic *Bacillus cereus* disease result from the action of other potent exotoxins that function as proteases, phospholipases, lecithinases, and hemolysins, producing extensive tissue damage and necrosis of visceral organs, including the brain, lungs, and eyes. *Bacillus cereus* also has the ability to produce β-lactamases, which render it resistant to penicillins and cephalosporins (Sankararaman and Velayuthan,2013).

Virulence factors associated with non gastrointestinal infections include hemolysins and phospholipase C. Hemolysin III causes erythrocyte lysis (Hoffmaster *et al.*, 2006). Phospholipase C causes tissue damage by stimulating degranulation of human neutrophil, and breaks down the sub epithelial matrix affecting the healing of tissue in infections (Kotiranta *et al.*, 2000).

2.2. Epidemiology

2.2.1. Distribution and outbreaks

The distribution of *Bacillus cereus* is worldwide (Logan *et al*., 2006).Diseases caused by *B. cereus* are commonly found in places where there is improper food handling. The prevalence of *B. cereus* induced food-borne illnesses is difficult to determine, because the symptoms associated with *B. cereus* infections or intoxication are generally mild. Therefore, it is conceivable that many *B. cereus* infections are not reported and that the prevalence of these infections is largely underestimated. Generally, *B. cereus* cases and outbreaks are only reported when either a large number of patients acquired symptoms or when the symptoms are more severe than usual. Despite the possible underestimation, *B. cereus* was the cause of most incidents with an identified agent in the Netherlands in 2007 (Doorduyn *et al.*, 2008; Venkitanarayanan *et al.*, 2008)). Between 2004 and 2007, *B. cereus* was the causative agent of food-borne illnesses in percentages ranging from 13% to 41% (Table 2)

Table 2: Number of reported *B. cereus* infections in The Netherlands in 2004-2007

	2007	**2006**	**2005**	**2004**
Bacillus cereus caused incidents[1]	23	22	19	17
Total incidents with known cause	57	56	148	95
Total incident	564	474	387	506
Percentage of *Bacillus cereus* incidents[2]	41%	39%	13%	18

1 An incident is a food-borne outbreak with more people or a single case

2 Percentage given is number of B. cereus incidents in relation to the total incidents with a known cause

Source: (Maarten,2009)

Besides this, comprehensive surveillance study in Europe has shown that in some countries, most notably Norway and The Netherlands, *B. cereus* is an important causative agent of food-borne infections (Schmidt and Gervelmeyer, 2003). In Norway similar data were reported as for The Netherlands, 35% and 32% of outbreaks were caused by *B. cereus* in 1999 and 2000, respectively. In other countries, lower relative incidences of outbreaks of *B. cereus* were reported. England, Italy, Germany, and France reported numbers ranging from 0.5% to 5% for *B. cereus*-attributed outbreaks in 1999 and 2000, respectively. The differences in reported incidences between countries may be caused by different food consumption patterns or different food handling. However, differences in local procedures for *B. cereus* detection and awareness of *B. cereus* infections are more likely causing the large differences in reported *B. cereus*-attributed cases between countries (Maarten, 2009).

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2.2.2. Occurrence of B. cereus in different products and milk

As a soil bacterium, *Bacillus cereus* can spread easily to many types of foods such as plants, eggs, meat, and dairy products (FDA, 2007), ice cream, herbs and spices (Klietmann *et al.*, 2002) and is known for causing 25 % of food-borne intoxications due to its secretion of emetic toxins and enterotoxins (FDA, 2007). Many of these foods may contain *Bacillus cereus* since spores of this organism are heat-resistant and can survive cooking (Klietmann *et al*., 2002).

The diarrhoeal type is commonly associated with proteinaceous foods, meat and milk products (Kotiranta *et al.*, 2000; Granum, 2007). Paradoxically, the emetic type *Bacillus cereus* strains are thus more often found in starch-rich foods, although their metabolic capacities are not necessarily well adapted to the nutrient composition of these foods, as strains of this type are generally not able to hydrolyse starch (Ehling-Schulz *et al.*, 2004b).

Bacillus cereus spores in raw milk have been reported as the main source of contaminants of milk products (Lin *et al.,* 1998).

Table 3: prevalence of *B. cereus* in milk and other dairy products

Authors and year	Prevalence	Samples types	Methods	Countries
(Christiansson *et al.*, 1999)	10-880 spores/L	Dairy products	Culture and spore count	Sweden
(Schlegelova *et al.*, 2003)	31% (66 /215)	Dairy products	Culture and SPC	Czech Republic
(Hempen *et al.*, 2004)	35.2% (206)	Raw and sour milk	Standard Plate Count (SPC)	Senegal
	33.3% (268)	Raw and sour milk	Standard Plate count	Guinea
(Mogessie, 2006)	$>10^9$ cfu/ml Mesophilic bacteria	Fermented dairy and beverages products	Culture filtrates SPC	Ethiopia
(El-Tabiy *et al.*, 2009)	25% (10/40)	skim milk	Culture and SPC	Egypt
	47.5% (19/40)	pasteurized full cream	Culture and total viable count	Egypt
(Muhamed *et al.*, 2010)	10% (8/80)	Raw milk sample	Culture and total viable count	South India
(Alemneh ,2012)	15.4% (59/384)	Raw milk sample	Culture and total viable count	Ethiopia
(Seblewengele ,2013)	15.86%(59/372)	Raw milk sample	Culture and total viable count	Ethiopia

Source :(Seblewengele,2013**).**

2.2.3. Host Range

Animals and humans, specifically those who are immunocompromised, intravenous drug users, or neonates (murray *et al*.,2007; Logan *et al*., 2006; Rosovitz *et al* .,1998) are described as being of special risk for *Bacillus cereus* food borne disease. However, individuals with lowered stomach acidity, for example elderly people, may be more susceptible to *Bacillus cereus* diarrhoeal disease, because a larger number of cells are expected to survive gastric acid (Kotiranta *et al.*, 2000).

2.2.4. Mode of Transmission

The primary mode of transmission is via the ingestion of *B. cereus* contaminated food (murray *et al*.,2007; Logan *et al.*, 2006) emetic type of food poisoning has been largely associated with the consumption of rice and pasta, while the diarrheal type is transmitted mostly by milk products, vegetables and meat. It forms spores and spreads easily (Kotiranta *et al.*, 2000). In hospitals, *B. cereus* can be transmitted via contaminated linen (Barrie *et al*.,1992).

2.2.5. Risk factors for contamination of milk

Feed, Soil, Pasture and feces are sources of milk contamination (Magnusson *et al.*, 2007). Spores are present in high concentrations in deep sawdust bedding of housed animals (Magnusson *et al.*, 2007) and in the soil of grazed areas (Christiansson *et al.*, 1999). The concentration of spores in air, in feed, feces, soil and milking equipment are important sources of raw milk contamination. Foremilk had a much higher spore concentration than mid or late-stream milk (Magnusson *et al.*, 2007). The spore content of milk in cows is strongly associated with degrees of soil contamination with teats, dirtiness of the cow and its concentration (Christiansson *et al.*, 1999).

Milk from a cow with an infected udder is likely to contain a large number of organisms. Mastitis, which is a disease causing inflammation of the udder, contributes considerable number of organisms, sometimes even blood cells into the milk. The hair, dirt and dust often fall from the animal body into the milking pails or the teat cups of milking machines. Dried dirt and filth is picked up by all movements and carried about as dust in the atmosphere. For this reason, dust may be the source of almost every kind of contamination (Christiansson *et al.*, 1999).

Utensils and equipments are known to be the greatest sources of contamination. They may account for as much as 100, 000 to a billion organisms per milliliter. Pails, strainers, milking machines, cans, pipes, bottles, and other equipments used for the handling of milk are sometimes not properly washed and sanitized. All persons involved in the milking process must be in good health and careful in their personal cleanliness. Probably the majority of milk borne epidemics of disease was started by workers who were carriers, or who had mild cases or who were in close contact with others so affected. Water from surface supplies is contaminated by dust, animals, plants, people, and other agents (Magnusson *et al.*, 2007).

2. 2 .6. Lifestyles

Bacillus cereus exists in two forms: spores and vegetative cells. Natural reservoirs for *Bacillus cereus* include decaying organic material, vegetables, and fomites (Sankararaman and Velayuthan, 2013), many types of soils, sediments, dust and plants (Kotiranta *et al.*, 2000). When adverse conditions prevail, the bacillus sporulates and the endospores are highly resistant to harsh environmental conditions (Sankararaman and Velayuthan, 2013).

Bacillus cereus is capable of adapting to a wide range of environmental conditions. It is distributed widely in nature and is commonly found in the soil as a saprophytic organism (Vilain *et al.*, 2006). *Bacillus cereus* is also a contributor to the micro flora of insects, deriving nutrients from its host (Vilain *et al.*, 2006). Spores may be passively spread and thus found also outside natural habitats. It is believed that *Bacillus cereus* exists in soil as spores, and germinates and grows when brought in contact with organic matter or an insect or animal host. Interest in the

ecology of this bacterium spurred a study showing that *Bacillus cereus* could germinate, grow and sporulate in soil, thus demonstrating a saprophytic life cycle (Jensen *et al.*, 2003).

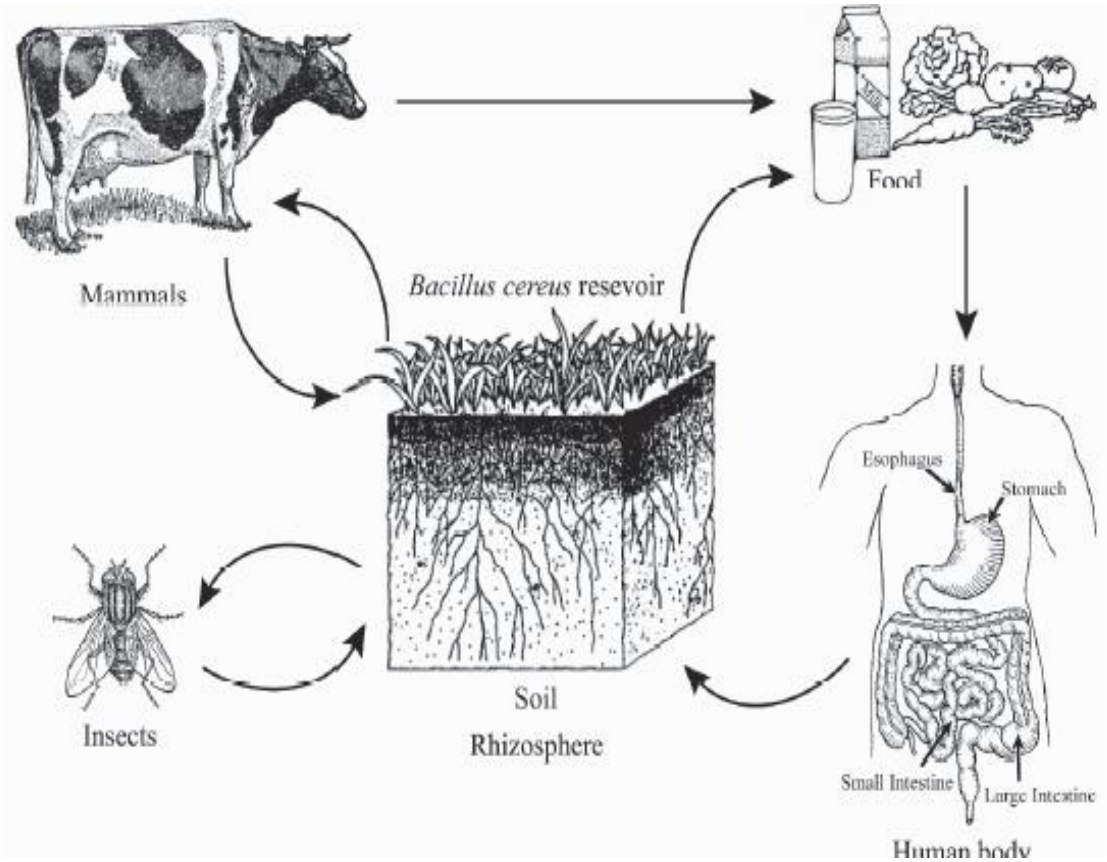

Figure 2: Illustration of environments from which vegetative cells and/or spores of *B. cereus* can be isolated.

