Original Report

Feed intoxication in chinchilla induced by bacillus cereus enterotoxin

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Summary

The purpose of this investigation was to find the causes of death in chinchilla with clinical symptoms of strong watery diarrhoea, which were given feed from which was isolated Bacillus cereus strain.

For the investigation were used 10 chinchilla of the standard race. 12 feed components were studied. Fragments of the large and small intestine, and gastric and intestine contents with PEMBA medium B. cereus strains were obtained. The strains produced diarrhoeal enterotoxin. The strains with identical biochemical tests were obtained from feed ($5,4x10^5$c.f.u./g), premix ($1,8x10^7$c.f.u./g), wheat ($1,4x10^1$c.f.u./g), barely ($1,4x10^1$c.f.u./g), and oats ($1,1x10^1$c.f.u./g). The strains, which were obtained from the animals and from the feed, produced enterotoxin in BCET-RPLA test. Strains from 4 feed components survived in an acid environment (pH 4,0-1,5). With disc diffusion testing both strains were sensitive to: amikacin, ciprofloxacin, enterofloxacin, imipenem and norfloxacin.

Key words:
antibiotic - sensivity, Bacillus cereus, chinchilla, contamination, diarrhoeal enterotoxin, enterotoxin, extremely acid environmental, feed, intestine, intoxication, watery diarrhoea.

Bacillus cereus is a Gram positive, relative anaerobic bacillus that produces endospores. This bacterium is widely distributed in the natural environment, being frequently isolated from soil, water and air. Due to its general occurrence, there is a possibility that feed products and animal feeds are contaminated with it. B. cereus is frequently isolated from grain (in particular rice) and cereals, from milk (fresh and pasteurised) and its products, as well as from raw meat (*Boron-Kaczmarska et al., 1999, Molska I., 1996*). Very often feed products are contaminated due to the addition of herbs containing heat-resistant endospores of Bacillus cereus *(Turnbull P. et al., 1990).* During the frequently used thermal processes that are connected with the preparation of feed as well as animal feeds at not very high temperatures, the endospores of B. cereus germinate and the

vegetative cells develop emetic toxin and (or) diarrhoeal enterotoxin. The human emetic toxin is ethiologically connected with the emetic syndrome of typical feed poisoning *(Boron-Kaczmarska et al., 1999, Zaremba M. L. et al., 1997)*. It is a thermostabile protein with a low molecular weight, which is exceptionally effectively synthetised during endospore production. On the other hand, the diarrhoeal toxin that is responsible for the diarrhoeal syndrome (frequently of bacterial feed infection character) is a protein with a molecular weight of approximately 50,000. Intoxication with that toxin resembles that with Cl. perfringens or Vibrio cholerae. This bacteria also produces a number of other toxins including cereolysin with a strong necrotic character, hemolysin II, and lecithinase (of stable metalloenzyme complex character), not connected however with feed intoxications (*Boron-Kaczmarska et al., 1999, Turnbull P. et al., 1990).*

To demonstrate that this bacterium is the ethiological agent of intoxication, one should determine whether the strain isolated from the feed is the same one which isolates from a sick animal. The mere isolation of B. cereus from the feed does not suffice since this bacterium occurs very often in the natural environment and does not produce clinical symptoms of disease. In case of isolation of B. cereus from vomit, feces, or internal organs, one should keep in mind that it is then essential to determine the number of cells, the serotype or phage type, and the virulence of the isolated strains, first their ability to produce toxins (*Turnbull P. et al., 1990).* As a rule, feed intoxication with B. cereus is found in humans. Feed intoxications with enterotoxins produced by Bacillus cereus have not been found in phytophagous rodents.

The aim of the study was to determine the cause of deaths in chinchillas with severe symptoms of diarrhoea that were given a feed from which Bacillus cereus was isolated.

Material and methods

The study material consisted of 10 dead chinchillas of Standard breed with severe symptoms of diarrhoea, as well as the feed and 12 of its components, with which the animals were fed.

