Prevalence and Characterization of *Bacillus cereus* in Meat and Meat Products in and around Jammu Region of Jammu and Kashmir, India

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A B S T R A C T

K e y w o r d s
B. cereus, Biochemical characterization, Incidence, Meat, Meat products.
Article Info
Accepted: 10 October 2017 *Available Online:* 10 December 2017

The present study was carried out to isolate *B. cereus* from meat and meat products collected from Jammu city. In this study 150 samples comprising of raw mutton (n=50), raw chicken (n=50) and chicken biryani (n=50) were collected from various parts of Jammu and analyzed for the presence of *B. cereus*. 52 isolates of *B. cereus* were confirmed morphologically by colony characteristics and biochemically by using conventional biochemical methods. The 52 presumptive isolates were further subjected to PCR by targeting species specific *gyrB* gene. Out of 52, 44 presumptive isolates possessed *gyrB* gene and were confirmed as *B. cereus*. Thus the incidence of *B. cereus* in meat and meat products was found to be 29.33 % while as Incidence of *B. cereus* in raw mutton, raw chicken and chicken biryani was found to be 28%, 24% and 36% respectively. Hence it was concluded that the occurrence of *B. Cereus* in foods especially in meat and meat products was frequent with more incidence in cooked meat (36%) than raw meat (26%) which is a serious public health concern and need to be addressed.

Introduction

The major cause of morbidity and mortality all over the world are reported to be due to food borne diseases which pose a serious threat to public health. Both developed as well as developing countries suffer from large number of food borne outbreaks and the incidences are showing increasing trend on global level. The increasing trans-border movement of people, animals and animal products, rapid urbanization, rapid increase in number of immune-compromised people, food handling and consumption pattern changes and pathogens showing antibiotic resistance play significant role in increasing

the outbreaks all over the world (Unnevehr, 2003). The pathogens transmitted through food contribute 30 per cent to globally emerging infections (Carlin *et al.,* 2009). Majority of the cases go unnoticed as symptoms are mild often associated with diarrhea and vomiting.

Bacillus cereus is a Gram positive, motile, rod shaped, aerobic and spore forming bacteria belonging to the family *Bacillaceae*. The vegetative cells are typically 1.0- 1.2 μm by 3.0-5.0 μm in size. The spores are ellipsoidal in shape and are formed in a central or

paracentral position without swelling the sporangium. *B. cereus* grows in the temperature range 10-48^{0}C, with optimum growth occurring between 28^{0}C and 35^{0}C. Minimum pH for growth in meat is 4.35.

B. cereus has been identified as a cause of food borne illness since 1950's. Hauge in 1950 reported the first well documented case of *B. cereus* food poisoning in Norway. *B. cereus* food borne outbreaks have been reported by many countries viz., Finland, Netherland, Canada and in Hungary it has been found to be third most common cause of food poisoning cases. In India, cases of food poisoning have been reported by Hussain *et al.,* (2007) who reported an episode of gastrointestinal illness due to consumption of *Bacillus cereus* contaminated food in a fast food (Chola-puri) restaurant in May 2006. Abbas *et al.,* (2014) isolated *B. cereus* from milk and milk products. Ashraf *et al.,* (2015) detected the presence of *B. cereus* in samples of meat products like luncheon, sausage, minced meat and beef burger. Similarly Ibrahim-Hemat *et al.,* (2014) isolated *B. cereus* from the samples of meat products represented by frozen rice kofta, kobiba-shami, oriental sausage and beef luncheon. In Kolkata (India), presence of this organism has been reported in 3.5 % cases in acute diarrhea for a period of 2-years (October 2006 to September 2008) (Banerjee *et al.,* 2011). There is a frequent association of *B. cereus* with diarrheal and emetic types of food borne illnesses and the diarrheal syndrome is caused by an enterotoxin produced in the lower gastrointestinal tract during vegetative growth of *B. cereus* after 8-16 hours of ingestion of contaminated food whereas, the emetic type syndrome is caused by a preformed toxin produced by growing cells in the food with an incubation period of 1-5 hours (Granum, 1994). The infective dose of *B. cereus* in diarrheal food poisoning is 10^4-10^{11}cfu/g or ml while in emetic food poisoning it is 10^3 to

10^{10}cfu/g or ml (Granum and Lund, 1997; Lund, 1990). *B. cereus* plays a significant role in food borne illness along with other bacteria as it was responsible for seventy three outbreaks out of 5141 food borne outbreaks during a period between 1982 and 1986 involving 1323 cases (Shinagawa, 1990).