Source: (Maarten,2009)

2.2.7 .Public Health significance of Bacillus cereus

Over the last 3 decades, *Bacillus cereus* has been recognized increasingly as a cause of potentially fatal systemic infections outside the GI tract, particularly in high-risk patients, such as neonates, patients with central lines, immune compromised individuals, injection drug users, and chronically debilitated patients. Serious systemic infections caused by *Bacillus cereus* include bacteremia, septicemia, meningitis, cerebral abscess, pneumonia, and endocarditis. In vulnerable high-risk patients, prompt recognition and aggressive management of these systemic infections are required to reduce both morbidity and mortality. Localized infections with *Bacillus cereus* have been reported in posttraumatic wounds, surgical wounds, and burns. Ocular infections, such as panophthalmitis, endophthalmitis, and corneal abscess, also have been documented (Kotiranta *et al.*, 2000, Wijnands *et al.*, 2006; Sankararaman and Velayuthan, 2013)

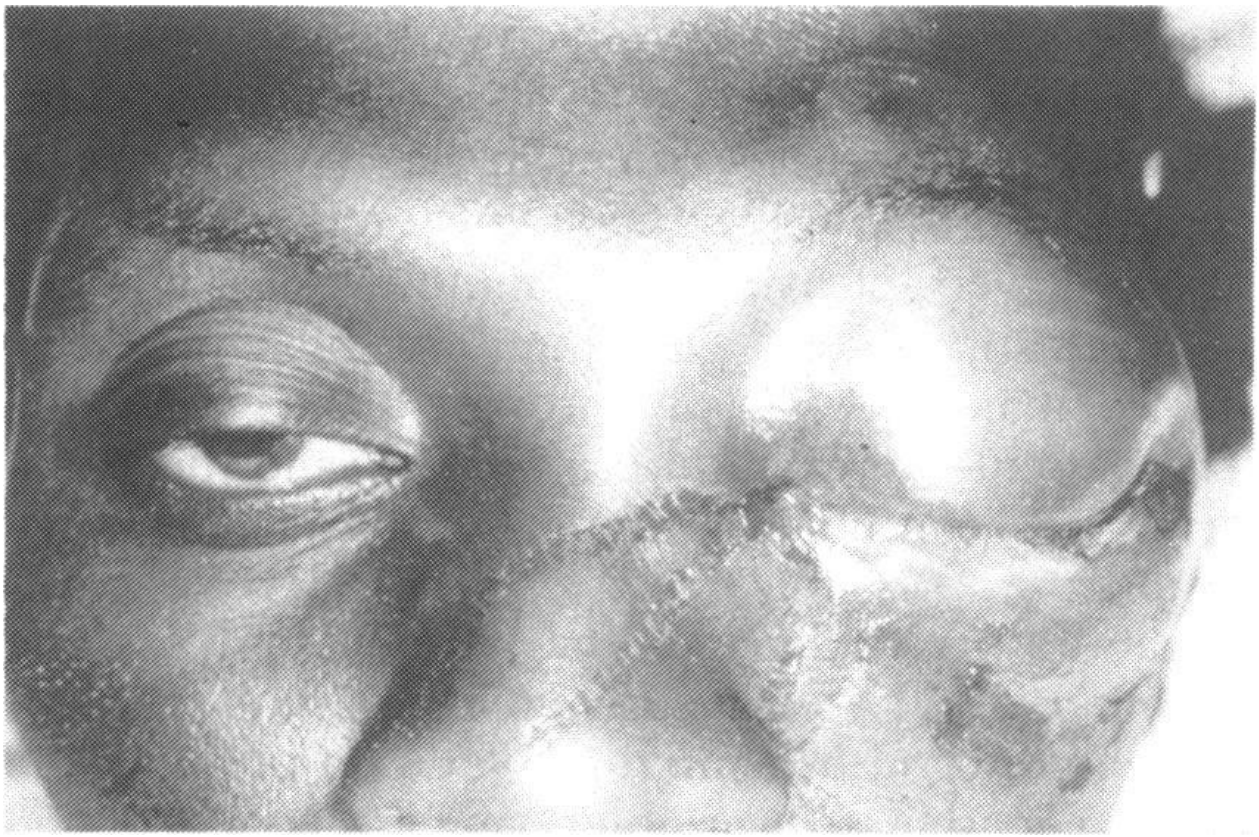

Figure 3: Patient with *B. cereus* panophthalmitis. Note periorbital swelling.

Source: (Al-Hemidan *et al.*, 1989).

Bacillus cereus causes two types of food poisoning in humans including diarrheal syndrome and emetic syndrome. Food poisoning results from its production of enterotoxins in the gastrointestinal tract. The dosage of ingested *Bacillus cereus* spores leading to diarrheal syndrome is 10^5–10^7 g 1 of ingested food, and 10^5–10^8 g 1 of ingested food for emetic syndrome (Quinn *et al.*, 1999; Hoffmaster *et al.*, 2006).

Enterotoxins associated with diarrhoeal syndrome are un resistant to the acidic conditions of the stomach. However, the cereulide peptide toxin associated with emetic syndrome is more resistant to acidic conditions and remains active at 121 °C (Hoffmaster *et al.*, 2006).

The main virulence factor in *Bacillus cereus* endophthalmitis is HBL which can result in the detachment of the retina and blindness. In addition, *Bacillus cereus* can cause gangrene, pyogenic infections, cellulitis, infant death, septic meningitis, lung abscesses, and endocarditis (Hoffmaster *et al.*, 2006). However, these infections are less common.

The most common is a diarrheal illness caused by a heat-liable toxin, accompanied with abdominal pain with incubation period of 4-16 hrs and symptoms lasting 12-24 hrs (Wijnands *et al.*, 2006).

The second type of emetic illness: characterized by vomiting and nausea that usually occurs within 1-5 hrs after ingestion of the contaminated food (FDA, 2007). Bacillus food borne illnesses occur due to survival of the bacterial endospores when food is improperly cooked (Turnbull, 1996).

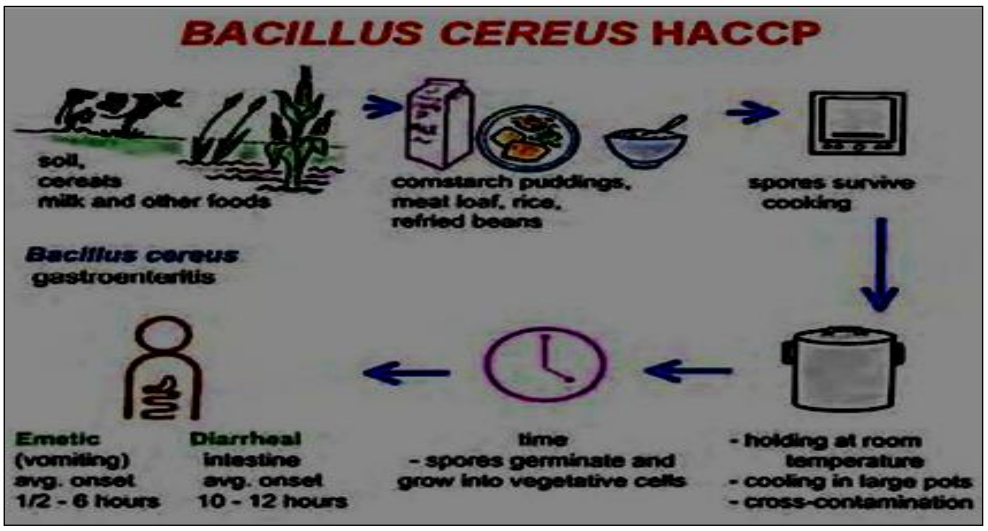

Figure 4: The public health significance of *Bacillus cereus* species

Source : (Elina, 2008)

2.2.8 Economic importance of Bacillus cereus

Besides being an important food-borne pathogen, *B. cereus* is also a notorious food spoilage organism. Food spoilage is caused by growth of unwanted bacteria in food and causes enormous expenses for food industry (Gram *et al.*, 2002). *B. cereus* causes mainly spoilage of milk and dairy products, thereby shortening the shelf-life of these products Raw milk is often contaminated with *B. cereus* spores (Te Giffel and Beumer, 1998) and these spores can easily survive pasteurization and germinate in milk (Wilkinson and Davies, 1973). Furthermore, spores and vegetative cells of *B. cereus* can attach to processing equipment and food contacting surfaces and subsequently form biofilms. Biofilms are multi-cellular complexes within a matrix of polysaccharides that are attached to a surface. Cells that are embedded in a biofilm tend to be more resistant to cleaning agents, making them hard to eradicate from processing equipment (Peng *et al.*, 2002; Stoodley *et al.*, 2002). Furthermore, biofilms may lead to equipment failure and corrosion (Beech *et al.*, 2005). From biofilms, cells and spores are easily dispersed and therefore biofilms are a continuous source of (re)contamination (Wijman *et al.*, 2007). Especially when dispersed cells and spores can germinate and grow spoilage can occur. Several *B. cereus* strains are known to grow at low temperatures and growth and subsequent spoilage may occur rapidly when the refrigerator temperature is abused. Therefore, the economic impact of unwanted *B. cereus* growth in food and food processing lines is substantial (Te Giffel, 2001).

2.3. Isolation and Enumeration of *Bacillus cereus*

2.3.1. *Isolation of B. cereus from raw milk*

The most commonly used selective medium for the *B. cereus* are *Bacillus cereus* Selective Agar Base (CM0167), Bacillus cereus selective Agar , Mannitol-Eggyolk- polymyxine (MYP) Agar, Bacillus cereus HiCrome™ Agar, Bacillus cereus Rapid Agar (BACARA®). KG medium is another widely used selective medium.Low level of peptone and the absence of carbohydrate in KG medium facilitated formation of spore (Johnson, 1984). A Columbia base 5% blood agar surface-spread with polymyxin B is recommended by Kramer *et al*.(1982) for better colony characteristics and differentiation.

