NOTE *Public Health*

Bacillus cereus from the environment is genetically related to the highly pathogenic *B. cereus* in Zambia

Hirohito OGAWA[1,2)], Miyuki OHNUMA[3)], David SQUARRE[4)], Aaron Simanyengwe MWEENE[2)], Takayuki EZAKI[5)], Daisuke FUJIKURA[3)], Naomi OHNISHI[3)], Yuka THOMAS[1,2)], Bernard Mudenda HANG'OMBE[6)] and Hideaki HIGASHI[1,2,3)]*

[1)]*Hokudai Center for Zoonosis Control in Zambia, School of Veterinary Medicine, University of Zambia, P.O. Box 32379, Lusaka 10101, Zambia*

[2)]*Department of Disease Control, School of Veterinary Medicine, University of Zambia, P.O. Box 32379, Lusaka 10101, Zambia*

[3)]*Division of Infection and Immunity, Research Center for Zoonosis Control, Hokkaido University, Kita-20 Nishi-10, Kita-ku, Sapporo, Hokkaido 001–0020, Japan*

[4)]*Zambia Wildlife Authority (ZAWA), Private Bag 1, Chilanga 10101, Zambia*

[5)]*Department of Microbiology, Regeneration and Advanced Medical Science, Gifu University Graduate School of Medicine, 1–1 Yanagido, Gifu 501–1194, Japan*

[6)]*Department of Paraclinical Studies, School of Veterinary Medicine, University of Zambia, P.O. Box 32379, Lusaka 10101, Zambia*

(Received 27 January 2015/Accepted 10 March 2015/Published online in J-STAGE 23 March 2015)

ABSTRACT. To follow-up anthrax in Zambia since the outbreak in 2011, we have collected samples from the environment and the carcasses of anthrax-suspected animals, and have tried to isolate *Bacillus anthracis*. In the process of identification of *B. anthracis*, we collected two isolates, of which colonies were similar to *B. anthracis*; however, from the results of identification using the molecular-based methods, two isolates were genetically related to the highly pathogenic *B. cereus*, of which clinical manifestation is severe and fatal (e.g., pneumonia). In this study, we showed the existence of bacteria suspected to be highly pathogenic *B. cereus* in Zambia, indicating the possibility of an outbreak caused by highly pathogenic *B. cereus*.

KEY WORDS: *Bacillus anthracis*, *Bacillus cereus* group, *Bacillus thuringiensis*, epidemiology

doi: 10.1292/jvms.15-0059; *J. Vet. Med. Sci.* 77(8): 993–995, 2015

Bacillus cereus is mainly known as a cause of emetic and diarrheal food poisoning (typical *B. cereus*) [6]. In the past few decades, in humans, there have been reports of severe manifestations and fatal cases (e.g., pneumonia) resembling anthrax due to *B. cereus* (highly pathogenic *B. cereus*) [5, 10]. *B. cereus* belongs to the *B. cereus* group that includes *B. anthracis*, which is a well-known causative agent of anthrax worldwide; *B. thuringiensis*, which is a source of effective insecticide; and *B. mycoides*, *B. pseudomycoides* and *B. weihenstephanensis* [6]. *B. cereus*, *B. anthracis* and *B. thuringiensis* are considered to belong to a single species on the basis of the genome similarity [4]. Highly pathogenic *B. cereus* harbors *B. anthracis* genes in its genome [2, 5, 7]. Accordingly, it is highly pathogenic and is genetically closer to *B. anthracis* than typical *B. cereus* and *B. thuringiensis*. Indeed, it is difficult to distinguish highly pathogenic *B. cereus* from typical *B. cereus*, *B. anthracis* and *B. thuringiensis*, and these genetic situations have raised questions regarding the species to which the origin is and the species to which it should be classified.

