INTRODUCTION

Members of the genus *Bacillus* can cause illness and should not be ignored when isolated form multiple blood cultures **(Hugo and Russell, 1983).**

The genus *Bacillus* consists of a heterogenic group of Gram positive, endospore forming, rod-shaped, facultative anaerobic bacteria that are widely distributed in nature. Due to their endospore-forming abilities, these bacteria tolerate adverse conditions better than the non sporulating bacterial entero-pathogens and may proliferate in a wide range of environments including water, processed and untreated foods **(From *et al.*, 2005).**

Bacillus cereus is a widely distributed bacterium that is commonly found in the soil and has been isolated from a variety of foods, including rice, species, meat, eggs and dairy products **(Wong *et al.*, 1988).** The bacterium, which is best known as the causal organism of two distinct food poisoning syndromes, has also been implicated in a wide variety of illness, including systemic infections and panophthalmitis. The virulence factors of this bacterium remain ill defined partially because it produces a large number of proteins that potentially possess toxigenic activity because these metabolites are difficult to isolate **(Turnbull, 1981).**

There is some evidence to suggest that phospholipase C might play an axillary pathogenic role by breaking down host

cell membrane phospholipids exposed by the action of other enzymes or toxins **(Turnbull and Kramer, 1983).**

Enterotoxicity is due to single protein with vascular permeability activity and mouse lethal activity. It is still not clear whether the other two proteins are enterotoxic, cytotoxic or non-toxic **(Shinagwa *et al.*, 1992**; **Granum and Nissen, 1993).**

The molecular nature of virulence of *B. cereus* is poorly understood **(Beecher and Wong, 1994a)**. The organism is the common cause of highly fluminant post traumatic and metastatic endophthalmitis. Exotoxins or enzymes likely contribute to the severity of infection.

Concerning intestinal pathologies associated to *B. cereus,* two main syndromes have been reported: emetic and diarrhoeic. Whereas emetic syndrome is associated to cereulide, a thermostable dodecadepsipeptide **(Agata *et al.*, 1994; Horwood *et al.*, 2004; Ehling-Schulz *et al.*, 2005),** the diarrhoeic syndrome has been related to plethora of extracellular factors with different biological effects **(Granum, 1997; Alouf, 2000; Beecher *et al.*, 2000; Fagerlund *et al.*, 2004).**

Scientific data suggest toxin production occurs among *Bacillus* species other than *Bacillus cereus* and more specifically, among strains of *Bacillus licheniformis.* Suspiction of toxin production in strains of these species originates from

reports implicating *B. licheniformis* occasionally and *Bacillus subtilis* more rarely as a cause of food poisoning **(Kramer and Gilbert, 1989; Drobniewski, 1993; Turnbull and Kramer, 1995).** Cross-reactions with *B. cereus* enterotoxins have been shown by use of commercial immunological kits for some strains of *B. licheniformis* **(Beattie and Williams, 1999),** and homology between enterotoxin genes in -*B. cereus* and strains of other *Bacillus* spp. was indicated by polymerase chain reaction (PCR) **(Chaithong *et al.,* 1999).** Further in vitro cytotoxicity has been demonstrated in culture broths of some strains of *B. licheniformis* and *B. subtilis* **(Beattie and Williams, 1999; Salkinoja-Salonen *et al.,* 1999; Mikkola *et al.,* 2000).**

It is widely recognized that a pharmaceutical product contaminated with microorganism may present a potential health hazard to the patient and the contaminants may alter physical characteristics, stability and even the activity of the medicament itself **(Hugo and Russel, 1983).**

AIM OF THE WORK

This study aimed to evaluate the microbial load of *Bacillus* spp. contaminated some pharmaceutical products, and trying to get rid of the contamination to solve this important problem which represents health hazard.