In addition to selective compounds like polymyxin, these media utilize the bacterium's lecithinase production, (egg-yolk reaction giving precipitate zones) and lack of mannitol fermentation (Fricker *et al*., 2008). Principally in medium, Peptone provides nitrogen and amino acids. Sodium chloride maintains the osmotic balance of the medium; Mannitol is the carbon source that serves as the fermentable carbohydrate, fermentation of which can be detected by the pH indicator. Mannitol fermenting organisms like *Bacillus Megateruim* yield yellow colour colonies (Fricker *et al.*, 2008). When chromogenic mixture present in the medium is cleaved by the enzyme β-glucosidase found in resulting in the formation of blue colonies. Magnesium sulphate provides ions; disodium phosphate and mono-potassium phosphate constitute the buffer system of the medium. Sodium pyruvate stimulates the growth of microorganisms, improve egg yolk precipitation and enhance sporulation (Mossel *et al.*, 1967). Bromothymol blue is the pH indicator. Agar is the solidifying agent. Polymyxin B is the selective agent and has a bactericide activity against Gram-negative microorganisms. The egg yolk emulsion is incorporated to detect the proteolytic activity (ICMSF, 1996). For further identification and confirmation several biochemical reactions are in use like glucose fermentation, nitrate reduction and Voges Proskauer reaction, hemolytic activity, motility, growth characteristics (rhizoidity), and protein toxin crystal identification may be prescribed (Borge *et al*., 2001).

After 18-24 hours of incubation at 30°C in aerobic conditions, *Bacillus cereus* shows crenated, colonies about 5 mm in diameter, turquoise blue in colour, surrounded by a distinct opaque zone of egg yolk precipitation of the same colour as the colonies (Fricker *et al.,*2008). The medium is made selective by addition of Polymyxin B Supplement (SR99) which gives a final concentration of 100IU of polymyxin B per ml of medium. Polymyxin B, as a selective agent for the isolation of *Bacillus cereus* has been previously suggested by Donovan (1958) and found to be satisfactory (Mossel *et al.*, 1967). It is recommended that, where large numbers of fungi are expected in the inoculums, cycloheximide (SR222) is added to the medium at a final concentration of 40mg/l (Mossel *et al.*, 1967)

2.3.2. Sampling and sample processing

If the quantity of food to be examined is large, take representative samples of 10 g/mL each from different parts of the suspect food because contamination may be unevenly distributed. Using aseptic technique weigh 10 g/ml of sample into sterile blender jar/universal bottle. A sample could be serially diluted as follows by transferring one milliliter from the sample into 9 ml of sterile peptone broth in tube A and mixed well. This is the first 1/10 serial dilution. Next one milliliter from tube A is transferred to 9 ml of sterile media in tube B and mixed. This is another 1/10 serial dilution yielding total dilution up to this point 1/100. Then 1 ml from tube B is transferred to 9 ml of sterile media in tube C and mixed. This is the third 1/10 serial dilution making total dilution up to this point is 1/1000. At the end, 0.1 ml from each tube (A, B and C) should be plated on Bacillus cereus selective agar base (CMo167, Oxoid) and incubated for enumeration (FDA, 2013).

2.3.3. Presumptive Bacillus cereus Colony Identification

The primary diagnostic features on the medium are colonial morphology, precipitation of hydrolyzed lecithin around colonies and the failure of *Bacillus cereus to* utilize mannitol sugar. The typical colony of *Bacillus cereus* on *Bacillus cereus* selective agar base (CM0167, Oxoid) is crenate, about 5mm in diameter and have a distinctive turquoise to peacock blue colour surrounded by egg yolk precipitation of the same colour. These features distinguish *Bacillus cereus* group from other Bacillus species (Mossel *et al.*, 1967).

Other egg yolk reacting organisms which can grow on the medium, including *Staphylococcus aureus* and *Proteus vulgaris,* are distinguished from *Bacillus cereus* group by colony form and colour. These organisms also produce an egg yolk-clearing reaction in contrast to egg yolk precipitate produced by *Bacillus cereus* group. Microscopic examination for presence of lipid globules in the vegetative cells is recommended as a rapid and confirmatory test for *Bacillus cereus* and replaces the need for biochemical testing (Holbrook and Anderson, 1980). They confirmed that only *Bacillus cereus* from *Bacillus* species possess lipid globule in its vegetative cell. One further advantage of this test is that strains of *Bacillus cereus* that react only weakly or not at all with egg yolk can be detected and confirmed microscopically after staining (Mossel *et al.*, 1967).

To interpret results, bacteria that ferment mannitol produce acid products and form yellow colonies. Bacteria that possess lecithinase hydrolyze lecithin and a zone of white precipitate forms around the colonies; therefore, *Bacillus cereus* is known by mannitol non fermentation (blue colonies) and lecithinase system (forming zone of precipitation around colonies) producing colonies which are flat, crenate to slightly rhizoid, turquoise to peacock blue in colour and having a ground glass surface appearance. Therefore, Enumeration of presumptive *Bacillus cereus* colonies can be carried out according to (ICMSF, 1996) by surface plating technique (0.1 ml of prepared dilutions) onto *Bacillus cereus* Selective Agar Base (CM0167), incubating at 30 ^{0}C for

24 hrs. This may be transferred to nutrient agar or nutrient agar slant and blood agar for further confirmatory and differential tests according to (APHA, 1992).

2.3.4. Confirmatory and Differential tests for Bacillus cereus

To observe glucose fermentation by *B. cereus,* inoculate 3 ml Phenol red glucose broth with 2 mm loop full of culture. Incubate tubes anaerobically 24 hrs at 35 °c in GasPak anaerobic jar. Shake tubes vigorously and observe for growth as increased turbidity and color change from red to yellow, which indicates that acid has been produced anaerobically from glucose (USDA, 1998).

To observe nitrate reduction to nitrite, inoculate 5 ml Nitrate broth with 3 mm loop full of culture. Incubate tubes 24 hrs at 35 °c. To test for nitrite, add 0.25 ml each of nitrite test reagents A and C to each culture. An orange color, which develops within 10 min, indicates that nitrate has been reduced to nitrite (USDA, 1998)..

For Voges Prorkauer reaction test, inoculate 5 ml modified VP medium with 3 mm loop full of culture and incubate tubes 48 ± 2 hrs at 35°C. Test for production of acetylmethyl-carbinol by pipetting 1 ml culture into 16 x 125 mm test tube and adding 0.6 ml alpha-naphthol solution (R8921) and 0.2 ml 40% potassium hydroxide (R8922). Shake and add a few crystals of creatine. Observe results after holding for 1 hrs at room temperature. Test is positive if pink or violet color develops (USDA, 1998).

To observe tyrosine decomposition, inoculate entire surface of tyrosine agar slant with 3 mm loop full of culture. Incubate slants 48 hrs at 35°C. Observe for clearing of medium near growth, which indicates that tyrosine has been decomposed. Examine negative slants for obvious signs of growth, and incubate for a total of 7 days before considering as negative (USDA, 1998).

To observe growth on Lysozyme broth, inoculate 2.5 ml of nutrient broth containing 0.001% lysozyme with 2 mm loop full of culture. Also inoculate 2.5 ml of plain nutrient broth as positive

control. Incubate tubes 24 hrs at 35°C. Examine for growth in lysozyme broth and in nutrient broth control. Incubate negative tubes for additional 24 hrs before discarding (USDA, 1998)..

Results obtained with these confirmatory tests are recorded tentatively as *B. cereus.* The isolates abilities to produce large Gram-positive rods with spores; produce lecithinase and do not ferment mannitol; grow and produce acid from glucose anaerobically; reduce nitrate to nitrite; produce acetylmethyl carbinol (VP-positive), decompose tyrosine and grow in the presence of 0.001% lysozyme are common features of all *B. cereus* group (*B. cereus, B. mycoides, B. thuringiensis, B. megaterium* and *B. anthracis*. Therefore, strains that produce atypical features should be differentiated by further analysis of differentiation before classified as *B. cereus* (FDA, 2013).

Rhizoid growth test: to test rhizoid growth nature, pour 18-20 ml nutrient agar into sterile 15 x 100 mm Petri dishes and allow agar to dry at room temperature for 24-48 hrs. Inoculate by gently touching surface of medium near center of each plate with 2 mm loop full of 24 hrs culture suspension. Allow inoculums to be absorbed and incubate plates for 48 -72 hrs at 30 °c. Examine for development of rhizoid growth which is characterized by production of colonies with long root-like structures that may extend several centimeters from site of inoculation. Rough galaxy-shaped colonies are often produced by *B. cereus* strains but not other group members. (USDA, 1998).

Haemolysis test: to do this differential test, prepare sheep blood agar plate that has been divided into 4 - 6 segments is used for inoculation. A 2 mm loop should be used to deposit the inoculums in the center of the segment. Incubate plates for 24 hrs at 35°c. Examine plates for hemolytic activity. *B. cereus* cultures usually are strongly hemolytic and produce 2-4 mm zone of complete (β) hemolysis surrounding growth(USDA, 1998).

Motility test: this differential test can be done on motility medium by making a center line stab inoculation with a 3 mm loop and incubating the tube at 30 °C for 18-24 hrs. A motile organism is indicated by observing diffuse growth into the medium away from the stab. Alternatively a microscopic motility test may be used. The slide motility test is done by adding 0.2 ml of sterile water to a nutrient agar slant and then inoculating the aqueous phase with a 3 mm loopful of a 24

hrs slant culture, and incubating for 6-8 hrs at 30 °C. Place a loopful of the liquid culture on a glass slide and overlay with a cover slip. *Bacillus cereus* and *Bacillus thuringiensis* are actively motile while *Bacillus anthracis* and the rhizoid strains of *Bacillus cereus* are non-motile (USDA, 1998).

Para-sporal crystal formation(Protein toxin crystal stain) is differential stain, a smear is made on a microscope slide with sterile water from a 2-3 day old nutrient agar plate or slant. The slide is allowed to air dry and then gently heat fix. After cooling, flood the slide is flooded with methanol, wait for 30 seconds and poured off. Then flood the slide is flooded with 0.5% aqueous solution of basic fuchsin; the slide heated gently until steam is observed. After the removal of the heat, wait for 1-2 minutes and repeat the procedure. Then the slide is cooled and rinsed well with water, examined under oil immersion for free spores and darkly stained, diamond shaped toxin crystals. Toxin crystals should be present if the cells have lysed and free spores are observed. The presence of toxin crystals is strongly indicative that the organism is *Bacillus thuringiensis* (Borge *et al.*, 2001).

Finally, from the above tests, the bacterium which is lecithinase positive, strongly hemolytic on sheep blood agar, actively motile, does not produce rhizoid colonies and does not produce protein toxin crystals (diamond shaped) as para- sporal body, non mannitol fermenter, Gram positive, rod shaped producing endospore can be confirmed as *Bacillus cereus* (USDA,1998).

2.3.5. Enumeration of Bacillus cereus colonies

Viable counts involve counting cells that can be cultured and/or are metabolically active. Total counts involve counting all cells including dead or inactive cells. Direct methods of enumeration involve counting actual cells or colonies and indirect methods involve estimating the number of cells based on cell mass by scattering light through a culture (spectroscopy) or by a statistical method called the MPN (most probable number) technique (USDA, 1998).

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2.3.6. Plate count of B. cereus

This is done by preparing serial dilutions from 10^{-1} to 10^{-6} by transferring 1 ml homogenized sample (1:10 dilution) to 9ml dilution blank, mixing well with vigorous shaking and continuing until 10^{-6} dilution is reached. Inoculate duplicate on Bacillus cereus selective agar base (CM0167, Oxoid) plates with each diluted sample (including 1:10) by spreading 0.1 ml onto surface of each plate with sterile glass spreading rod. Incubate plates for 24 hrs at 30 °C and observe for colonies surrounded by precipitate zone, which indicates that lecithinase is produced by microbes. *B. cereus* colonies is crenate, about 5mm in diameter and have a distinctive turquoise to peacock blue colour surrounded by egg yolk precipitation of the same colour. These features distinguish *Bacillus cereus* group from other Bacillus species (Mossel *et al.*, 1967).