B. cereus is equally important in animals as it has been reported as a cause of gangrenous mastitis in dairy cows (Schiefer *et al.,* 1976). In addition to mastitis it is also responsible for various pathological conditions including abortion in animals (Wohlgemut *et al.,* 1972). The ubiquitous nature of *B. cereus* and the contamination of meat during meat production or processing make meat an ideal medium for the growth of this microorganism and meat further stimulates the growth of *B. cereus* and its toxin secretion. The spore forming and psychotropic nature of certain *B. cereus* strains ensures the survival and multiplication of bacteria during meat processing. Thus, the meat industry often reports food poisoning outbreaks due to *B. cereus* (Granum *et al.,* 1994). The organisms isolated from meat and meat products in different regions might have enough diversity at genetic level and can be classified at genetic level by using molecular techniques like polymerase chain reaction. The cereals also provide the perfect growing conditions for *B. cereus* when unhygienic cooking is practiced. *Bacillus cereus* has also been reported in cereals in significant levels (Mgbakogu and Eledo, 2015).

Multiple drug resistant isolates of *B. cereus* due to production of beta-lactamase pose a significant threat to Public Health. Beta-lactamases, being one of the potential virulence factors make these strains resistant to penicillin, ampicillin, and even to third generation cephalosporins (Cormian *et al.,* 1998). Resistance to erythromycin and tetracyclines reported from United States and

Europe adds further to the threat. The driving force in the development of antibiotic resistance is the selective pressure exerted by indiscriminate use of antibiotics. Hence need of the hour is to study the potential transmission of antibiotic resistant bacteria from food chain to humans (Faria-Reyes *et al.,* 2001).

Keeping in view the above facts, the present study was carried out to study the incidence of *B. cereus* in meat and meat products, its isolation by using various conventional biochemical methods and characterization of isolates of *B. cereus*.

Materials and Methods

Bacterial strain

In the current study a reference strain of *B. cereus* was used. The reference strain MTCC 1272 was obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India for standardization of techniques. The strain was maintained by periodic sub culturing on nutrient agar slant.

Sampling

Meat

A total of 100 samples of raw mutton and raw chicken were collected in sterile containers from various butcher shops and restaurants in and around Jammu city. The samples were brought to the laboratory on ice and processed within two hours of collection.

Meat products

A total of 50 samples of chicken biryani were collected aseptically from various retail outlets and restaurants in and around Jammu city. The samples were brought to the

laboratory on ice and processed within two hours of collection.

Isolation and phenotypic characterization of *Bacillus cereus*

Isolation

Approximately 30 g of meat sample was collected in a clean plastic zip lock sachet and brought to laboratory and processed immediately kept at 4^0C till processed. About 10 g from each sample was weighed aseptically and homogenized in 30 ml of peptone water. Polymyxin-pyruvate-Egg yolk-Mannitol-Bromothymol blue agar (PEMBA) media was used for isolation of *B. cereus*.

The samples were processed as per the method described by Shinagawa (1990). The samples were inoculated into brain heart infusion broth (BHIB) containing polymyxin (100 units/ml). The BHIB tubes were then incubated at 37^0C for 24-48 hours. After enrichment a loopful was streaked on PEMBA plates and incubated at 37^0C for 24 h.

The fimbriate peacock blue coloured colonies (3-5mm) surrounded by blue zone of egg yolk hydrolysis against green/greenish yellow back ground were presumed to be *B. cereus*. The presumed isolates were preserved on nutrient agar slants for further characterization.

Phenotypic characterization

All presumptive colonies of *B. cereus* were purified and subjected to morphological and biochemical tests for identification and confirmation as described in Bacteriological Analytical Manual of United States, Food and Drug Administration (Rhodehamel and Harmon, 2001)

Morphology

Gram stained smears were prepared from slants and examined microscopically. *B. cereus* appeared as large Gram positive bacilli in short to long chains, whereas, spores were ellipsoidal, central to subterminal and did not swell the sporangium

Genotypic characterization of *Bacillus cereus* isolates by targeting *gyrB* gene

The genotypic characterization was carried out by PCR which was used for detection of species specific *gyrB* gene by following methodologies described by Park *et al.,* (2007). The optimized PCR was carried out in a final reaction volume of 25µl and the cycling conditions consisted of an initial denaturation at 94^0C for 5minutes, 30 cycles of amplification with denaturation at 94^0C for 30 seconds, annealing at 63^0C for 30 seconds, an extension at 72^0C for 30 seconds and final extension of the incompletely synthesized DNA at 72^0C for 5 minutes. The primers used for detection of *gyrB* gene for characterization of *B. cereus* isolates were obtained from Promega Corporation, U. S. A., Madison (Table 1). The confirmation and resolution of the PCR product was done by agarose gel electrophoresis.

