(NOTE)

Cholera toxin production by El Tor variant of *Vibrio cholerae* O1 as compared to prototype El Tor and classical biotypes

J. Ghosh-Banerjee[1]*, M. Senoh[2]*, T. Takahashi[2], T. Hamabata[3], S. Barman[1], H. Koley[1], A.K. Mukhopadhyay[1], T. Ramamurthy[1], S. Chatterjee[4], M. Asakura[4], S. Yamasaki[4], G.B. Nair[1] and Y. Takeda[2]

[1]National Institute of Cholera and Enteric Diseases, Kolkata, India
[2]Collaborative Research Center of Okayama University for Infectious Diseases in India, Kolkata, India
[3]Research Institute, International Medical Center of Japan, Tokyo, Japan
[4]Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Sakai, Japan.

*Equal contribution

Corresponding Author
Yoshifumi Takeda, Collaborative Research Center of Okayama University for Infectious Diseases in India, Beliaghata, Kolkata 700100, India
Tel: 91-33-2350-1176
Fax: 91-33-2350-5066
E-mail: takeda.yoshi@pheasant.pharm.okayama-u.ac.jp

Short title; Cholera toxin production by El Tor variant

Key word: cholera toxin, El Tor variant, *Vibrio cholerae* O1

Abstract

Vibrio cholerae O1 El Tor variant strains produced much more cholera toxin than that produced by prototype El Tor strains. The amount of cholera toxin produced by El Tor variant strains both *in vitro* and *in vivo* was more or less equivalent to that produced by classical strains.

Vibrio cholerae O1 is classified into classical and El Tor biotypes. Among other genetic, biochemical and physiological differences, each biotype has unique gene sequences encoding cholera toxin B subunit (CTB), that is, classical *ctxB* and El Tor *ctxB*. Besides these two prototype biotypes of *V. cholerae* O1, Nair *et al*. (9) in 2002 in Bangladesh isolated strains that possess phenotypic properties of both classical and El Tor biotype carrying classical *ctx*B. The same group also isolated El Tor strains that had classical *ctx*B (10). For these new types of strains of *V. cholerae* O1, we have recently proposed the designation of hybrid and El Tor variants, respectively (13). Subsequent to the isolation of El Tor variant in Bangladesh by Nair *et. al*. (10), El Tor variant strains were isolated from several countries and areas in Asia and Africa (1, 11, 15-18). In Kolkata, India, we showed that El Tor variant strains appeared in 1990 and a complete replacement of prototype El Tor strains by El Tor variant strains occurred since 1995 (14).

In this study, we investigated the amount of cholera toxin (CT) produced both *in vitro* and *in vivo* by *V. cholerae* O1 El Tor variant strains isolated in Kolkata during a period from 1996 to 2007. It was found that El Tor variant strains produced much higher amount of CT than prototype El Tor strains and that the amount of CT produced by El Tor variant strains was more or less equivalent to that produced by classical strains.

V. cholerae O1 strains used in this study are listed in Table 1. AKI (3) and Syncase medium (2) were used for culturing the test strains. The rationale for selecting these media was that AKI preferentially supports the production of El Tor CT (3) while Syncase medium is reported to be the best medium supporting the production of CT by classical biotype (2). Measurement of CT concentration produced by *V. cholerae* O1 strain was carried out as follows. Each strain was cultured either in AKI medium at 37ºC for 20 hours without shaking or in Syncase medium at 37ºC for 20 hours with shaking, and OD of the culture was measured at 600 nm. After centrifugation, the

supernatants were collected and the concentration of CT (ng/ml/OD_{600}) in the samples was measured by bead-ELISA. The method of the bead-ELISA employed was essentially as described by Oku *et al*. (12). In brief, a polystyrene bead (6.5 mm in diameter) was coated with anti-CT IgG and used as a solid phase. The coated bead was first incubated with the sample, and then incubated with anti-CT IgG (Fab')-horseradish peroxidase conjugate. Peroxidase activity was determined colorimetrically with 3, 3', 5, 5'-tetramethylbenzidine as the substrate. The absorbance at 450 nm (OD_{450}) was linear between 0 and 0.5 that represented CT concentration of 0 – 20 ng/ml. The sample prepared as described above (the supernatant of the culture of the strain) was appropriately diluted so that the OD_{450} fell in the range of 0.1 and 0.5 and the amount of CT produced by the strain was expressed by ng/ml/OD_{600}.