In 2011, there was an outbreak of human anthrax in Zambia [3]. We have followed-up anthrax in Zambia since 2011. In the process of *B. anthracis* isolation during the surveillance study, we isolated a large number of *Bacillus* spp. from soil in Lower Zambezi National Park (15°40.931′S, 29°27.635′E). *Bacillus* spp. were isolated using the standard procedure. In brief, an aliquot of 1 g of specimen suspended in 10 m*l* of sterilized saline was incubated at 75°C for 20 min and then spread and cultured on 10% (v/v) sheep blood agar. In the process of identification of *B. anthracis*, several flat, "medusa head" and dry colonies formed by gram-positive spore-forming large-rod bacteria were harvested. Hemolysis on blood agar helps in the differentiation of *B. anthracis*, which is almost always nonhemolytic, and *B. cereus*, which is usually strongly hemolytic. In the hemolysis test, *B. anthracis* CZC5, which was isolated in Zambia [8], and *B. cereus* JCM2152 were used as standards. We collected two interesting colonies, named LZ77-2 and LZ78-8, which displayed weak hemolysis at 35°C for 48 hr, but were similar to *B. anthracis* at 37°C for 24 hr. In the detection of *B. anthracis* genes by conventional PCR [1], it is conceivable that LZ77-2 and LZ78-8 were *B. anthracis* isolates lacking plasmids or other bacteria that have similar properties to *B. anthracis* (Table 1). Furthermore, we performed the following: (i) determination of the 640th nucleotide of PlcR, which is a transcriptional regulator of extracellular virulence factors; (ii) PCR detection of four lambda phage

*CORRESPONDENCE TO: HIGASHI, H., Division of Infection and Immunity, Research Center for Zoonosis Control, Hokkaido University, Kita-20 Nishi-10, Kita-ku, Sapporo, Hokkaido 001–0020, Japan. e-mail: hidea-hi@czc.hokudai.ac.jp

Table 1. Characterization of *Bacillus* spp. analyzed in this study

Species	Strain	Hemolysis	Conventional PCR[b]			*plcR* nt640	Lambda phage				*dnaJ* group[c]
			pag	*cap*	Ba813		01	02	03	04	
Bacillus cereus	LZ77-2	Hemolytic[a]	–	–	+	G	–	–	–	–	BA
Bacillus cereus	LZ78-8	Hemolytic[a]	–	–	+	G	–	–	–	–	BA
Bacillus cereus	JCM2152	Hemolytic	–	–	–	G	–	–	–	–	BC 2
Bacillus anthracis	CZC5	Nonhemolytic	+	+	+	T	+	+	+	+	BA

a) After incubation at 35°C for 48 hr, weak hemolytic activity was displayed. b) *pag*, *cap* and Ba813 indicate protective antigen encoded by plasmid pXO1, capsule protein encoded by plasmid pXO2 and chromosome gene in *Bacillus anthracis*, respectively. c) BA and BC indicate *Bacillus anthracis* and *Bacillus cereus*, respectively.