Antibiogram of *Bacillus cereus*

All the *B. cereus* isolates were examined for their antibiogram pattern by disc diffusion technique as described by Bauer *et al.,* (1966) against a panel of 10 antibiotics. Isolates were inoculated in Muller Hinton broth and incubated at 37^0C for 16-24 hours. Each broth culture was smeared on Muller-Hinton agar plates with help of a sterile cotton swabs. Plates were allowed to dry for few minutes and antibiotic discs were placed on the agar surface and plates were incubated for 12-24 hours at 37^0C. The sensitivity or resistance of an isolate for a particular antibiotic was determined by measuring the diameter of the zone of inhibition of growth. The results were interpreted as sensitive or resistant based on CLSI interpretive standards (2007). The antibiotics and their concentration used in Antibiotic sensitivity assay are given in table 2.

Results and Discussion

Isolation and phenotypic characterization of *Bacillus cereus*

For isolation of *B. cereus* samples were triturated and enriched in Brain Heart infusion Broth followed by streaking on Polymyxin pyruvate Egg yolk Mannitol Bromothymol Blue Agar (PEMBA). On PEMBA medium, typical crenate to fimbriate peacock blue coloured colonies (3-5mm) surrounded by blue zone of egg yolk hydrolysis against greenish yellow back ground were presumed as *B. cereus* (Figure 1). Out of 150 samples 63 (42 %) samples showed characteristic peacock blue coloured colonies on selective PEMBA. On Gram staining all these isolates were Gram positive rods and sometimes spores were also seen.

Biochemical characterization to identify *B. cereus* isolates was done. Sixty three (63) isolates were subjected to various biochemical tests but only 52 (34.6%) isolates showed characteristic biochemical features as that of *B. cereus*. All the 52 isolates were positive for catalase test, citrate utilization test (Figure 2) and motile (Figure 3). Forty seven (91%) *B. cereus* isolates also hydrolyzed starch (Figure 4) and 40 (77.27%) *B. cereus* isolates produced beta-hemolysis on sheep blood agar (Figure 5). All the 52 *B. cereus* isolates were positive for nitrate reduction test and Voges-Proskauer test and but negative for oxidase test. These 52 isolates were further subjected to confirmation by PCR assay.

Genotypic characterization of *Bacillus cereus* isolates

Phenotypically identified presumptive *B. cereus* isolates were further analysed by PCR. The 52 presumptive isolates were subjected to PCR for obtaining a product size of 475 bp by targeting species specific *gyrB* gene. Out of 52, 44 presumptive isolates possessed *gyrB* gene and were confirmed as *B. cereus* (Figure 6).

Prevalence of *B. cereus* in meat and meat products

Out of 150 samples of meat and meat products, which comprised of 50 samples each of raw mutton, raw chicken and chicken biryani were assessed for the presence of *B. cereus*.

Out of 150 samples, 44 samples were found positive for *B. cereus* with an overall incidence of 29.33 per cent. Among 44 isolates, 14 were found in raw mutton, 12 in raw chicken and 18 in chicken biryani with an incidence of 28 per cent, 24 per cent and 36 per cent in raw mutton, raw chicken and chicken biryani, respectively (Table 3).

Antibiogram of *B. cereus* isolates

All the 44 isolates of *B. cereus* were tested against ten different commonly used antibiotics and sensitivity pattern results was obtained on the basis of zone of inhibition (Plate-13 and Plate-14). All the isolates were resistant to penicillin G, although all the isolates were sensitive to streptomycin. Out of 44 isolates of *B. cereus* 63.6, 86.3 and 77.2 per cent isolates showed resistance against amoxicillin, ampicillin and carbencillin, respectively. All the isolates were sensitive to neomycin and chloramphenicol except 5 (11.3%) isolates. 68.1 per cent *B. cereus* isolates showed sensitivity to gentamicin

while 45.4 per cent isolates showed sensitivity to tetracycline. For erythromycin 40.9 per cent isolates were sensitive while 27.2 per cent isolates were resistant (Table 4).