Select plates that contain an estimated 15-150 distinctive turquoise to peacock blue lecithinase producing colonies. Mark bottom of plates into zones with black felt pen to facilitate counting and count colonies that are typical of *B. cereus*. This is the presumptive plate count of *B. cereus* (Tallent *et al.*,2012.,Holbrook and Anderson, 1980).

2.4. Prevention and Control

2.4.1. Prevention

Since *Bacillus cereus* is ubiquitous in the environment, preventing contamination of food with its spores is almost impossible. Thus, measures to inhibit spore germination and prevent the growth of vegetative cells in cooked, ready-to-eat foods might be the approach to effectively prevent and control the spread of this pathogen. Thorough cooking is most likely to destroy the vegetative cells and spores. However, temperatures under 100°C might allow spore survival. Non-refrigerated storage of foods and especially rice should be avoided. Also, foods that require heating or cooling should undergo that process rapidly (Andersson *et al.*, 1998a).

The following conditions Suggest to Prevent *Bacillus cereus* Outbreaks, Maintain dairy products below 4°C, effectively wash equipment, avoid re-pasteurizing product, Never rework products that have left the plant, ensure returned product is well marked and stored well away from saleable product to prevent accidental shipment of returned product (Nguyen-the and Carlin, 2003).

2.4.2. Control

Control measures during an outbreak of *Bacillus cereus* from contaminated linens in a Japanese hospital included autoclaving the linens, increased use of gloves in patient care and use of strong alkaline detergents to clean the washing machines. Under favorable conditions, such as contact with organic material or animal or human hosts, the spores germinate into their vegetative form (Silo-Suh *et al.*, 1994).

Control of *Bacillus cereus* during food processing can be achieved by heating up to proper temperature in an appropriate time. Decreasing pH values to ≤4 and increasing levels of sodium chloride to ≥1.0% decreases growth rate and increased the lag phase of *Bacillus cereus*. The combination of decreasing pH, increasing salt concentration and setting storage temperatures

below 12 ^{0}C is sufficient to inhibit *Bacillus cereus* growth after heat treatment at 90 ^{0}C for 10 min (Ernst *et al.*, 2006;Martinez *et al.*, 2007,;Park *et al.*, 2009).

In general, food production and foodservice establishments must use heating methods that destroy vegetative cells and most spores. Cooked food products should not be stored at room temperature. Foods should not be cooled in large, deep pans or kettles. Foodservice personnel should be trained to use good personal hygiene and proper methods of hand washing when handling food products. Methods that adequately clean and sanitize surfaces, equipment and utensils should be used. As a routine practice, clean all bench tops, cutting boards and utensils with detergent and follow up with a sanitizer which kills bacteria (Akbas and Ozdemir, 2008).

Heat resistance is an important character of *Bacillus* spp. spores. Appropriate temperature selection and duration of heating are crucial for the required microbial quality of the milk product, as with a linear temperature increase the time needed to achieve nearly complete abiosis becomes shorter exponentially. When determining the heat resistance and thus the sufficient duration of thermic treatment, it is useful to define the D value (decimal reduction time), i.e., a temperature value resulting in a decrease of the number of live microorganisms by one order (Kyzlink, 1980)

2.5. Treatment of *Bacillus Cereus*

The microbe is susceptible to vancomycin, aminoglycosides, clindamycin, carbapenems, chloramphenicol, and erythromycin. In high-risk populations, early aggressive treatment reduces further dissemination of the bacilli and subsequent production of its toxins. In view of the fulminant course and poor outcome associated with systemic disease in the context of severe immune compromise, the use of two antibiotics should be considered in particularly vulnerable patients (Sankararaman and Velayuthan, 2013).

When the microbiological laboratory preliminarily reveals gram-positive bacilli in a gram stain or blood culture, *Listeria monocytogenes* often is presumed to be the likely pathogen because

physicians are more familiar with *Listeria monocytogenes* causing serious infections than with *Bacillus cereus*. Unfortunately, ampicillin, typically used to treat *Listeria monocytogenes*, is ineffective against *Bacillus cereus*. Clinicians should be aware also that penicillins and third-generation cephalosporins, often chosen as empirical therapy for serious infections, are not effective against *Bacillus cereus*. Because of its ubiquity, *Bacillus cereus* often is ignored or dismissed as a contaminant when found in a culture specimen. Whenever in doubt, a culture positive for *Bacillus cereus* should be repeated, and appropriate antimicrobial therapy should be started expectantly in high-risk patients. Persistent recovery of the bacillus from the same site or source confirms true infection rather than contamination and mandates continued aggressive treatment (Sankararaman and Velayuthan, 2013).

Because antibiotic therapy typically destroys the planktonic bacilli but not the biofilm, prolonged antibiotic therapy is indicated in high-risk patients, along with the removal of the infected devices. For deep-seated visceral abscesses (e.g., cerebral abscesses), surgical drainage often is needed in addition to antimicrobial treatment (Sankararaman and Velayuthan, 2013).

3. MATERIALS AND METHODS

3.1. Study Area

Assosa is the capital city of the Benishangul-Gumuz Regional State and composed of 74 administrative peasant associations (Assosa District Agricultural and Rural development office, personal communication), which is located at 8°30'and 40°27' N latitude and 34°21' and 39°1' E longitude 687 kms Northwest of Addis Ababa. The altitude of Assosa ranges from 580 to over 1544 meter above sea level. The area is characterized by low land plane agro- ecology according to National Meteorological Service Agency (NMSA, 2007) with average annual rainfall of 1316 mm with uni-modal type of rainfall that occurs between April and October. Its mean annual temperature ranges between 16.75°C and 27.9°C. Assosa zone has 35.6% of the livestock population of the region constituting 61,234 cattle, 191,383 goats, 19,729 sheep, 25,137 donkeys, 439,969 poultry and 73,495 beehives (CSA,2011), and the Assosa District has 16,990 cattle,30,728 shoat,57,089 poultry and 5,240 donkey (Assosa District Agricultural and Rural development department, personal communication).

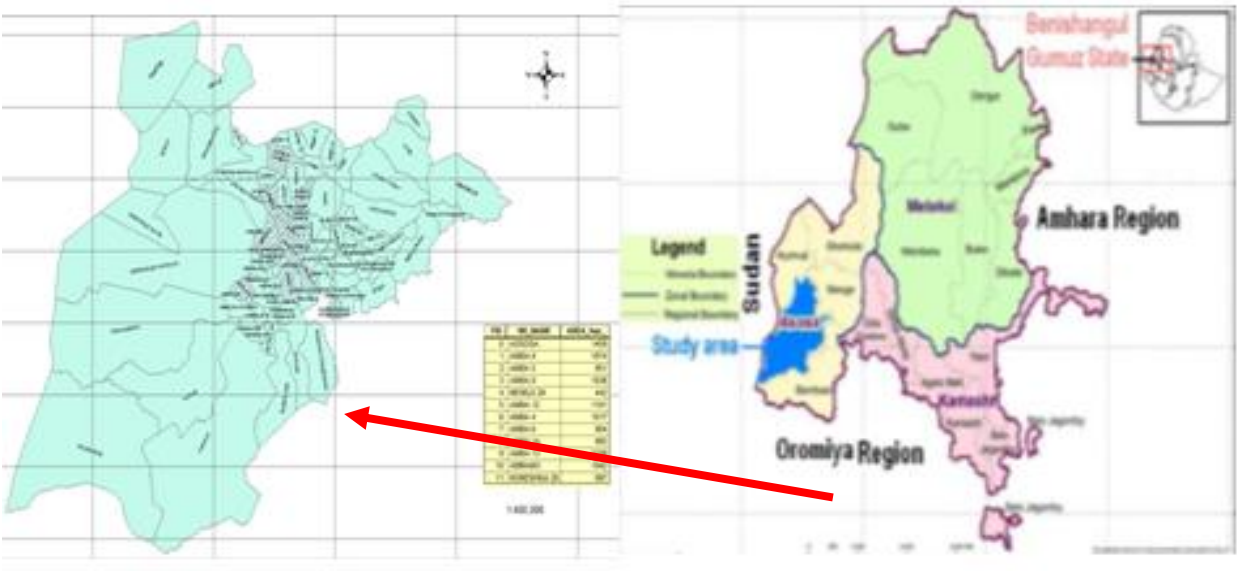

Figure 5: Map of the study area showing relative location of Assosa in Benshangul Gumuz Regional State.

Source: (Mulaw *et al*.,2011)

3.2. Study Design

A cross-sectional study was carried out between September 2013 to June 2014 on bovine raw milk samples collected from purposively selected 12 peasant Associations (PAS) of the District, on lactating dairy cows belonging to small household farmers in and around Assosa District. A multistage sampling technique was employed. Accordingly, among the 74 administrative PAs in the District, 12 PAs were purposively selected based on accessibility to transport and milk sample were collected from lactating Zebu cows owned by selected households.Then, simple

random sampling technique was employed to select households and the animals, i.e., one lactating cow was selected from each households . Similarly, for those farmers having more than one lactating dairy cow simple random sampling technique was applied. Prior to sample collection, information was gathered from milkers by using semi-structured questionnaire survey which was designed to assess the risk factors for milk contamination with the organism like the situation of lactating dairy cow management, milking procedures and hygienic status and addressing public health significance of the organism. All respondents of questionnaire survey were selected purposively based on their voluntariness and owning lactating dairy cow; thus, a total 120 respondents were interviewed.

3.3. Study Population

The study population was lactating dairy cows owned by purposively selected Peasant Associations of small household farmers in Assossa District.

3.4. Sample Size Determination

The total sample size for raw milk collection, isolation and enumeration of *B. cereus* was assigned according to statistical formula of Thrustfield (2005). A 5% absolute precision at 95% confidence interval was used during determining the sample sizes. Since there was no previous work in the study area for *B. cereus* prevalence on raw milk, the expected prevalence of this bacterium on raw milk was taken as 50% according to Thrustfield (2005). Therefore, the total sample size for this study was calculated as follows:

$$n = \frac{(1.96)^2 x\ P\ (1-P),}{d^2}$$

Where: n = the total sample size

P = expected prevalence (50%)

d = desired absolute precision (0.05) at 95% CI

$$n = \frac{(1.96) \times (1.96) \times (0.5) \times (1-0.5)}{(0.05) \times (0.05)} = 384$$

From the above equation, the total 384 lactating cows from small house hold farms were sampled in this study.

3.5. Sampling Procedures

From purposively selected Peasants Associations, sampling was practiced on all lactating dairy cows owned by randomly selected small households, i.e., one lactating cow was selected from each household, if farmers have more than one lactating dairy cow similarly random sampling was applied. Before sampling, the udder and teats were washed with potable water and disinfected with clean cotton soaked in 70% alcohol solution wearing latex glove and rinsed by clean towel separately for each cow. The first two to three streaks of milk were flushed out into the ground and then representative milk sample (10 ml) was collected aseptically from all teats (about 2-3 ml from each teat) into a single screw capped container or universal bottle of 15ml. The cap was removed from the universal bottle without touching the inside and it was held in such way that the inner surface faces down to prevent sample contamination. The universal bottle was kept at 45^0 angles so that debris did not fall into it during sampling. The cap was immediately replaced after the sample was obtained (For detail procedures refer Appendix-VI). The collected milk samples were clearly labeled by permanent ink, using coding system for each lactating dairy cow with small household owner name and dates to differentiate the samples and additional secondary data and sensible physical dirt and milk abnormalities were assessed by using organoleptic tests and documented in to pre-prepared tables. Finally, the milk samples were transported immediately to Regional veterinary Laboratory of Benishangul Gumuz, Assosa, in the ice box.