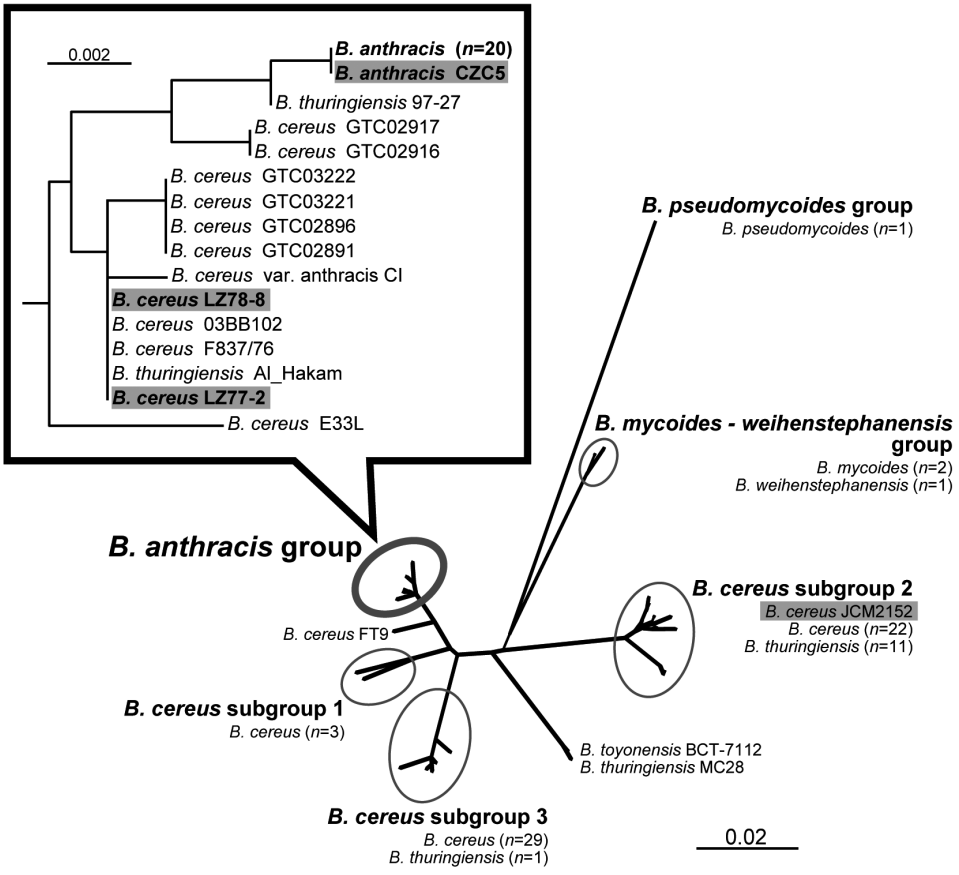

Fig. 1. Partial *dnaJ* phylogeny among the *Bacillus cereus* group. *DnaJ* sequences of *B. cereus* strains LZ77-2 and LZ78-8 as well as published data for 107 isolates were used to construct a neighbor-joining tree. The tree was constructed according to a published method [10]. The strains shaded with grey are *Bacillus* strains used in this study. The scale bar represents evolutionary distance in substitutions per site.

genes specifically integrated into the chromosome of *B. anthracis*; and (iii) phylogenetic analysis of the *dnaJ* sequence encoding heat shock protein 40. These are known as useful methods to differentiate *B. anthracis* from the *B. cereus* group at the genetic level [1, 10]. In *B. cereus* and *B. anthracis*, the 640th nucleotides are guanine/cytosine and thymine, respectively. In *B. anthracis*, a nonsense mutation caused by thymine inactivates the function of PlcR and ensuing nonhemolytic activity [9]. The 640th nucleotide in *plcR* of LZ77-2 and LZ78-8 was guanine (Table 1). Furthermore, four lambda prophage genes (lambda phage 01–04) specific to *B. anthracis* were not detected in LZ77-2 and LZ78-8 (Table 1). These results indicated that both the strains were not *B. anthracis* lacking plasmids. The *dnaJ* sequence is a potential molecular marker to discriminate highly pathogenic *B. cereus* strains from typical *B. cereus* strains [10]. As a result of phylogenetic analysis, LZ77-2 and LZ78-8 were grouped into the *B. anthracis* group, and they clustered with highly pathogenic *B. cereus* strains, including *B. cereus* GTC02891, GTC02896, GTC03221 and GTC03222, which

were isolated from severe infection outbreaks [10]; *B. cereus* 03BB102, which was isolated from a fatal pneumonia case [5]; and *B. cereus* var. anthracis CI, which was isolated from lethal anthrax in a chimpanzee [7] (Fig. 1). Taken together with previous reports [2, 5, 7], *B. cereus* and *B. thuringiensis* classified in the *B. anthracis* group are highly pathogenic *B. cereus* and *B. thuringiensis* isolated from humans. Therefore, it is suggested that LZ77-2 and LZ78-8 are highly pathogenic *B. cereus* because of its genetic properties.

We show here that bacteria suspected to be highly pathogenic *B. cereus* are present in Zambia. It is generally assumed that animals are infected with *B. anthracis* by ingesting its spores from the environment. Humans are infected on contact with infected animals. Indeed, human anthrax caused by zoonotic transmission occurred in 2011 on contact with anthrax-infected hippopotamus carcasses [3]. Cases of anthrax-suspected deaths in wildlife have been reported every year in Lower Zambezi National Park; however, our examinations showed that most cases were not caused by anthrax. Therefore, Zambia may be a high-risk country for highly pathogenic *B. cereus* infection outbreaks. Our results indicate the possibility of an outbreak caused by a highly pathogenic *B. cereus* in Zambia.

ACKNOWLEDGMENTS. We thank Evans Mulenga, Ladslav Moonga, Drs. Emiko Nakagawa, Yasuko Orba and Hirofumi Sawa for their tremendous support. We also thank the Zambia Wildlife Authority (ZAWA) and the Conservation Lower Zambezi (CLZ) for providing permission and the enabling environment for sampling in the national park and game management area. This work was supported by the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