Meat and meat products may serve as a potential medium for many bacterial pathogens including *B. cereus*. The abilities of the spores and toxins of *B. cereus* to survive high temperatures and of certain psychotolerant strains to flourish at low temperatures make *B. cereus* a unique meat-borne pathogen. In comparison to other microorganisms, *B. cereus* is well versed with diversified characteristics like aerobic and facultative anaerobic growth, motility, psychrotrophic (4^0C) and thermophilic (50^0C) nature.

These characteristics make *B. cereus* a unique and one of the most important food poisoning organisms. *B. cereus* makes its presence either in the form of vegetative cells or spores or toxins and is hence, anticipated from almost all foods of domestic consumption owing to its ubiquitous nature and adaptation to the environmental changes, thus posing a great public health threat.

Meat may act as a good source of *B. cereus* either directly in raw form or in the form of hot served meat products. The chances of its presence would be high in meat and meat products when meat is temperature abused.

When meat is transported from the production areas to the consumer sites, the meat and meat products get continuously exposed which may facilitate growth and toxin production especially when the ambient temperature favours the growth requirements of the organism. Thus the entry of toxins, emetic and diarrheic could lead to onset of emetic and diarrheic syndromes in human beings when such contaminated meat and meat products are consumed.

Table.1 Sequences of oligonucleotide primers

Target gene	Primer	Sequence(5-3)	Amplicon Size (bp)	Reference
gyrB	BCJH-F	TCATGAAGAGCCTGTGTACG	475	(Park *et al.,* 2007)
	BCJH-R	CGACGTGTCAATTCACGCGC		

Table.2 Antibiotics and their concentration used in antibiotic sensitivity assay

S.No.	Name of antibiotic	Concentration(µg)
1.	Amoxicillin (Am)	30
2.	Ampicillin (A)	10
3.	Neomycin (N)	30
4.	Chloramphenicol (C)	30
5.	Carbencillin (Cb)	100
6.	Erythromycin (E)	15
7.	Gentamycin (G)	10
8.	Penicillin G (P)	10
9.	Streptomycin (S)	10
10.	Tetracycline (T)	30

Table.3 Prevalence of *B. cereus* in meat and meat products

S. No.	Type of sample	Number of samples tested	Samples positive	Prevalence (%)
1	Raw mutton	50	14	28
2	Raw chicken	50	12	24
3	Chicken biryani	50	18	36
	Total	**150**	**44**	**29.33**

Table.4 Antibiogram of *B. cereus* isolates

Antibiotics	Sensitive	Intermediate	Resistant
Amoxicillin	16 (36.3%)	0 (0%)	28 (63.6%)
Ampicillin	6 (13.6%)	0 (0%)	38 (86.3%)
Carbencillin	7 (15.9%)	3 (6.8%)	34 (77.2%)
Chloramphenicol	27 (61.3%)	12 (27.2%)	5(11.3%)
Erythromycin	18 (40.9%)	14 (31.8%)	12(27.2%)
Gentamicin	30 (68.1%)	8 (18.1%)	6 (13.6%)
Neomycin	34 (77.2%)	10 (22.7%)	0 (0%)
Penicillin	0 (0%)	0 (0%)	100 (100%)
Streptomycin	35 (79.5%)	9 (20.4%)	0 (0%)
Tetracycline	20 (45.4%)	10 (22.7%)	14 (31.8%)

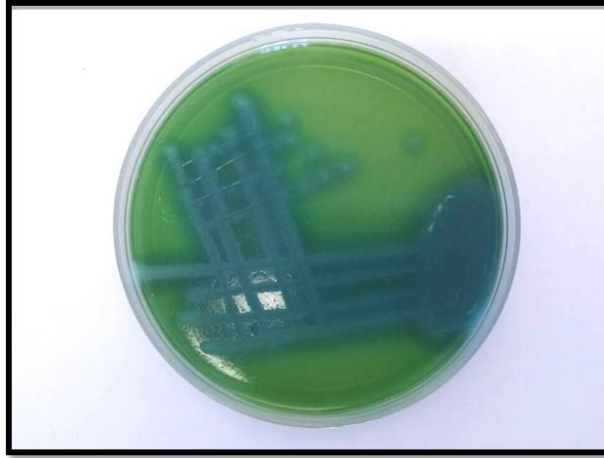**Fig.1** B*acillus cereus* colonies on PEMBA

Fig.2 Citrate utilization test (blue colour production indicates positive test)