3.6. Sample Processing and Plating

Upon arrival on the laboratory from each raw milk sample 0.1 ml, without dilution, was plated immediately on the *Bacillus cereus* selective agar base for determination of prevalence. The remaining 9.9ml was stored in a refrigerator at 4 ^{0}C only for about less than 24 hrs. If there is growth of presumptive *Bacillus cereus* colony sample processing for *B. cereus* load determination was proceeded immediately. Sample processing was done by diluting 1 ml milk from 10 ml milk sample and pipetting this into sterile universal bottle filled with 9 ml of 0.1% peptone water (CM0009, Oxoid Ltd) in safety cabinet. The diluted sample was mixed manually by moving gently about half arc 10 – 15 times. From this initial dilution (10^{-1}), serial dilutions from 10^{-2} to 10^{-6} were made using-sterile peptone water. Following this, 0.1 ml milk sample was spread on to *Bacillus cereus* selective agar (CM0167; Oxoid Ltd, Basingstoke Hampshire, England) in duplicates; that is, two plates were used for each dilution factor. The plates were incubated aerobically at 30 ^{0}C for 18 – 24 hrs and checked for presumptive colony growth. If no colonies grew, the incubation was extended for another 24 hrs and rechecked for colony growth.

3.7. Presumptive Bacillus cereus Colony Count

The presumptive *Bacillus cereus* colonies were identified based on colony colour, morphology and precipitation of egg yolk. After 18 – 24 hrs of incubation at 30 ^{0}C in aerobic conditions, *Bacillus cereus* colonies were identified as colonies about 5 mm in diameter, turquoise blue in colour, surrounded by a distinct opaque zone of egg yolk precipitation of the same colour as the colonies.

Based on morphological characteristics on selective media, presumptive colonies were counted. As a rule, plates having colonies below 15 were not considered as the true representatives of the sample and recorded as too small to count; whereas, plates with more than 150 colonies were difficult for counting and reported as too numerous to count. Therefore, the actual numbers of colony forming units (CFU) reported in this study were colonies from plates falling between 15 and 150 colony counts. Finally, the number of CFU/ml of samples was calculated using the standard equation of Nicoletta and Royston (2008).

$$N = \frac{\Sigma C}{(n_1 + 0.1 \times n_2) \times d}$$

Where: N = total viable colony count; Σc = sum of colonies counted from all plates; n_1 = number of plates counted at first dilution; n_2 = number of plates at second dilution; C= number of colonies counted; d = dilution factor from which the first counts obtained (least counted dilution).

3.8. Confirmatory and Differential Tests

For confirmation and differentiation 2 – 3 presumptive colonies were picked from positive plates and transferred to nutrient agar slants. These were incubated for 24 hrs at 30 ^{0}C aerobically. Using Gram staining *Bacillus cereus* group was identified as large Gram-positive rod shaped cells with short to long chains. Most biochemical tests are confirmatory but not differential because they are common for *Bacillus cereus* group members namely, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus thuringiensis*, and *Bacillus anthracis* with identical characteristics; therefore, additional tests were performed for differentiation.

By inoculating isolates on sheep blood agar (CM0854; Oxoid Ltd), *Bacillus cereus* colony grow as with flat and irregular shaped, 2–5 mm in diameter forming creamy to white colour on a ground glass appearance with strong β-haemolysis. This colonial appearance was used for differentiating *Bacillus cereus* from its group members. Since *Bacillus anthracis* forms non haemolytic gray/white colonies where as *Bacillus mycoids* forms colonies with rhizoid/hairy like projections. Alternatively, *Bacillus cereus* was differentiated from other non motile group members forming diffuse growth in semisolid SIM medium (M181; HiMedia Ltd) except from *Bacillus thuringiensis.* In addition, from *Bacillus cereus* group only *Bacillus mycoids* can form rhizoid growth on pre-dried nutrient agar (CM0003; Oxoid Ltd) or blood agar (CM0854; Oxoid Ltd).

Rapid staining methods using warm 0.5% basic Fuchsin (212545; BD Difco BBL Stains), Malachite green (90903; Fluka) and Sudan Black B (199664; Sigma-Aldrich), give characteristic morphology of pale green endospores without bulged sporangium and with no parasporal crystal bodies in red stained cytoplasm. This was used to differentiate *Bacillus cereus* from *Bacillus thuringiensis.*

3.9 .Questionnaire Survey

In addition to collecting samples, questionnaire survey was conducted on form, frequency, clinical signs and symptoms of milk consumption, illness of consuming milk, awareness on milk born disease, habitat and housing of dairy cow, cleanness of udder, equipment used for milking ,source of water to clean milking equipment and wash hands of milker to assess public health implication and milk contamination. A structured questionnaire was prepared and pre-tested.120 voluntary respondents were interviewed. The questions were originally written in English and translated into Amharic Language when administered. The answers were than translated to English and entered into the original form.

3.10. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility tests were conducted on 25 isolates of *Bacillus cereus*. The isolates were tested on 8 antimicrobials using the Kirby-Bauer disks diffusion method by 0.5 McFarland standards on Muller Hinton agar plates (NCCLS., 1997; Quinn *et al.,* 2004).

The antibiotic discs were applied on to the surface of the inoculated Muller Hinton agar plate using aseptic techniques. Each disc was pressed down to ensure complete contact with the agar surface. The discs were deposited with centers at least 24 millimeters apart (Quinn *et al*., 2004).

The inhibition zone was reported as the diameter of the zone surrounding the individual disc in which bacterial growth was absent. Based on this, the isolates were defined as resistant,

intermediate and susceptible according to the guidelines of the manufacturer manual and NCCLS (1997).

The following antimicrobial disks (Oxoid,Basing Stoke,UK) with their corresponding concentration were used in the study: polymixin B (300µg),tetracycline(10 µg),ampicilin (10µ),penicillin G(10u), kanamycin (30µg) , clindamycin (10µg), chloramphnicol (30 µg) and vancomycin (30µg) .

3.11. Data Analysis

The data was entered into Microsoft Excel (MS-Excel, 2007). These data were analyzed using Statistical Package for Social Science (SPSS version 20.0, Inc., 2007, Chicago, Illinois) software. The log_{10}-transformed values of raw milk standard plate count (log_{10}CFU/ml) were computed using mean values as continuous variable and parities, lactation stage, hygienic condition, laboratory result and CMT result as categorical variables. The descriptive and analytic statistics called Pearson Chi-Square test and logistic regressions were used to see statistical significance associations for categorical data. The degree of association was computed using Odds ratio (OR) and for all statistics 95% CI with 5% degrees of freedom ($P < 0.05$) was considered to say significant.

4. RESULTS

In the present study a total of 384 lactating cows belonging to small households were sampled for raw milk at Assosa District, and milk samples were processed microbiologically for isolation and identification of *Bacillus cereus*. Variations in parity, lactation stage, hygienic condition and CMT result were used as risk factors for assessing contamination rates of milk by *Bacillus cereus*.

4.1. Prevalence of *Bacillus cereus*

The overall prevalence of *Bacillus cereus* in raw bovine milk samples was 22.4% at cow level (Table4). The *Bacillus cereus* load from raw milk samples ranged from 3.1505 to 7.1672 logarism of colony forming unit per milliliter (log CFU/ml). The bacterial loads in CFU/ml of most of milk samples were above legal limit ($>10^5$CFU/ml) in raw milk. From positive samples, counts above and below legal limit in cow raw milk intended for human consumption were 75.58% (65/86), 24.42%(21/86), respectively.

Table 4: Overall prevalence and bacterial load above and below legal limits in raw bovine milk at Assosa District

	No of examined animals	**No of positive animals**	**Prevalence (%)**
Bacillus cereus	384	86	22.4%
Bacterial load above legal limit	384	65	16.9%
Bacterial load below legal limit	384	21	5.5%

4.2. Association of *Bacillus Cereus* Contamination of Raw Milk

Analyses were made to look at the association of *Bacillus cereus* occurrence in raw milk with various host and management factors.

The Statistical analysis in table 5 below shows hygienic status of the barn, lactation length, and California mastitis test in small house holder dairy farms were significantly associated with the positivity of *Bacillus cereus*. As the laboratory result revealed the prevalence *B. cereus* was significantly ($p<0.05$) higher as the hygienic practice declines, increasing the chance of contamination of raw bovine milk with B*acillus cereus*. Lactation length also had a significant ($p<0.05$) effect on the occurrence of *bacillus cereus*, it was higher around early and mid-lactation stages. Statistically significant association was also observed between California mastitis test

values and *Bacillus cereus* prevalence, whereas there was no statistical significance (p>0.05) between parity and positivity of *Bacillus cereus*.

Table 5: Association between risk factors and positivity of *Bacillus cereus* contamination of bovine raw milk using chi-square ($\chi 2$) at Assosa District.

Risk Factors	**cat**	**N**	***Bacillus Cereus* Positivity (%)**		**$\chi 2$**	**df**	**P-Value**
			Positive	Negative			
Hygienic Condition	Poor	135	62(45.9%)	73(54.1%)	66.609	2	.000**
	Intermediate	127	14(11.0%)	113(89.0%)			
	Good	122	10(8.2%)	112(91.8%)			
Parity in number	1-3	289	57(19.4%)	232(80.6%)	5.682	2	.058
	4-6	77	25(33.8%)	52(66.2%)			
	>6	18	4(22.2%)	14(77.8%)			
Lactation length	≤3	149	37(24.8)	112(75.2%)	11.394	3	.010
	4-6	138	39(28.3)	99(71.7%)			
	7-9	48	5(10.4)	43(89.6%)			
	>9	49	5(10.2)	44(89.8%)			
CMT reaction	Reactive	89	2(2.2%)	87(97.8%)	27.061	1	.001*
	Non Reactive	295	84(28.5%)	211(71.5%)			

N=Number of observation, Cat=Category, $\chi 2$= chi-square, df=degree of freedom

Results of univariate logistic regression revealed that hygienic condition (OR=10.040, 95% CI: 4.584-21.989) and California mastitis test (CMT) (OR=15.939, 95% CI 3.676-69.101) had significant effect on the prevalence of *Bacillus cereus*. The prevalence rate was higher in poor hygienic condition (OR=10.040) than better hygienic conditions and also cows with California mastitis test positive values had higher chance (OR=15.939) of acquiring *Bacillus cereus* contamination than California mastitis test negative ones (Table 6). Whereas the results of univariate logistic regression showed that parity and lactation length had no significant effect.

Table 6: Indicators of positivity to Bacillus cereus as revealed by univariate logistic regression analysis

Categories		**N**	**Prevalence %**	**P=value**	**OR**	**CI of OR**	
Hygienic	Poor	135	62(45.9%)	.000	10.04	4.584	21.989
Condition	Intermediate	127	14(11.0%)	.540	1.319	.543	3.204
	Good	122	10(8.2%)				
Parity in number	`1-3	289	57(19.7%)	.599	.698	.182	2.674
	4-6	77	25(32.5%)	.679	1.351	.325	5.616
	>6	18	4(22.2%)				
Lactation	≥3	149	37(24.8%)	.282	1.857	.602	5.731
stage(Months)	4-6	138	39(28.3%)	.195	2.091	.686	6.378
	7-9	48	5(10.4%)	.479	.594	.141	2.513
	>9	49	5(10.2%)				
CMT Reaction	Reactive	89	2(2.2%)	.000	15.939	3.676	69.101
	Non Reactive	295	84(28.5%)				

N=Number of observation, Cat=Category, OR=odds ratio, CI=confidence interval.