REFERENCES

1. Ågren, J., Hamidjaja, R. A., Hansen, T., Ruuls, R., Thierry, S., Vigre, H., Janse, I., Sundström, A., Segerman, B., Koene, M., Löfström, C., Van Rotterdam, B. and Derzelle, S. 2013. *In silico* and *in vitro* evaluation of PCR-based assays for the detection of *Bacillus anthracis* chromosomal signature sequences. *Virulence* **4**: 671–685. [Medline] [CrossRef]
2. Han, C. S., Xie, G., Challacombe, J. F., Altherr, M. R., Bhotika, S. S., Brown, N., Bruce, D., Campbell, C. S., Campbell, M. L., Chen, J., Chertkov, O., Cleland, C., Dimitrijevic, M., Doggett, N. A., Fawcett, J. J., Glavina, T., Goodwin, L. A., Green, L. D., Hill, K. K., Hitchcock, P., Jackson, P. J., Keim, P., Kewalramani, A. R., Longmire, J., Lucas, S., Malfatti, S., McMurry, K., Meincke, L. J., Misra, M., Moseman, B. L., Mundt, M., Munk, A. C., Okinaka, R. T., Parson-Quintana, B., Reilly, L. P., Richardson, P., Robinson, D. L., Rubin, E., Saunders, E., Tapia, R., Tesmer, J. G., Thayer, N., Thompson, L. S., Tice, H., Ticknor, L. O., Wills, P. L., Brettin, T. S. and Gilna, P. 2006. Pathogenomic sequence analysis of *Bacillus cereus* and *Bacillus thuringiensis* isolates closely related to *Bacillus anthracis*. *J. Bacteriol.* **188**: 3382–3390. [Medline] [CrossRef]
3. Hang'ombe, M. B., Mwansa, J. C., Muwowo, S., Mulenga, P., Kapina, M., Musenga, E., Squarre, D., Mataa, L., Thomas, S. Y., Ogawa, H., Sawa, H. and Higashi, H. 2012. Human-animal anthrax outbreak in the Luangwa valley of Zambia in 2011. *Trop. Doct.* **42**: 136–139. [Medline] [CrossRef]
4. Helgason, E., Økstad, O. A., Caugant, D. A., Johansen, H. A., Fouet, A., Mock, M., Hegna, I. and Kolstø, A. B. 2000. *Bacillus anthracis, Bacillus cereus*, and *Bacillus thuringiensis*—one species on the basis of genetic evidence. *Appl. Environ. Microbiol.* **66**: 2627–2630. [Medline] [CrossRef]
5. Hoffmaster, A. R., Hill, K. K., Gee, J. E., Marston, C. K., De, B. K., Popovic, T., Sue, D., Wilkins, P. P., Avashia, S. B., Drumgoole, R., Helma, C. H., Ticknor, L. O., Okinaka, R. T. and Jackson, P. J. 2006. Characterization of *Bacillus cereus* isolates associated with fatal pneumonias: strains are closely related to *Bacillus anthracis* and harbor *B. anthracis* virulence genes. *J. Clin. Microbiol.* **44**: 3352–3360. [Medline] [CrossRef]
6. Jensen, G. B., Hansen, B. M., Eilenberg, J. and Mahillon, J. 2003. The hidden lifestyles of *Bacillus cereus* and relatives. *Environ. Microbiol.* **5**: 631–640. [Medline] [CrossRef]
7. Klee, S. R., Brzuszkiewicz, E. B., Nattermann, H., Brüggemann, H., Dupke, S., Wollherr, A., Franz, T., Pauli, G., Appel, B., Liebl, W., Couacy-Hymann, E., Boesch, C., Meyer, F. D., Leendertz, F. H., Ellerbrok, H., Gottschalk, G., Grunow, R. and Liesegang, H. 2010. The genome of a *Bacillus* isolate causing anthrax in chimpanzees combines chromosomal properties of *B. cereus* with *B. anthracis* virulence plasmids. *PLoS ONE* **5**: e10986. [Medline] [CrossRef]
8. Ohnishi, N., Maruyama, F., Ogawa, H., Kachi, H., Yamada, S., Fujikura, D., Nakagawa, I., Hang'ombe, M. B., Thomas, Y., Mweene, A. S. and Higashi, H. 2014. Genome Sequence of a *Bacillus anthracis* Outbreak Strain from Zambia, 2011. *Genome Announc.* **2**: e00116–e14. [Medline] [CrossRef]
9. Slamti, L., Perchat, S., Gominet, M., Vilas-Bôas, G., Fouet, A., Mock, M., Sanchis, V., Chaufaux, J., Gohar, M. and Lereclus, D. 2004. Distinct mutations in PlcR explain why some strains of the *Bacillus cereus* group are nonhemolytic. *J. Bacteriol.* **186**: 3531–3538. [Medline] [CrossRef]
10. Zhang, J., van Hung, P., Hayashi, M., Yoshida, S., Ohkusu, K. and Ezaki, T. 2011. *DnaJ* sequences of *Bacillus cereus* strains isolated from outbreaks of hospital infection are highly similar to *Bacillus anthracis*. *Diagn. Microbiol. Infect. Dis.* **70**: 307–315. [Medline] [CrossRef]