1. REVIEW OF LITERATURE

1.1. Pathogenicity and Virulence of *Bacillus* species

There has been an increasing number of reports about infections with *Bacillus* spp other than virulent *Bacillus anthracis* **(Pearson, 1970).**

Bacillus spp caused systemic diseases such as meningitis, pneumonia, and sepsis **(Ihde and Armstrong, 1973).**

However there has been increased appreciation of the pathogenicity of these organisms, particularly *Bacillus cereus* **(Craig *et al.*, 1974; Turnbull *et al.*, 1977; Turnbull *et al.*, 1979; Mittel and Lusins, 1980; Biasioli *et al.*, 1984; Bekemeyer *and* Zimmerman, 1985).**

Bacillus organisms, except for *B. anthracis*, are generally regarded as saprophytes but can become pathogenic if the host's local or systemic defense mechanism are altered **(Tabbara and Tarabay, 1979).**

The most common *Bacillus* isolates implicated in post traumatic endophthalmitis include: *Bacillus cereus, Bacillus subtilis,* and *Bacillus licheniformis* **(Kotiranta *et al.*, 2000; Gerri *and* Hall, 2006)**.

Endophthalmitis caused by *Bacillus* in the setting of ocular trauma can cause complete corneal opacification within

24 hours of injury **(Spalding and Sternberg, 1990).** In cases of post-traumatic endophthalmitis caused by *Bacillus cereus* within 18 to 24 hours after injury, patients may begin to experience severe pain and may have chemosis, periorbital edema, proptosis, and peripheral corneal edema **(O'Day *et al.*, 1981).** In an experimental model of *Bacillus cereus* endophthalmitis, mouse eyes were injected with this microbe intravitreally, and polymorphonuclear leukocyte infiltration into the vitreous cavity began by 4 hours after inoculation **(Ramadan *et al.*, 2006).** The presence of a corneal ring abscess is particularly suggestive of infection with *Bacillus* as is the presence of systemic signs such as fever **(Hemady *et al.*, 1990).**

Bacillus species spores are abundant in soil **(Fekete, 2005),** which may explain why *Bacillus* endophthalmitis occurs relatively more commonly in cases of rural post-traumatic endopthalmitis. This species is rarely a part of the normal human conjunctival flora **(Parrish and O'Day, 1987).**

Endophthalmitis caused by *Bacillus* species often results in poor visual outcome **(Miller *et al.*, 2008).**

Clinical infections caused by *B. cereus* fall into six broad groups: (i) local infections, particularly of burns, traumatic or post surgical wounds and the eye **(Davey and Tauber ,1987; Al-Hemidan *et al.*, 1989; Beer *et al.*, 1990; Akesson *et al.*, 1991);** (ii) bacteremia and septicemia **(Cotton *et al.*, 1987; Banerjee *et al.*, 1988)**; (iii) central nervous system infections, including meningitis, abscesses, and shunt – associated

infections **(Colpin *et al.,* 1981; Feeder *et al.,* 1988; Barrie *et al.,* 1992);** (iv) respiratory infections **(Bekemeyer and Zimmerman, 1985; Carbone andStauffer,1985; Funada *et al.,* 1991; Gascoigne *et al.,* 1991);** (v) endocarditis and pericarditis **(Craig *et al.,* 1974; Block *et al.,* 1978);** and (vi) food poisoning, characterized by toxin induced emetic and diarrheagenic syndromes **(Kramer and Gilbert, 1989; Shingawa, 1990; Abo-State, 1996).**

Endogenous panophthalmitis due to *B. cereus* has been reported with intravenous drug abuse **(Tuazon *et al.,* 1974)** and with contaminated intravenous medication **(Bouza *et al.,* 1979).**

It is therefore of interest that *B. cereus* was recovered from the conjuntiva of one patient at the initial examination **(Patrick *et al.,* 1982).**

Bacillus cereus is a major cause of severe keratitis, endophthalmitis, and panophthalmitis **(Ansell *et al.,* 1980; Al-Hemidan *et al.,* 1989).**

In one US. Study of post traumatic endophthalmitis, *Bacillus* species were the second most common organism isolated after *Staphylococcus epidermidis* **(Davey and Tauber, 1987).** More alarming, ocular infections due to *B. cereus* appear to have increased over the last 15 years, particularly among the immunocompromised and intravenous drug abusers **(Young *et al.,* 1980; Greenwald *et al.,* 1986).**