During post mortem examination, a severe enterocolitis was found in all dead animals, both in the small and the large intestine. From all 10 chinchillas small segments of altered intestines were taken for microbiological analysis. Since chinchillas are animals in which the vomiting reflex is very rare, it was not possible to take vomit for further analysis (important material in feed intoxication in the human), and microbiological analysis included only the gastric and intestine contents. The chinchilla feed consisted of oats, barley, soya, wheat, wheat bran, flax-seed, dried grass, methionine, behladol, premix, molasses and animal meal. All feed components were mixed together at 50°C. For microbiological analysis, 10 g of feed and of each of its components were taken, followed by decimal dilutions made in peptone water. All samples (intestine segments, gastric and intestine contents, feed, and its components) were inoculated on nutrient agar with egg yolk emulsion, blood agar, and McConkey, Chapman, Listeria Selective Agar Base, Edwards, Wilson-Blair, and PEMBA media. The bacterial cultures were incubated for 24 hours at 37°C. From bacterial colonies that had been grown on selective PEMBA culture medium, microscopic preparations were made that were stained using the Gram method as well as the method of Holbrook and Anderson in which the staining of endospores according to Ashby was connected with the intercelluar staining of lipids according to Burdon. For staining, malachite green and Sudan black and safranin solution were used. The basic characters of B. cereus had been determined, based first of all on the presence of lipide globules, the kind of endospores produced, the lecithinase synthesis, the growth in anaerobic

conditions and at 50°C, the acid production from glucose, mannitol, xylosis, the nitrate reduction, and the starch, gelatin and tyrosine hydrolysis. To detect diarrhoeal enterotoxin of B. cereus, the reversed passive latex agglutination test - Bacillus cereus RPLA Toxin Detection Kit BCET-RPLA TD 950 (Oxoid) - was applied. This is a sensitive, semiquantitive method enabling detection of the presence of protein enterotoxin in the feed and in the filtrate from bacterial culture (*Boron-Kaczmarska et al., 1999*). To examine the presence of enterotoxin, 10 strains isolated from the intestines of dead chinchillas and 5 strains isolated from the feed, premix, barley, oats and wheat were used. Agglutination results had been read according to the formulae published by the test manufacturer.

Moreover, the survival rate of B. cereus strains that were isolated from the premix, barley, wheat, oats, and the complete feed had been tested in a highly acidic environment (pH 1.7 to 4.0). The chosen range was dictated by two reasons: pH of cardia in phytophagous rodents is 1.5 (*Gibaszewicz W., 1989*), and the survival rate in a very acidic environment (pH = 4.3) is a diagnostic feature typical for the cells of B. cereus, distinguishing them from B. licheniformes and B. subtilis for which the most extreme pH is 5.0. 1 ml of bouillon culture of B. cereus strain with a density of 1.0 on the Mc Farlands scale was inoculated to 10 ml of bouillon, and particular samples were acidified to pH: 4.0, 3.0, 2.0, and 1.5. The culture was incubated (24h/37°C), followed by sieving on PEMBA culture medium. The character of growth on that culture medium was analyzed after 24 hours.

The antibiotic-sensitivity of B. cereus that had been isolated from the intestines and the feed was determined by disc diffusion testing.

Results and discussion

After incubation for 24 hours at 37°C on culture mediums on which inoculations were made from the intestines (small and large) and their contents as well as from the gastric content, apart from single bacterial colonies of E. coli, P. vulgaris, Staphylococcus spp., Streptococcus spp., a very profuse growth was found of large, flat bacterial colonies with irregular edges that produced hemolysis β (sheep or horse erythrocytes) on blood agar and lecithinase (on yolk agar), that had manifested in the presence of white precipitate around the bacterial colonies grown. On PEMBA medium, a profuse growth of large, flat bacterial colonies was found of peacock blue colour with a large, blue zone of yolk precipitation. The PEMBA is a selective medium, used to isolate B cereus, consisting of agar with polymyxin, pyruvate, mannitol, and bromothymol blue. The inhibitor suppressing the growth of bacterial flora that contaminates the analysed material is polymyxin (*Boron-Kaczmarska et al., 1999).*