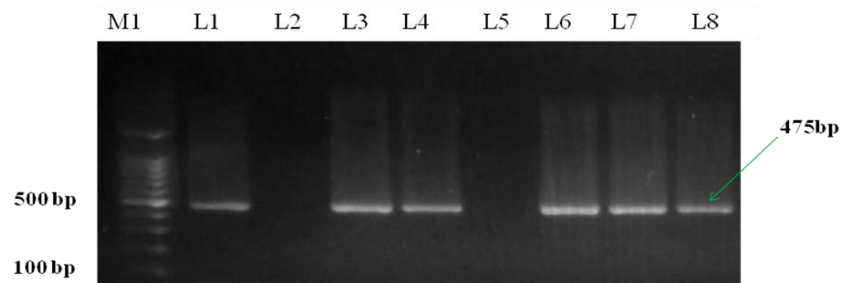**Fig.3** Motility test (haze around stab by motile bacteria)

Fig.4 *B. cereus* showing starch hydrolysis (zone around streak)

Fig.5 *B. cereus* showing β-haemolysis on sheep blood agar

Fig.6 Agarose gel electrophoresis depicting amplified PCR product of *gyrB* gene from genomic DNA of *B. cereus* (isolated from samples) for confirmation

M1-100 bp DNA ladder (Marker)

L2 and L5– Negative controls

L8-Positive control

L1, L3, L4, L6 & L7-Samples positive for *gyrB* gene

In this study, 150 samples of meat and meat products were subjected to standard microbiological procedures for detection of *B. cereus* as described by Rhodehamel and Harmon (2001). Further, the morphological and biochemical profiles of the 52 isolates closely resembled with the standard strain. All the 52 isolates were positive for Voges-Proskauer, nitrate reduction, citrate utilization but some degree of variability was seen in hemolysis and starch hydrolysis. In the present study, 52 isolates were positive for VP reaction which is in accordance with results of Priest *et al.,* (1988), who reported 91 per cent isolates positive for VP. However, Haque and Russel (2004) reported that all *B. cereus* isolates of Bangladeshi rice were VP negative but reduced nitrogen.

The reason behind this variation in VP reaction may be the strain variation of *B. cereus.* All the 52 isolates reduced nitrate to nitrite which are almost similar to the results of Chopra *et al.,* (1980), who observed 87 per cent of *B. cereus* isolates reduced nitrate to nitrite. Out of 52 *B. cereus* isolates, 40 (77.27 %) showed beta-hemolysis and 52 (100 %) were catalase positive which is in agreement to the results of Schiemann (1978), who also reported that 96 per cent isolates were positive for beta–hemolysis and catalase.

The variation in the hemolysis pattern may be because 28 isolates carried at least one gene of HBL complex. However, other 12 (twelve) isolates might be carrying other toxins which are having hemolytic effect like cereolysin O (Coolbaugh and Williams, 1978), hemolysin iii (Baida and Kuzmin, 1996) hemolysin ii (Miles *et al.,* 2002) or Cyt K (Hardy *et al.,* 2001). All the *B. cereus* isolates were motile, utilized citrate and 47 (91 per cent) isolates hydrolyzed starch which was supported by Gilbert *et al.,* (1981), who reported that 80-100 per cent *B. cereus* strains hydrolyze starch and utilize citrate.

In the present study results revealed the incidence of *B. cereus* isolates as 29.33 per cent in meat and meat products which are in agreement with similar incidence reported by Schlegelova *et al.,* (2003) and Tewari *et al.,* (2013), who too reported an incidence of 28 per cent and 30.9 per cent respectively in meat samples. However, Bedi *et al.,* (2004) and Kamat *et al.,* (1989) reported an incidence of 63.2 per cent and 80 per cent respectively in chicken and meat products.

The probable reason behind this variation may be the better hygienic qualities maintained by restaurants and meat shops nowadays. Similarly Eluma *et al.,* (2015) reported an incidence of 16.9 per cent in meat by using nutrient agar from 450 meat samples. This incidence was lower than the present study incidence, the probable reason behind this may be the more selective approach adopted in the present study i.e., PEMBA which might have increased the recovery of bacteria from the samples as compared to nutrient agar used in their study.

In the present study, results revealed the contamination of raw meat to be as 26 per cent with *B. cereus* which was lower than cooked meat products 18 (36 %). This per cent finding are in accordance to the previous reports of Bachhil and Negi (1984) who reported more contamination of cooked meat 65 per cent than raw meat 35 per cent and Bachhil and Jaiswal (1988) who reported contamination of raw meat as 35 per cent and cooked meat as 100 per cent.