Barnham (1980); White (1980) and Melling and Gilbert (1980) reported that *B. cereus* was the causal organism of pathogenicity in eight cases of wounds; a case of stickey eye, a case of pyrexia after caesarian section and local infections. **Davey and Tauber (1987)** indicated that *B. cereus*, an especially virulent pathogen, caused **a** fulminant endophthalmitis characterized by rapid destruction of intravitreal contents and a uniformly poor visual outcome. **Gigantelli *et al.* (1991)** mentioned that *B. cereus* had emerged as one of the most virulent bacteria affecting the eye, causing a destructive endophthalmitis following trauma or intravenous drug use. **Beecher *et al.* (1995)** reported that, in vivo, *B. cereus* toxins caused endophthalmitis clinically characteristic of *B. cereus* within 4 hours. Histological changes included rapid retinal necrosis and detachment, choroidal edema, detachment and disruption of retinal pigment epithelium and rapid infiltration of polymorphonuclear leukocytes.

Post-traumatic endopthalmitis is an uncommon yet devastating complication of an open globe injury. Risk factors include presence of an intraocular foreign body (IOFB), lens rupture, delayed primary globe repair, rural trauma, and trauma with contaminated objects. Visual prognosis in post-traumatic endopthalmitis is affected by the virulence of the microbe, the presence of a retinal break or detachment, the timing of treatment, the presence or absence of an IOFB, and the extent of initial injury **(Bhagat *et al.*, 2011).**

Several species of the *Bacillus* group recovered from eye infections have been identified as causative; for example *B. laterosporus* **(Tabbara *et al.*, 1977),** *B. cereus* **(Bratcher, 2003),** and *B. subtilis* **(Pearson, 1970).** Although *B. licheniformis* has been reported as a cause of sepsis in a patient with no known immunologic defect **(Tabbara and Tarabay, 1979)**. Various *Bacillus* species were found in skin ulcers, conjunctival ulcers, traumatic and surgical wounds, burns, bone fracture sites and sites of osteomyelitis, urogenital infection **(Pearson, 1970)**, also *Bacillus* spp. can be opportunistic pathogens in granulocytopenic patients **(Ihde and Armstrong, 1973).**

Bacillus spp. present a threat to the compromised patient under gnotobiotic care **(Colpin *et al.*, 1981).**

Richard *et al.* (1988) described 11 cases of *Bacillus* bacteremias of which *B. subtilis* was isolated in eight patients. Four of these suffered from cancer disease and four others had head trauma, stroke or had undergone surgery.

B. licheniformis should be considered as a potential pathogen in immunocompromised patients, especially when bacteremia is associated with the presence of long term central venous catheters **(Blue *et al.*, 1995).**

Although normally considered soil organisms, members of the spore forming genus *Bacillus* can inhibit the gastrointestinal tract (GIT) of insects and animals **(Hong *et al.*, 2005)**, pathogens such as *Bacillus anthracis, Bacillus cereus,*

Bacillus thuringiensis and *Bacillus sphaericus,* entry into the GIT is an essential part of their virulent life cycle **(Nicholson, 2002; Jensen *et al.*, 2003).**

Periodically, over the following millennia, there were outbreaks of anthrax world-wide. For example, there was a substantial outbreak in Germany in the 14th century. During the 17th century, there were large outbreaks in Russia and one in Europe that killed more than 60.000 head of cattle. During the mid 18th century, a panzootic consumed half of the sheep in Europe **(Witkowski, 2002).**

B. anthracis was found in various tissues that were tested at the time of autopsy. The presence of *B. anthracis* was found in the lungs (9 cases); mediastinum (7 cases), spleen (6 cases); brain and liver (5 cases each); lymph nodes (4 cases); small intestine, kidneys, and cerebrospinal fluid, and brain in different descriptions **(Kenneth Alibek *et al.*, 2005)**.