On the other hand, the microbiological analysis of the feed and its components revealed the presence of B. cereus in the premix in the amount of 1.8×10^7 c.f.u./g, and in oats, wheat and barley in an amount not exceeding 1.4 c.f.u./g. In the feed, 5.4×10^5 cells of B. cereus were found in 1 g (Table 1). The premix percentage in the feed appropriated for chinchillas is small and amounts to 1%. Moreover, bacteria of the coli group were found in the feed (Table 1). When culturing in anaerobic conditions on Wilson-Blair culture medium, anaerobes - Cl. perfringens, that also might be the cause of diarrhoeal syndrome observed in chinchillas - were not found. The number of bacteria cells isolated from the feed was important evidence that Bacillus cereus was the cause of feed intoxication. According to Kramer and collaborators (see *Turnbull P. et al., 1990*), strong proof for B. cereus being the ethiological agent causing feed intoxication is to demonstrate that a symptomatic number of B. cereus cells, i.e. $>10^5$ c.f.u./g, is found in the suspected product, or to find that the strain simultaneously isolated from feces and feed belongs to the same serotype. On the one hand, to cause dysfunction of the alimentary tract requires a significant number of B. cereus cells with a high virulence (toxin synthesis), and, on

the other hand, the presence of illness predisposing factors, such as the reduced level of local cellular or humoral immunity. It might be that such problems had occurred in the animals examined. Chinchillas belong to a mammal species in which many bacterial intoxications (infections with L. monocytogenes, Y. pseudotuberculosis) cause inflammation of the alimentary tract and persistent diarrhoea (*Emirsajlow-Zalewska W. et all, 1997, Furowicz A. J. et al., 1987, Furowicz A. J. et al., 1996, Furowicz A. J. et al., 1999).*

Table 1. Microbiological analysis of feed and feed components (c.f.u./g)

Feed and feed components	Total number of aerobic bacteria	Total number of bacteria of coli group	Number Bacillus cereus
Total feed	$9,4x10^6$	$2,7x10^1$	$5,4x10^5$
Oats	$3,2x10^7$	$1,6x10^2$	$1,1x10^1$
Barley	$2,4x10^5$	$1,1x10^2$	$1,4x10^1$
Soya	$7,9x10^2$	0	0
Wheat	$5,0x10^3$	0	$1,4x10^1$
Wheat bran	$2,9x10^4$	0	0
Flax-seed	$2,8x10^4$	$3,2x10^3$	0
Dried grass	0	0	0
Methionine	0	0	0
Animal meal	$5,1x10^3$	$4,0x10^2$	0
Bahladol	$1,7x10^2$	0	0
Premix	$8,2x10^7$	0	$1,8x10^7$
Molasses	$6,4x10^3$	0	0

It had been assumed that mixing of the feed components at 50°C destroys the vegetative forms and at the same time stimulates the sporulation of B. cereus strains. Endospores, germinating at room temperature (i.e. at which the feed is stored), produce toxins. Also the mere storage of feed is propitious for bacterial cells to multiply. B. cereus strains grow in a broad temperature range - 10 to 48°C, but their optimal growth is 28 to 35°C (*Turnbull P. et al., 1990*). The growth of bacterial cells is also stimulated by the addition of a feed component that is untypical for phytophagous animals, namely animal meal. According to many authors, these elements play an important role in the stimulation of growth of B. cereus (*Boron-Kaczmarska et al., 1999, Turnbull P. et al., 1990*).

It is worth stressing that the feed contaminated with the cells of B. cereus was unchanged organoleptically. In feed intoxications with B. cereus reported in humans, no changes in the organoleptic properties of contaminated products has ever been observed (*Turnbull P. et al., 1990*).