4.3. Results of the Questionnaire Survey on Public Health and Source of Milk Contamination

In addition to the laboratory survey, issues of public health implication arising from *B. cereus* and possible sources milk contamination with *B. cereus* were assessed using structured questionnaire survey on 120 respondents.

In the current study risk factors for public health such as milk consumption, form of milk consumption, illness of consuming milk, clinical signs and symptom of milk born disease, awareness on milk born disease and frequency of milk consumption were assessed.

As observed during the current study (Table 7) 97.5% of interviewed personnel consume milk and out of these respondents 29.1 % consume Raw (fresh whole milk). 41.7% of the respondents consume milk as a common diet. About 42.5 % of the respondents reported that their family had faced illness after consumption of milk with 31.4%, 25.5% and 19.6% clinical sign and symptoms of diarrhea, vomiting and abdominal cramp and pain, respectively. 34.2% of the respondents had awareness in milk born disease but no one described *Bacillus cereus* food poisoning directly rather they explained the disease indirectly through the illness faced as diarrhea, vomiting and abdominal cramp and pain. About 27.1% of the respondents consume milk stayed for long time.

Table 7: Results of questionnaire survey on public health significance of *Bacillus cereus* at Assosa District

Public health implication issues raised	**Response category**	**No of respondents (%)**
Milk consumption	Yes	117(97.5%)
	No	3(2.5%)
Form of milk consumption	Raw(fresh whole milk)	34(29.1%)
	Ergo(naturally fermented milk)	32(27.3%)
	Ayib (Ethiopian cottage cheese)	23(19.7%)
	Boiled	28(23.9%)
Acquiring Illness	Yes	51(42.5%)
	No	69(57.5%)
Knowledge on Signs of illness	Diarrhea	16(31.4%)
	Vomiting	13(25.5%)
	Both diarrhea and vomiting	10(19.6%)
	abdominal pain and cramp	12(23.5%)
Awareness on milk born illness	Yes	41(34.2%)
	No	79(65.8%)
Description of the illness	Diarrhea and Vomiting	7(17.1%)
	abdominal pain and cramp	5(12.2%)
	TB-Tuberculosis	17(41.5%)
	Internal parasitism	12(29.3%)
Feeding Habits	For breakfast	44(36.6%)
	As common diet	50(41.7%)
	Frequently	11(9.2%)
	As required	15(12.5%)

Table 7 (continued)

Public health implication issues raised	Response category	No of respondents (%)
Time gap between taking and using milk	Yes	111(92.5%)
	No	9(7.5%)
Average time gap between taking and using milk.	1-4hrs	26(23.4%)
	4-10hrs	26(23.4%)
	10-16hrs	29(26.1%)
	more than 16hrs	30(27.1%)

Furthermore, possible sources of milk contamination with *B. cereus* such as habitat of dairy cow, housing and milking area floor type, time length of cleaning dairy house and cows' udder, equipment used for milking, source of water to clean milking equipment wash hands of milker were assessed through semi-structured questionnaire.

Accordingly, 95% dairy cows stay day time in field and night at home and 92.5% were used natural floor for housing and milking. About 50% of dairy cow was cleaned monthly and only 24.2% respondents clean the udder of lactating dairy cow the remaining let calves to suckle before milking (Table 8).

Almost all milkers wash their hand before milking and about 81.7% used plastic equipment for collecting and transporting their milk. Although 67.5% respondents use non-chlorinated well water for washing purpose (udder, equipment and hand).

Table 8: Results of survey on possible sources of raw bovine milk contamination at Assosa District

Milk contamination assessment issues raised	**Response category**	**No of respondents (%)**
Habitat of dairy cow	Day time in field and night at home	114(95.0%)
	Day and night in the house	6(5.0%)
	Day and night in the field	-
	In fenced barrels	-
Housing area floor type	Concrete ground	7(5.8%)
	Natural floor	111(92.5%)
	Straw bedded floor	-
	Stone and sand	2(1.7%)
Milking area floor type	Concrete ground	7(5.8%)
	Natural floor	111(92.5%)
	Straw bedded floor	-
	Stone and sand	2(1.7%)
Time length of cleaning dairy house	daily	19(15.8%)
	weekly	41(34.2%)
	monthly	60(50.0%)
	yearly	-
Time length of cleaning milking cows' udder	Only calve suckle it	91(75.8%)
	Only before milking	29(24.2%)
	Only after milking	-
	Before and after milking	-

Table 8 (continued)

Milk contamination assessment issues raised	**Response category**	**No of respondents (%)**
Equipment used for milking	Aluminum cans	20(16.7%)
	Plastic beaker	98(81.7%)
	clay pot	2(1.7%)
	other traditional	-
Source of water to clean milking equipment	River	23(19.2)
	well water	81(67.5)
	tap water	7(5.8)
	stagnant water	9(7.5)
Hand washing	Yes	100
	No	0
Source of water to clean milker hand	River	23(19.2)
	well water	81(67.5)
	tap water	7(5.8)
	stagnant water	9(7.5)

4.4. Antibiotic Susceptibility Profiles of *Bacillus Cereus* Isolates

According to NCCLSs' Standard on zone size interpretation chart (Appendix-x) Almost all Bacillus cereus isolates displayed high resistance to Polymixin B (100%), tetracycline (88%), Ampicilin (80%), penicillin G (76%), and kanamycin (72%); the isolates were highly susceptible to clindamycin (84%), chloramphnicol (88%), and vancomycin (92%) (Table 6).

Table 9:Antibiotic susceptibility profiles of Bacillus cereus isolates from raw milk at Assosa District

Antimicrobial agent	**Content**	**Zone diameter nearest whole mm(%)**		
		Resistant	Intermediate	Susceptible
Penicillin G	10u	19/25(76%)	2/25(8%)	4/25(16%)
Tetracycline	10µg	22/25(88%)	1/25(4%)	2/25(8%)
Chloramphincol	30µg	2/25(8%)	1/25(4%)	22/25(88%)
Kanamycin	30µg	18/25(72%)	-	7/25(28%)
Clindamycin	10µg	4/25(16%)	-	21(84%)
Ampicilin	10µ	20(80%)	-	5(20%)
Vancomycin	30µg	1/25(4%)	1/25(4%)	23/25(92%)
Polymixin B	300µg	100%	-	-

5. DISCUSSION

Bacillus cereus is an emerging human food-borne pathogen and has long been recognized increasingly as a cause of potentially fatal systemic infections in individuals recovering from surgery, particularly in high-risk patients, such as neonates, patients with central lines, immune compromised individuals, injection drug users, and chronically debilitated patients (Turnbull *et al.*, 1979; Kamar *et al*., 2013).and causes animal infections like , bovine mastitis and bovine abortion (Tumbuii and Krarner,1991; Melling ***et*** *al*., 1976). Besides being an important food-borne pathogen, *B. cereus* is also a notorious food spoilage organism, mainly spoilage of milk and dairy products, which lead to economic impact on dairy industry (Te Giffel and Beumer, 1998; Gram *et al.*, 200; Beech *et al.*, 2005).

Previous laboratory surveys in Ethiopia showed that the *Bacillus cereus* contamination of raw bovine milk is prevalent in different dairy cow rearing areas of the country (Alemneh , 2012; Seblewengele ,2013).

In the present study, prevalence of 22.4% was recorded in raw bovine milk at Assosa district small household lactating dairy cows using cultural isolation, confirmatory and differential tests. This finding is in agreement with the results of contamination recorded by (Slaghuis *et al*.,1997),(Ayuob *et al*. ,2003) and (Yobouet *et al*., 2014) who found isolation rate of 23%, 26.7%,27% respectively. However, the result of this study is lower contaminated than previous reports by (Abdel-khalek and EI-sherbini ,1996),(Te Giffel and Beumer ,1998), (Schelegelova *et al.*,2003), (EI- Shinawy ,2004),(Ombui and Nduhiu,2005), (Rezende-lago *et al.* ,2007), *(Hassan et al*.,2010), (Haughton *et al*.,2010) and (Adesina *et al.*,2011) at rates of 40%,35%,31%, 62%, 35.2%, 50%, 59% and 46.7%, respectively.But finding is higher than previous reports by (Ahmed *et al*., 1983), (Mosso *et al.*, 1989), (Gilles *et al., 2002*), (Hempen *et*

al., 2004) ,(Parkash *et al.*, 2007), (Muhamed *et al.*, 2010), (Alemneh ,2012), and (Seblewengel,2013) who reported isolation rates of 9%, 0%, 15.4%,17% 6.5%, 10% , 15.4% and 15.86%, respectively.

The higher prevalence in some finding may be due to poor hygienic practices conducted on lactating dairy cow ,sources of higher milk contamination unique to certain climatic ,small household lactating dairy cow ,housing conditions; in the result which is reported as lower prevalence than the present study may be due to good hygienic practices in the dairy farms.

In the present study bacterial load was range 3.1505 to 7.1672 logarism of colony forming unit per milliliter (log CFU/ML).Majority of raw milk samples had higher *Bacillus cereus* load (75.58 % (65/86)) than acceptable limit of raw milk ($>10^5$CFU/ml)(Council Directives 92/46/EEC,1992) which higher than the result reported by (seblewengele,2013) 38.98%. This may be due to the differences in management systems, poor hygienic condtions practiced in the milk production by the producers who have no awareness about proper milk production between the current study conducted at Assosa small household dairy farms (traditional dairy farms) and that of Seblewengele ,conducted in Alage ATVET college dairy farm, this is may be due better management and good hygienic practices conducted at Alage farm compared to Assosa district small households farms. In this study, the CFU/ml of *Bacillus cereus* ranged from $1.09x10^3$-$1.10x10^7$CFU/ml ($5.50x10^6$ave.). This finding is lower when compared with the work of (DeGraff *et.al.,* 1997) ($3.88x10^7$CFU/ml), (Godifay and Molla ,2000) ($1.9x10^8$CFU/ml) and (Esther *et.al.* 2004) ($3x10^7$CFU/ml) and higher the finding of (Alemneh ,2012) from Ethiopia ($2.8x10^5$), (Rai and Dawvedi,1990) from India ($7.7x10^5$CFU/ml), (Kurwijilla *et al.* ,1992) from Tanzania (10^5CFU/ml), (Ombui *et al.* ,1995) from Kenya ($5x10^4$CFU/ml), *(Hassan e tal.*,2010) from United stated of America ($9.11x10^2$) ,however, it is in the same range with the finding of (Bonfoh *et.al.*,2003) from Mali (10^6CFU/ml) from raw bovine milk. This high bacterial count is good indicator for monitoring hygiene and management practices during production

,collection,and handling of raw milk (Murphy,1996; Yilma and Faye,2006; Lingathurai and Vellathurai, 2010)

The presence of *Bacillus cereus* were significantly variable with hygienic status of the barn ($p<0.05$).Hygienic status of the barn of smallholder dairy farms has a high implication on the positivity of *Bacillus cereus.* As there is poor hygienic practice conducted, the chance of being contamination raw bovine milk with *Bacillus cereus* increase. As the laboratory result revealed in the current study 45.9%, 11.0%, 8.2% were found in poor, intermediate and good hygienic practices respectively. Therefore, it is likely that raw milk may be contaminated with *Bacillus cereus* from manure, soiled bedding and soil. The present finding was supported by (Connell *et al.*, 2013) from USA, (Yobouet *et al.*, 2014) from Abidjan, Côte d'Ivoire.