A recent study has identified *Bacillus* spore – formers in human feaces **(Fakhry *et al.*, 2008).** It has been shown recently that spores of a laboratory strain of *Bacillus subtilis*, are able to germinate in the jejunum and ileum of mice dosed orally with spores **(Cansula and Cutting, 2002; Tam *et al.*, 2006).** Surprisingly, germinated spores could outgrow and then, as they progressed into the upper colon, re-sporulate. This phenomenon was also observed with other, natural isolates of *B. subtilis* that had been recovered from human feaces, suggesting that *B. subtilis* could use the GIT for both growth and sporulation.

1.1.1. *Bacillus* spp. toxin(s)

Bacillus sp. capable of producing a toxin (Lecithinase) that is cytotoxic, thrombotic, and lethal in experimental animals **(Turnbull *et al.,* 1979),** and it is well known to produce extracellular protease **(Pedersen *et al.,* 2002).** The toxin is an extremely stable dodecadepsipeptide which acts as potassium ionophore **(Mikkola *et al.,* 1999).**

Bacillus cereus is the aetiological agent of two distinct types of food poisoning. One is the diarrhoeal syndrome, which is characterized by abdominal pain with diarrhea after 12-24h. four different heat labile enterotoxins have been implicated in the diarrhoeal syndrome and have been described from various strains: two protein complexes, haemolysin BL (HBL) and non haemolytic enterotoxin (NHE) **(Beecher and Wong, 1994; Lund and Granum, 1996).** The second kind of illness, the emetic syndrome, is characterized by nausea and vomiting occurring 1-5 h after ingestion of, predominantly, rice dishes **(Kramer and Gilbert, 1989);** and is caused by a heat stable dodecadepsipeptide. The emetic toxin, named cereulide **(Agata *et al.,* 1995a)** is produced during bacterial growth in contaminated foods. The emetic syndrome is potentially more dangerous than the diarrhoeal syndrome **(Mahler *et al*., 1997)** and there other three *B. cereus* enterotoxins are monomeric. Enterotoxin T (bce T) **(Agata *et al.,* 1995b; Ombui *et al.,* 1997)** exhibits toxicity in laboratory tests although it's enterotoxin **(Choma and Granum, 2002).** Cytotoxin K (cyt K)

was implicated in a food poisoning outbreak shows necrotic and haemolytic activity, and is highly toxic to epithelial cells **(Hardy *et al.*, 2001; Lund *et al.*, 2002).** There are two forms of cyt K: cytK 1 is highly toxic to human epithelial cells and cytK2 is more common but for less toxic to human epithelial cells **(Fagerlund *et al.*, 2004).** Enterotoxin FM (enFM) is a relatively unknown hemolytic enterotoxin which is cytotoxic to vero cells **(Asano *et al.*, 1997).**

Didelot *et al.*, (2009) found that *Bacillus cereus* group of bacteria that cause wide range of disease in humans, including food poisoning systemic infections and highly lethal forms of anthrax.

Enterotoxins of *Bacillus cereus* are known to cause two different types of food poisoning **(Schoeni and Wong, 2005; Stenfors *et al.*, 2008),** which are characterized by either emesis or diarrhea. The diarrheal type has been linked to a single protein cytotoxin K1, **(Lund *et al.*, 2000)** as well as two enterotoxin complexes as causative agents **(Beecher *et al.*, 1995; Lund and Granum, 1996).** Enterotoxin production is a key factor in *Bacillus cereus*.

Bacillus cereus is a recognized human pathogenic bacterium that causes local or systemic infections and is frequently implicated in cases of food-borne poisoning **(Schoeni and Wong, 2005).**

Cereulide is a cyclic dodecadepsipeptide, a potassium ionophore and mitochodriotoxin **(Sakurai *et al.,* 1994; Agata *et al.,* 1995a; Shingawa *et al.,* 1995; Mikkola *et al.,* 1999; Hoornstra *et al.,* 2003; Teplova *et al.,* 2006).** It is one of the most toxic substances among the known heat-stable toxins of microbial origin **(Andersson *et al.,* 2007)** and has caused even fatalities in human **(Mahler *et al*., 1997; Dierick *et al.,* 2005).**

The effect of carbohydrate on enterotoxin production was tested at the growth rate giving the maximal enterotoxin production on sucrose. The results present here in provide compelling evidence that toxin production of both enterotoxins involved in diarrheic *B. cereus* food borne poisoning is strongly influenced by carbohydrate **(Ouhib *et al.,* 2006)**.