Rabbit ileal loop test was carried out essentially as described by Koley *et al*. (7). Eight intestinal loops of about 10 cm, separated by uninoculated segments of 1-2 cm, were prepared in each animal. Test loops were inoculated with 1 ml of bacterial suspension containing approximately 10^9 cells. Negative control loops were inoculated with 1 ml of phosphate buffered saline. The loops were replaced in the peritoneal cavity and the cavity was closed. After about 20 hours the animal was sacrificed by intravenous injection of sodium pentobarbital and the loops were taken out. The volume of the accumulated fluid in ml and the length of the loop in cm were measured and the extent of the fluid accumulation (FA) was expressed by ml/cm.

All 19 strains of *V. cholerae* O1 El Tor variant belonged to the El Tor biotype as evident from phenotypic traits such as resistance to 50 units of polymyxin B and positive Voges-Proskauer test (19). All harbored El Tor biotype specific alleles of *tcpA* and *rstR* when examined as described (5, 6). The *ctxB* gene of all strains was of classical type by mismatch amplification mutation assay (MAMA)-PCR carried out as described by Morita *et al*. (8). Further, the CTB produced by all strains was confirmed to be the classical type by Western blotting by using monoclonal antibody against either classical CTB or El Tor CTB, which was prepared by immunizing rats with synthesized peptide (either NTQIYTLNDKC for El Tor CTB and NTQIHTLNDKC for classical CTB). Approximately 50-100 ng of CT (measured by bead ELISA) in the culture supernatant of each strain were analyzed. The result of the Western blotting of a representative strain (strain AM157) is shown in Fig. 1.

Fig. 2 shows the distribution of the amount of CT produced by strains examined.

Each strain of El Tor variant, prototype El Tor and classical biotype was cultured in 2 ml of AKI medium in 10 ml test tube at 37°C for 20 hours without shaking, the supernatant of the culture was collected by centrifugation and was measured to determine the amount of CT by the bead-ELISA. It was found that most strains of El Tor variant produced much more CT than most strains of prototype El Tor strains. All 19 El Tor variant strains produced more than 1,000 ng/ml/OD_{600} of CT and among them 5 strains (AM157, 06-049, IDH60, BD200 and 06-098) produced more than 2,500 ng/ml/OD_{600}, the highest (strain AM157) producing 4,656 ng/ml/OD_{600}. The amount of CT produced varied, but was not related to the year of the isolation. Among 11 El Tor strains, 8 strains (V113, VC60, M14716, V7, VC64, V54, V24, V32) produced less than 100 ng/ml/OD_{600}, and among them 3 strains (V54, V24, V32) produced less than 20 ng/ml/OD_{600}. Rest of the strains (N16961, V100, V114) produced more than 100 ng/ml/OD_{600} and the standard strain N16961 produced the highest amount (345 ng/ml/OD_{600}). All 7 classical strains produced more than 900 ng/ml/OD_{600} and 2 of them (L362 and GP15) more than 2,000 ng/ml/OD_{600}, the highest being L362 (3,028 ng/ml/OD_{600}).

The amount of CT produced was measured during the growth of the strains in AKI medium with the representative strains of El Tor variant, prototype El Tor and classical biotype and it was found that the difference of the amount of CT produced among these 3 biotypes were observed from the beginning of the growth (early logarithmic phase) till the late stationary phase (data not shown).

Table 2 shows the mean CT amount produced by the strains of different biotypes with standard deviations. The amount of CT produced by El Tor variant strains was about 20 times more than that produced by prototype El Tor strains, and it was more or less equivalent to that produced by classical strains. A difference of the CT production between El Tor variant strains and prototype El Tor strains was statistically analyzed by Microsoft Excel 2004 for Mac, the p-value being <0.05.