The positivity of *Bacillus cereus* was not significantly different with in parities classes in raw milk ($p>0.05$) but there is higher positivity at the middle class of parity. This may be due to high milk yield and large udder size at the mid-parity, so while the cow rests there may be direct contact with the ground, urine, and dung and feed refusals which increase the chance of contamination (Galton *et al*., 1986). The present finding was supported the work done by (Seblewengel, *2013*) in Ethiopia.

The presence of *Bacillus cereus* were significantly variable with CMT reaction ($p<0.05$). It indicates *Bacillus cereus* is one of the causative agents for the occurrence of mastitis. The present finding supports the work done by (Brown and Scherer, *1957*) from California, (Jones and Turnbull, 1981) from British, (Schiefer *et al*., 1976) from Canada,. Their report describes *Bacillus cereus* as the cause of mastitis in cows and (Girma *et al., 2012*) and (Gizaw,2004) in Ethiopia, also describes *Bacillus cereus* as one cause of mastitis. Therefore, frequent monitoring and treatment of dairy cows for presence of mastitis is very crucial to control shading of the bacteria into raw milk.

There was significant variation of lactation stage on the prevalence of *Bacillus cereus* ($p<0.05$). There was an increase in prevalence of *Bacillus cereus* at mid-class lactation stage. This may be due to high milk yielding capacity, large udder size, low attention given by the owner of the cow on the management practices, this directly exposes the cow to direct contact with the ground, urine, and dung and feed refusals which increase the chance of contamination (Galton *et al*., 1986). This indicates the middle stage of lactation is very important for the occurrence of *Bacillus cereus.* Therefore, consumers and milk producers need to be careful in handling milk originating from cows at their middle lactation stages since such cows are exposed to higher bacterial contamination. The present finding was supported by the work done by (Seblewengel, *2013*) in Ethiopia.

As observed during the current study 97.5% of interviewed personnel consume milk and out of these respondents 29.1% consume Raw(fresh whole milk). Cross-contamination with pathogenic micro organism can gain access to milk either in facal, soil, feed refusals contamination or direct excretion from the udder into milk (El-Ziney and Al-Turki, 2007).Thus consumption of raw milk with no treatment may pose public to *Bacillus cereus* food born poisoning as a result of contamination .Similarly numerous epidemiological reports have implicated non-heat treated milk and raw milk products as the major factors responsible for illness caused by food born pathogens (El-Ziney and Al-Turki, 2007)

About 41.7% of the respondents consume milk as a common diet and 42.5% of the respondents reported their family had faced illness after consumption of milk with 31.4%,25.5%,19.6 and 23.5% clinical sign and symptoms of diarrhea, vomiting, both diarrhea and vomiting and abdominal cramp and pain respectively.34.2% of the respondents had awareness in milk born disease but no one described bacillus cereus food poisoning directly rather they explained the disease indirectly through the illness faced as diarrhea, vomiting and abdominal cramp and pain. This finding was similarly reported by (Logan and Rodrigez,2006) .About 27.1% of the respondents consume milk stayed for long time, this might favor bacterial growth and

multiplication which leads to increase of pathogenic bacteria load .Similarly (Fara *et al*., 2007) reported as there is delay in storage time bacterial load show highest count.

The hygienic condition or quality of milk has serous implication on public health safety. The questionnaire result mainly gave broad understanding the milking and hygienic practice in the study area.Maintaining the hygienic conditions of dairy house, milking area, milking equipment and milker's hand is important for production of good quality milk (Slaghuis et al.,1997,Christiassen.,1999;Magnusson *et al.*,2007;Yilma,2012). As observed during the current study most of the farmers milk their animals under poor hygienic conditions of traditional barns, which are often contaminated with mud, animal dung, dust/sand, faecal materials and urine, and in study observation there are several flies usually surrounding around the milking area. Therefore, it is likely raw milk might be contaminated from manure, urine, soiled bedding, grazing pasture, feed refusals and soil and this report was supported by the findings (Conner and Charles, 1995).

It was reported by Galton *et al* .(1986) pre-milking udder preparations play an important part in the contamination of milk during milking. Cleaning the udder of cow before milking is important since it could have direct contact with the ground,urine dung,and feed refusals while resting.Not washing udder before milking can impart possible contaminants into the milk. As observed during the present study, only 24.2% of the respondents wash cow's udder before milking. The remaining respondents let the calves to suckle before milking.Such practice,however cannot replace washing.This is similarly reported by Yilma (2012).Farmers should,therefore,make udder washing a regular practice in order to minimize contamination and produce good quality milk.In most of the cases under smallholer conditions,the common hygienic measures taken during milk production especially during milking are limited to letting the calve to suckle for few minutes and or washing the udder only before milking .

In this study, about 67.5% of the respondents reported to use well water for cleaning purpose(udder, milk equipment, and hand) and this insecured water quality may increased the contamination probability. This was similarly reported by yilma (2003).

Equipments used for milking, processing and storage determine the quality of milk and milk products(Patsy *et al* .,2005;Yilma,2012). In this study 81.7% of the respondents were using plastic material for collecting and handling milk. Although, alumininum and clay pot are preferred, only 16.7%and 1.7% of farmers reported it respectively. Thus, plastic container scratch easily and provide hiding places for bacteria during cleaning and sanitization and are poor conductors of heat and hence hinder effective sanitization by heat .In the same way (Soomro *et al*.,2003).Donkor *et a*l.(2007) reported that plastic containers, which was the type used by almost all market agents was associated with high SPC(standard plate count)compared with other container type which include mainly metal and wood. Milking equipment should be easy to clean. Producers or farmers need; therefore, pay particular attention for the type as well as cleanliness of milking equipments.

Antibiotic resistant bacteria pose growing problems of concern, worldwide since the bacteria can be easily circulated in the environment. Effectiveness of current treatments and ability to control food born pathogens both in animal and human may become hazardous. A relatively high number of bacterial strain are resistant to antimicrobial used in therapeutic protocols of many human and animal infection (Normannon *et al*., 2007)

In this study, *bacillus cereus* isolates were most susceptible to Vancomycin(92%), chloramphnicol (88%) and clindamycin (84%) and the isolates relatively showed highest resistant to Polymixin B(100%), tetracycline(88%), Ampicilin (80%),penicillin G(76%), and kanamycin (72%). Since as the drug sensitivity test indicate that the microbe is highly susceptible to vancomycin,and chloramphnicol.Therefore,we can consider vancomycin and chloramphnicol as the drug of first choice . This result is similar with the findings of (Schlegelova *et al*.,2003),(Whong and Kwaga, 2007), (Agwa *et al*., 2012), (Sankararaman and Velayuthan, 2013),(Seblewegele,2013).The variability in susceptibility result partly arise from the difference of how frequent a drug was in use in the study area.

Therefore,infections with Bacillus cereus may not be cured by treatment regimes PolymixinB,tetracycline**,**Ampicilin,penicillin G and kanamycin.Unfortunately, intramammary infusions available in the country contain penicillin preparations; infusions containing vancomycin and chloramphenicol are not available yet. Therefore, proper communication of such

results to the relevant bodies is important to import Vancomycin and chloamphenicol containing intramammary infusions so as to prevent the public from health problems that may originate from *Bacillus cereus* and its toxin.

6. CONCLUSION AND RECOMMENDATIONS

The consumption of raw milk and its derivatives is common in Ethiopia, which is not safe from consumer's health point of view as it may lead to transmission of various diseases. In this study the results of bacteriological assessment showed that raw milk from 86 lactating dairy cows were highly contaminated with *B. cereus.* The majority of *Bacillus cereus* positive cows had bacterial load beyond the legal limits 65/86 for human consumption, this *B. cereus* load above acceptable limit in raw milk was an alarm for public health implication. Contamination rate of raw milk with *B. cereus* was associated with risk factors like hygienic conditions, lactation length, mastitis reactivity ($p< 0.05$). *Bacillus cereus* was found to be one of the causes for mastitis .Unclean milking and housing environment, improper hygienic condition and lack of awareness of the farmers may play a great role on the contamination of raw milk with the bacteria. *B.cereus* causes food poisoning in human and mastitis in cows. To treat the disease caused by *Bacillus cereus* Vancomycin and chloamphenicol are the drug of choices.

Therefore, based on the above conclusion the following recommendations are forwarded:

- The hygiene condition of small household dairy farms and milkers should be improved to reduce *B. cereus* load below acceptable limit ($<10^5$) for public consumption and prolong the keeping quality of raw milk.

- Milk for public consumption should be properly boiled at appropriate temperature and time.

- Treatments of *Bacillus cereus* infected cows should be done based on drug susceptibility testing.

- Awarness should be created among small house hold dairy farmowners on the public health importance, contaminatin sources of disease and proper hygienic practice implementation.

- Further study should be conducted in determining the status of *B. cereus in small house farms* and its toxins in raw milk in different parts of the country.

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8. APPENDICES

Appendix I: Questionnaires for:

A. Public Health Implication Assessment

Respondent name _____Date_____

Code for lactating dairy cow _____Address/kebele _____

1- Do you consume the milk at home?

A. Yes B. No

2-If Yes, by what form?

A. Raw (fresh whole milk) B. Ergo (naturally fermented milk)

C. Ayib(Ethiopian cottage cheese) D. boiled

3. Did one of your family members become ill after consuming the milk?

A. Yes B. No

4. If yes, which of the following signs did he or she showed?

A. Diarrhea B. Vomiting

C. both diarrhea and vomiting D. abdominal pain and cramp

5. Do you know any milk borne diseases?

A. Yes B. No

6. If, yes to question number 5 can you name them----------

7. How often do you and your family consume the milk and its products?

A. For breakfast B. As common diet

C. Frequently D. As required

8. Is there any time gap between taking & using the milk?

A. yes B. No

9. If yes how much is the average time gap?

A. 1-4hrs B .4-10hrs C.10-16hrs D. more than 16hrs

B. Milk Contamination Assessment

1. How do you keep your dairy cows?

A. day time in field and night at home B. day and night in the house

C. day and night in the field D. in fenced barrels

2. What kind of floor did you use for housing dairy cows?

A. concrete ground B. natural floor

C. straw bedded floor D. stone and sand

3. What about for milking areas?

A. concrete ground B. natural floor

C. straw bedded floor D. stone and sand

4. How often did you clean your cows' house?

A. daily B. weekly C. monthly D. yearly

5. How often did you clean your milking cows' udder?

A. Only calve suckle it B. Only before milking

C. Only after milking, D. Before and after milking,.

6. What kind of milking equipment did you use?

A. Aluminum cans B. Plastic beaker

C. clay pot D. other traditional

7. What is the source water do you use to clean your equipment to take the milk from the lactating dairy cow and the udder?