All bacterial colonies isolated from dead animals and from the feed that had grown on PEMBA culture medium were stained with the method of Holbrook and Anderson. In that method of staining, the endospores of B. cereus assumed a green colour, the cytoplasm of the vegetative cell acquired a red colour, and the lipide globules inside the cytoplasm turned black. When staining with the Gram method, bacilli were found stained positively. The

characteristics of these bacteria confirmed that they belonged to B. cereus (Table 2).

Morphological and biochemical similarity of the microorganisms that had been isolated both from the feed and 4 of its components and from the intestines and the gastric and intestine contents allowed us to assume that all isolates belonged to the same biotype and were causing the feed entoxication.

Table 2. Comparison properties of strains of B. cereus isolated from feed and from intestines

Character	Bacillus cereus isolated from	
	Feed (5 strains)	Intestine (10 strains)
Presence of lipid globules	+	+
Kind of endospores	1	1
Lecithinase synthesis	+	+
Grown in anaerobic conditions	+	+
Grown at 50°C	-	-
Decomposition of mannitol	-	-
Decomposition of xylosis	-	-
Acid from glucose	+	+
Nitrate reduction	+	+
Starch hydrolysis	+	+
VP	+	+
Catalase	+	+
Motility	+	+
Gelatin hydrolysis	+	+

Due to poisining, the chinchillas died. An important element in the diagnostics of B. cereus is to determine its virulence, primarily its ability to produce toxins. It has been stated that in the Bacillus cereus RPLA Toxin Detection Kit test, the antibodies bound with latex particles strongly reacted with the diluted antigen which was the enterotoxin of B. cereus, that is of the microorganism that had been isolated from the feed and the intestines of all the dead animals. The result of the presence of enterotoxin was a visible latex agglutination. Similar results were obtained in the case of enterotoxin produced by B. cereus strains that had been isolated from the feed, premix, wheat, oats, and barley.

In the examinations in which the sensitivity to low concentrations of pH ions had been determined, high survival rates (even at pH 1.5) of B. cereus isolated from the feed, premix, barley, oat and wheat were found, and thus its low sensitivity (Table 3). The obtained result had also confirmed that the strain isolated from the feed was B. cereus growing at pH below 4.0. It should be stressed that other Bacillus species do not survive in so extremely an acidic environment *(Turnbull P. et al., 1990*).

In the microbiological analysis was found a full sensitivity of both strains to amikacin, ciprofloxacin, enrofloxacin, imipenem, and norfloxacin and the resistance to ampicilin, amoxicillin, apramycin, bacitracin, carbenicilin, cefuroxime, cefradine, cefazolin, cloxacillin, colistin, doxacycline, erythromycin, gentamycin, lincomycine, neomycin, nitrofurantoin, oleandomycin, oxytetracyclin, penicillin, rifampicin, spectiomycin, streptomycin, sulfonamides, tobramycin, trimethoprim, and vankomycin. The use of directional antibiotic-therapy is justified only in the case of feed intoxications that follow the course of bacterial infections (most often the effect of enterotoxin produced in the alimentary tract by B. cereus cells). It refers also to animal and human infections with a severe course (bacteriemia, pneumonia, panophthalmitis, mastitis) that are caused by cereolysin produced by B. cereus (*Boron-Kaczmarska et al., 1999*). The form of antibiotic-therapy is very important considering the fact that B. cereus cells synthetise β-lactamases (*Carman J. A. et al., 1985*). For that reason, they are resistant to penicillin, ampicilin, and cephalosporines. Moreover, they are resistant to trimethoprim. However, the greater part of strains is sensitive to aminoglycosides, erythromycin, vankomycin, clindamycin, and to tetracycline and sulfonamides, the chemioterapeutics that are recommended in the therapy of diseases caused by B. cereus and B. cereus var. mycoides (*Boron-Kaczmarska et al., 1999, Turnbull P. et al., 1990).*

The strains examined showed sensitivity to 5 antibiotics only. The reason for that may be

connected with the growing resistance of bacterial strains considering a rather common fact that the breeders add antifungal and antibact-erial antibiotics to the feeds for chinchilla.