Similarly an incidence of 90.9 per cent *B. cereus* in cooked chicken as compared to 9.1 per cent in raw meat was reported (Aklilu *et al.,* 2015). However, Willayat *et al.,* (2007), who examined 200 raw mutton and cooked mutton samples found that 47 samples were positive for isolation and further results revealed that contamination of raw meat with

B. cereus was more than that of cooked meat as 30 per cent and 15 per cent respectively. The probable reason behind this may be the storage temperature abuse and improper cooking which leads to the germination of spores and also the present study involved chicken biryani which involves rice which might add to the occurrence of *B. cereus* as it is found in high numbers in starchy foods like rice and hence more occurrence of *B. cereus* in cooked meat products than raw meat.

The continuous emergence of new pathogens has made it mandatory to devise a technique or procedure which is of high value in differentiating closely related species as members of *B. cereus* group which include *B. anthracis*, *B. thuringenesis* and *B. mycoides* that are phenotypically too close. The traditional methods of differentiating closely related bacteria are time consuming and laborious and may lead to false results. To overcome this drawback, Park *et al.,* (2007) designed primers targeting *gyrB* gene for the differentiation of *B. cereus* group bacteria. In agreement to this, La-Duc *et al.,* (2004) characterized *B. cereus* group phylogenetically and found that *gyrB* gene more differential than 16s *rRNA* and other DNA hybridization techniques Out of 52 biochemically identified isolates only, 44 (84.6 %) isolates produced 475 bp products on agarose gel electrophoresis, which were specific for *B. cereus*.. The results are in accordance with the results of Rather *et al.,* (2011), who confirmed 59 (98.3 %) isolates out of 60 by targeting *gyrB* gene by using PCR. Similarly Tewari *et al.,* (2013) confirmed 29 (100 %) isolates by targeting species specific *gyrB* gene which supports the results of the present study.

The antibiotics have changed the idea of infectious diseases and human demography. The different types of antibiotics are promising much in reducing the major causes

of human and animal morbidity. However, bacterial antibiotic resistance which came into limelight due to extensive use of antibiotics needed to be studied (Wise *et al.,* 1998 and Okeke *et al.,* 2005). Various mechanisms have been devised and the genetic basis of the resistance was unfolded; the resilience of the bacterial species to antibiotic stress has been realized. With changing demographic patterns and extensive use of antibiotics which ultimately lead to 'antibiotic resistance' is creating issues globally especially in the persistence of infections which is a major cause of morbidity and mortality.

The antibiotic sensitivity of all the *B. cereus* isolates against a battery of ten commonly used antibiotics was carried out keeping in view the above facts. The antibiotics to which most of isolates were sensitive included streptomycin (79.5%), neomycin (77.2%), gentamicin (68.1%) followed by chloramphenicol (61.3%) which is similar to results of Whong and Kwaga (2007), Rather *et al.,* (2011) and Umar *et al.,* (2006). The isolates showed resistance to penicillin G (100%). However, less than 100 percent resistance was recorded for ampicillin (86.3%), carbencillin (77.2%), and amoxicillin (63.6%) which is similar to results of Hassan and Nabbut (1995) Anamika and Kalimuddin (2004). Less than 50 per cent resistance was observed towards erythromycin (27.2%) and tetracycline (31.8 %). In the present study, *B. cereus* was found susceptible to Aminoglycosides (gentamycin, neomycin and streptomycin) and chloramphenicol which is similar to the results of Luna *et al.,* (2007) who reported that *B. cereus* isolates were susceptible to aminoglycosides, chloramphenicol, clindamycin, erythromycin, tetracyline and vancomycin. Previous works on the antimicrobial susceptibility of *B. cereus* revealed higher sensitivity towards ampicillin, amoxicillin and less susceptible to

streptomycin, chloramphenicol, and neomycin as reported by Turnbull *et al.,* (2004), Meena *et al.,* (2000) and Schlegova *et al.,* (2003). However erythromycin showed a resistance of (27.2%) which is in agreement to Wong *et al.,* (1988) as some *B. cereus* isolates could attain resistance due to indescriminate use of these antibiotics in feeds and chemotherapy. Variations in the percentages may be due to the differences in the concentrations of antimicrobial agents used, differences in the source of isolates, drug resistance transfer and overall wide use of the antibiotics. The development of drug resistance against certain antibiotics may be due to the use of these drugs in Medical and Veterinary practice to treat infections and misuse of the drugs that could have lead to drug resistant strains.

Acknowledgement

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