A. River B. well water C. tap water D. stagnant water

8. Do you wash your hand before milking?

A. yes B. No

9. If yes, What is the source water you use to wash your hand before milking?

A. River B. well water C. tap water

Appendix II: Equipment and materials used for the study

1. Pipettes, 1, 5,10 and 15 ml, graduated in 0.1 ml units
2. Glass spreading rods (hockey stick) 3-4 mm diameter and 45-55 mm length
3. Incubators, 30 ± 2°C and 35 ± 2°C
4. Manual colony counter chamber
5. Marking pen, (waterproof or permanent ink)
6. Bunsen burner
7. Wire loops, platinum wire, 2-3mm
8. Vortex mixer
9. Microscope, microscope slides
10. Culture tubes, 13 x 100 mm sterile
11. Test tubes, 16 x 125 mm
12. Bottles, sterile
13. Water bath, 48-50°C
14. Culture tube racks
15. Staining rack
16. Petri dishes, sterile, 15 x 100 mm
17. Dilution bottles,
18. Refrigerator
19. Safty cabinent

Appendix III: Data collection sheet for each small household.

Appendix table 1: Data collection sheet for each small household.

No	Date	Owner name	Address	Animal code	Parity	Lactation length	Hygienic condition			Milk quality assessment
							poor	intermediate	good	

Appendix IV: Media and reagents used for the study

1. Bacillus cereus selective agar base (MC0167, Oxoid, Ltd.)
2. Egg yolk emulsion, 50% (M514)
3. Nutrient agar (CM0003)
4. Blood Agar (CM0854; Oxoid Ltd)
5. Motility medium (SIM) (M181)

6. dilution water (R1117) sterilized, with 450 ± 5 ml and 90 ± 2 ml
7. Gram stain reagents (R3219)
8. Basic fuchsin staining (R320)
9. Methanol 95%, Ethanol 70%

Appendix V: Composition and preparation of media used for the study

A. Bacillus cereus selective agar base (CM0167, Oxoid)

Typical Formula of *Bacillus cereus* Agar base

Typical formula	g/L
Peptone	1.0
Mannitol	10.0
Sodium Chloride	2.0
Magnesium Sulfate	0.1
Disodium Phosphate	2.5
Potassium dihydrogen Phosphate	0.25
Sodium Pyruvate	10.0
Bromothymol Blue	0.12
Agar	15.0
Supplement Polymyxin B (100,000IU)	0.015
Supplements Egg Yolk Emulsion	50 ml
Final Ph 7.2 $\pm$ 0.2 at 25 ^{0}C	

Instruction for use:

Suspended 20.5g in 475ml of distilled water and brought gently to the boil to dissolve completely. Sterilized by autoclaving at 121^{0}C for 15 minutes. Cooled to 50 ^{0}C and aseptically add the contents of supplements reconstituted as directed. Mix well and poured into sterile Petri dishes.

B.Blood agar (Oxoid, England)

Typical formula (g/l):

'Lab-Lemco' powder	10.0
Peptone	10.0
Sodium chloride	5.0
Agar	15.0

Final pH 7.3 ± 0.2 at 25^0C

Instructions for use:

Suspended 40g in 1litre of demineralised (distilled) water. Bring to the boil to dissolve completely. Sterilized by autoclaving at 121^0C for 15 minutes. Cool to 45-50^0C and add 7% sterile defibrinated blood and display on sterile petridish

C.Nutrient agar (Oxoid, England)

Typical formula (g/l):

'Lab-Lemco' powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0

Final pH 7.4 ± 0.2 at 25^0C

Instruction for use:

Suspend 28g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121^0C for 15 minutes and display on sterile petridish.

D.Muller Hinton Agar

Formula

Beef extract	2 g
Acid hydrolysate of casein	17.5 g

Starch	1.5 g
Agar	17.0 g

Instruction for use:

Suspended 38 g of the powder in 1 liter of purified water mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121 ^{o}c for 15 minute. Do not over heat. Display on sterile petridish.

Appendix VI: Detailed sampling procedures

a. Label tubes prior to sampling by (date, farm, cow, any changes in milk)

b. Brush loose dirt, bedding, and hair from the udder and teats and thoroughly wash

c. Discard several streams of milk from the teat (strict foremilk) and observe milk

d. Dip all quarters in an effective pre-milking teat disinfectant

e. Dry teats thoroughly with an individual towel.

f. Beginning cleaning teats on the far side of the udder, scrub teat ends vigorously

g. Begin sample collection from the closest teat and move to teats on the far side of the udder.

h. Remove the cap from the tube or vial but do not set the cap down or touch the inner surface of the cap.

i. Always keep the open ends of the cap facing downward. Maintain the tube or vial at approximately a 45 degree angle while taking the sample. Do not allow the lip of the sample tube to touch the teat end. Collect one to three streams of milk and immediately replace and tightly secure.

I. To collect a composite sample (milk from all four quarters in the same tube), begin sample collection with the nearest teats and progress to the teats on the far side of the udder. One to 2 ml of milk should be collected from each quarter of the udder.

II.When samples are taken at the end of milking or between milkings, teats should be dipped in an effective germicidal teat disinfectant following sample collection.

III. Store samples immediately on ice or in some form of refrigeration. Samples to be cultured at a later date (more than 48 hours) should be frozen immediately.
Source: NMC publication: Microbiological Procedures for the Diagnosis of Bovine Udder Infection and Determination of Milk Quality, Pp 24 (4th-Ed. 2004).
http://nmconline.org/articles/corynnotes.htm

Appendix VII: chemicals and procedures for staining

A. Gram Stain

Reagents: 1% aqueous Crystal violet
Gram's Iodine
Safranin
Distilled H_20
Alcohol (70%)

Procedures:

1. Prepare thin fixed smears of culture by adding 2 – 3 loopfulls of tap water onto slides, then using a flamed loop aseptically transfer a small amount of culture to a slide. Emulsify the

bacterial cells in the water over an area of approximately 1square cm. Allow smears to dry and fix heat fix gently passing on flame.

2. Place the prepared smears on the staining rack – apply crystal violet to just cover the smear – usually one or two drops. Leave the dye on for one minute, then rinse off in a gentle stream of water. Shake the slides to remove excess water.

3. Flood the smears with the gram's iodine solution; allow standing for one minute, washing with water.

4. Add alcohol to the smear and gently rock the slide, tip off alcohol and repeat. Contact time with the alcohol should be approximately for 30 seconds by this time most of the blue coloration should be removed. Wash in water.

5. Stain with safranin for 10 seconds, wash in water, shake off excess water and allow airing dry or gently blotting dry with tissue paper taking care not to remove the cells.

6. Examine microscopically, and record cell morphology and gram reaction.

B. Rapid Confirmatory Staining Procedure.

This staining method was developed by Holbrook and Anderson combining the spore stain of Ashby and the intracellular lipid stain of Burdon (Holbrook and Anderson, 1980).

Fuchsin solution preparation:

0.3 g of basic fuchsin
10 ml of ethanol, 95% (v/v)
5 ml of phenol, heat-melted crystals
95 ml of distilled water

Dissolve the basic fuchsin in the ethanol; then add the phenol dissolved in the water. Mix and let stand for 2-3 days. Filter before use.

Decolorizing solvent (alcohol) is 97 ml of ethanol, 95% (v/v)

Malachite green stain:

(0.5% (wt/vol) aqueous solution:

0.5 g of malachite green

100 ml of distilled water

Sudan black B is a lysochrome (fat soluble dye) predominantly used for demonstrating triglycerides in frozen sections. It is also valuable for demonstrating some protein bound lipids in paraffin sections. It may also stain other materials, not being completely restricted to lipids as the other dyes used.

Safranin counterstain:

Stock solution (2.5% (wt/vol) alcoholic solution)

2.5 g of safranin

100 ml of 95% ethanol

Working solution 10 ml of stock solution

90 ml of distilled water

Procedure:

1. Prepare films from the centre of a 1 day old colony or from the edge of a 2 day colony.
2. Air-dry the film and fix with minimal heating.
3. Flood the slide with aqueous 5% w/v malachite green and heat with a flaming alcohol swab until steam rises. Do not boil.
4. Leave for 2 minutes without re-heating.
5. Wash the slide with running water and blot dry.
6. Flood the slide with 0.3% w/v Sudan black in 70% ethyl alcohol. Leave for 15 minutes.
7. Wash the slide with running xylene from a wash bottle for 5 seconds.
8. Blot dry using filter paper.
9. Flood the slide with aqueous 0.5% w/v safranin for 20 seconds.
10. Wash under running water.

11. Blot dry and examine under the microscope using the oil immersion lens. A blue filter may be used to accentuate the appearance of the lipid granules but this will give a blue color cast to the red of the cytoplasm.

Appendix VIII: Differential characteristics of large-celled Group I *Bacillus* species

Appendix table 2: Differential characteristics of large-celled Group I *Bacillus* species

Feature	***B.Cereus***	***B.thuringiensis***	***B.mycoides***	***B.weihenstephanensis***	***B.anthracis***	***B.megaterium***
Gram reaction	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Motility	+	+	-	+	-	+/-*
Nitrate reduction	+	+	+	+	+	−
Tyrosine decomposing	+	+	+/−	+	−	+/−
Lysozyme resistance	+	+	+	+	+	−
Egg yolk reaction	+	+	+	+	+	−*
Anaerobic Glucose use	+	+	+	+	+	−
VP reaction	+	+	+	+	+	−
Ferment mannitol	−	−	−	−	−	+
Hemolysis on Sheep Blood agar	+	+	+	-	−	−
Para-sporal crystal formation	-	+	-	-	-	-
Known characterstics	Enterotoxin production	Endotoxin crystal (insecticide) production	Rhizoidal growth	Growth at 6 ^{0}C ; no growth at 43^{0}C	Pathogenic to animals and	

				human s

-*, most strains are negative +/−*, 50-50% of strains are positive.

Source**: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998, Chapter 14. Updated February 2012**

Appendix IX: Interpretation of CMT Scores and Corresponding SCC values

Appendix table 3: Interpretation of CMT Scores and Corresponding SCC values

CMT Score	**Visible reaction**	**SCC range (Cells per ml)**	**Somatic cell score**	**Approximate SCC midpoint**
Negative	Mixture remains liquid – no evidence of precipitate	0 – 200,000	0 1 2 3 4	12,500 25,000 50,000 100,000 200,000
Trace	Slight precipitate, best seen by tipping, disappears with continued movement	150,000 – 500,000	5	400,000
1	Distinct precipitate but no tendency toward gel	400,000-1,500,000	6	800,000

	formation			
2	Mixture thickness immediately, moves toward center	800,000 – 5,000,000	7 8	1,600,000 3,200,000
3	Gel forms and surface becomes convex	>5,000,000	9	6,400,000

Source: NCCLS, (1998)

Appendix x: Standards for interpretation

Appendix table 4: Zone Size Interpretive Chart for Antimicrobials

Antimicrobial agents	disc potency	resistance	intermediate	susceptible
Clindamycin	10µg	≤ 15	16-18	≥19
Tetracycline	10 µg	≤ 14	15-18	≥19
Penicillin for staph	10 U	≤ 20	21-28	≥ 29
Penicillin for others	10 U	≤ 11	12-14	≥ 22
Vancomycin	30µg	≤ 9	10-11	≥12
Chloramphenicol	30 µg	≤12	13-17	≥18

Gentamycin	10 mcg	≤12	-	≥13
Kanamycin	30 µg	≤13	14-17	≥18
Ampicillin	10µ	≤13	14-16	≥17
Polymixin B	300 µg	≤11	-	≥12

Source: NCCLS, (1998) and C LSI,(2012)