Table 3. Influence of reaction medium on growth of Bacillus cereus strains

Ordinal number	pH medium/time incubation at 37°C	Number B. cereus cells isolated from				
		feed	premix	wheat	barley	oats
1	4,0/24h	$7,9x10^3$	$2,7x10^3$	$7,7x10^3$	$4,9x10^3$	$8,1x10^3$
2	3,0/24h	$3,7x10^2$	$2,5x10^2$	$4,8x10^2$	$8,5x10^2$	$7,6x10^2$
3	2,0/24h	$9,1x10^1$	$5,1x10^1$	$4,9x10^1$	$2,9x10^1$	$3,21x10^1$
4	1,5/24h	$3,2x10^1$	$1,5x10^1$	$1,0x10^1$	$4,6x10^1$	$4,4x10^1$

When it comes to typical feed intoxication (effect of emetic toxin), the antibiotic-therapy has no effects. It has been shown in own studies that the B. cereus strain that caused the chinchillas to fall ill had synthetised enterotoxin. As a rule, such strains do not produce emetic toxin, or synthetise it exceptionally (*Turnbull P. et al., 1990).* Thus, one may assume that the application of antibiotics that have been found in vitro as the effective ones may be useful in the therapy of infections of that type.

To sum up, It was shown that B. cereus enterotoxic strain isolated from the chinchilla feed caused diarrhoeal syndrome and deaths in chinchillas.

References

Boron-Kaczmarska A., Furowicz A.J. 1999. Zoonosis transferred by alimentary track (in Polish: Choroby odzwierzcce przenoszone droga pokarmowa). Wyd. Lekarskie PZWL, Warszawa, p. 90.

Carman J.A., Hambleton P., Melling J.1985. Bacillus anthracis, w: Isolation and Identification of Micro-organisms of Medical and Veterinary Importance. Red. C.H. Collins, J.M. Grange, Academic Press, London, p. 207.

Emirsajlow-Zalewska W., Furowicz A.J. 1997. Evaluation of pathogenicity determinants of Yersinia pseudotuberculosis strains isolated from chinchilla (in Polish) Medycyna Wet. 53,51.

Furowicz A.J., Broda D., Loczewski P., Czernomysy-Furowicz D. 1989 . Therapeutic value of immunostimulation with Propionibacterium acnes in the treatment of listeriosis in chinchilla (in Polish), Medycyna Wet. 45,289.

Furowicz A.J., Czernomysy-Furowicz D., Kowalczewska M. 1996. Enterotoxigenic strain of Yersinia pseudotuberculosis as the cause of yersiniosis in chinchilla (in Polish), Medycyna Wet. 52,116.

Furowicz A.J., Czernomysy-Furowicz D. 1999. Elimination of yeriniosis (Yersinia pseudotuberculosiss) from chinchilla breeding (in Polish), Magazyn Wet. 8,130.

Gibaszewicz W.1989. Rabbit Diseases (in Polish: Choroby królików) PWN Warszawa, p.19.

Molska I.1996.Conditions of development and elimination Bacillus cereus from pasteurized milk (in Polish) :Przem. Spoz. 53,13.

Turnbull P., Kramer J., Melling J. 1990. Bacillus, in: Topley and Wilson's Principles of Bacteriology, Virology and Immunity. Red. M.T. Parker, B.J. Duerden. Vol. 2, E. Arnold, A division of Hodder and Stoughton, London-Melbourne-Auckland, p.187.

Zaremba M.L., Borowski J. 1997. Medical microbiology (in Polish: Mikrobiologia lekarska). Wyd. Lekarskie PZWL, Warszawa , p.727.