The second class of toxins expressed by *B. cereus* are phospholipases, which can induce hemolysis. The two major phospholipases are phsophatidyl choline specific phospholipase C (PC-PLC) **(Callegan *et al.,* 2002)** and sphingomyelinase (SPH) **(Pomerantsev *et al.,* 2003).**

The enterotoxin complex hemolysin (HbL) described by **Beecher and Wong (1994b)**, consisting of three components B (37-5 KDa), L_1 (38.2 kDa) and L_2 (43-5 KDa) shows hemolytic activity and has been characterized intensively in view of the biological activity **(Beecher *et al.,* 1995).**

Bacillus cereus enterotoxins test (Merck kGaA) is a newly developed gold-labeled lateral flow immunoassay for the

detection of *Bacillus cereus* enterotoxins. The test uses monoclonal antibodies (MAbs) against the L_2 component of hemolysin Bl (hbl) and NheB of the non-hemolytic enterotoxin (Nhe), respectively **(Krause *et al.*, 2010).**

Eleven *Bacillus* species isolated from veterinary samples associated with severe nongastrointestinal infections were assessed for the presence and expression of diarrheagenic enterotoxins and other potential virulence of factors - PCR studies revealed the presence of DNA sequences encoding hemolysin Bl (HBl)erotoxin complex and *B. cereus* enterotoxin T (BCeT) in five *Bacillus cereus* strain **(Rowan *et al.*, 2003).**

The response in the skin of rabbits or guinea pigs to intradermal injection of crude *B. cereus* culture filtrates is attributable to a combination of two distinct factors: (i) a partially characterized necrotic enterotoxin of a molecular weight of approximately 50000 daltons and (ii) the primary *B. cereus* haemolysin cereolysin **(Turnbull and Kramer, 1983).**

The enterotoxin, which has both dermonecrotic and vascular permeability effects has been shown to be the cause of diarrhea in *B. cereus* food. In an experimental model of *Bacillus cereus* endophthalmitis, mouse eye were injected with this microbe intravitreally, and polymorphonuclear leukocyte infiltration into the vitrous cavity began by 4 hours after inoculation **(Ramadan *et al.*, 2006).**

Members of the *B. cereus* group carry genes encoding several important virulence factors, including enterotoxins, phospholipases and exotoxins **(Sergeev *et al.*, 2006).**

The *B. cereus* group contains seven closely related species: *Bacillus anthracis, B. cereus, Bacillus thuringiensis, Bacillus mycoides, Bacillus pseudomycoides, Bacillus weihenstephanensis,* and *Bacillus medusa* **(Bavykin *et al.*, 2004).**

The toxin-producing strains could be assigned to four different species, *B. subtilis*, *B. majavensis*, *B. pumilus,* or *B. fusiforms,* by using a polyphasic approach including biochemical, chemotaxonomic, and DNA-based analyses **(From *et al.*, 2005).**

Several important toxins are produced only by *B. anthracis* and *B. thuringiensis* and are used as parameters for the classification of these two organisms. *B. thuringiensis* produces several endotoxins that are toxic to a wide variety of insects. *B. anthracis* secretes two exotoxins: lethal toxin (LT) a zinc-dependent metalloprotease **(Vitale *et al.*, 1998; Liddington *et al.*, 1999)** and edema toxin (ET) an adenylate cyclase exotoxin **(Leppla, 1982; Krantz *et al.*, 2004).** Both toxins are protein complexes with protective antigens (PA) and either lethal factor (LF) for LT or edema factor (EF) **(Petosa *et al.*, 1997; Lesieur *et al.*, 1997; Miller *et al.*, 1999; Nassi *et al.*, 2002).**

Diamondbackmoth, *Plutella xylostella,* Larvae were ected with a primary pathogen, *Bacillus thuringiensis kurstaki (Btk)* in a signel strain and mixed infections. Mixed infections