CT production by strains of El Tor variant, El Tor and classical biotype were also examined when they were cultured in Syncase medium (2 ml in 10 ml test tube) at 37°C for 20 hours with shaking. As shown in Table 2, although the amount of CT produced in Syncase medium was much less than those produced in AKI medium, El Tor variant strains produced much more CT than that produced by El Tor strains, and more or less equivalent to that produced by classical strains. The p-value of the difference of the

amount produced between El Tor variant strains and prototype El Tor strains analyzed by Microsoft Excel 2004 for Mac was <0.05.

Ileal loop test was performed with a representative strain of El Tor variant (strain NLC41 producing 1,606 ng/ml/OD_{600} in AKI medium) together with representative strains of El Tor biotype (VC60 producing 60 ng/ml/OD_{600} in AKI medium) and classical biotype (L362 producing 3,028 ng/ml/OD_{600} in AKI medium). As shown in Table 3, FA ratio of the El Tor variant NLC41 was almost the same to that of classical strain L362. On the other hand, El Tor strain VC60 did not cause measurable fluid accumulation. This is most probably number of inoculated cells were not enough. Number of *V. cholerae* in the accumulated fluid (cfu/ml) and the amount of CT in the loop (ng/ml and ng/cfu) were also measured, showing that the El Tor variant strain grew better than the classical strain in the loop, thus the amount of CT in the loop inoculated with El Tor variant strain was higher than that with the classical strain. Measurement of cfu/ml of the accumulated fluid of the prototype of El Tor strains was not possible as no fluid accumulation occurred.

It is known that clinical manifestation of cholera caused by classical strains is more severe than that caused by prototype El Tor strains (4). Although a definite evidence to explain this is still not available, it has been hypothesized that a significant difference between the amounts of CT produced by these two biotype strains may reflect severity of clinical manifestation. If we would accept the above hypothesis, a recent report by World Health Organization (20) that *V. cholerae* El Tor variant causes more severe episodes of cholera with higher case fatality rates might be explained by the results reported in this paper. However, Siddique *et al.* (16) reported that although El Tor variant strains appeared in 1998 in Bangladesh, severity of cholera became evident since around 2006. Therefore they concluded that it is not clear whether the observed higher proportion of severe dehydration is due to El Tor variants. Further study is needed to elucidate the role of CT produced by El Tor variant strains in the clinical manifestation in its infection.

We thank Koichiro Yamamoto for his valuable discussion during this work. We also thank Debarati Ganguly for assistance in editing the manuscript.
This study was supported by the Japan Initiative for Global Research Network on Infectious Diseases, Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1. Ansaruzzaman, M., N. A. Bhuiyan, G. B. Nair, D. A. Sack, M. Lucas, J. L. Deen, J. Ampuero, C. L. Chaignat, and the Mozambique Cholera Vaccine Demonstration Project Coordination Group. 2004. Cholera in Mozambique, variant of *Vibrio cholerae*. Emerg. Infect. Dis. **10:** 2057-2059.

2. Finkelstein, R. A., P. Atthasampunna, M. Chulasamaya, and P. Charunmethee. 1966. Pathogenesis of experimental cholera: biologic activities of purified procholeragen A. J. Immunol. **96:** 440-449.

3. Iwanaga M, and K. Yamamoto. 1985. New medium for the production of cholera toxin by *Vibrio cholerae* O1 biotype El Tor. J. Clin. Microbiol. **22:** 405-408.

4. Kaper, J. B., J. G. Morris, Jr., and M. M. Levine. 1995. Cholera. Clin. Microbiol. Rev. **8:** 48-86.

5. Keasler, S. P., and R. H. Hall. 1993. Detecting and biotyping *Vibrio cholerae* O1 with multiplex polymerase chain reaction. Lancet. **341:** 1661.

6. Kimsey, H. H., G. B. Nair, A. Ghosh, and M. K. Waldor. 1998. Diverse CTX phage and evolution of new pathogenic *Vibrio cholerae*. Lancet. **352:**457-458.

7. Koley H, R. Mitra, A. Basu, A. K. Mukhopadhyay, P. L. Saha, B. S. Ramakrishna, S. Krishnan, Y. Takeda, and G. B. Nair. 1999. Response of wild-type mutants of *Vibrio cholerae* O1 possessing different combinations of virulence genes in the ligated rabbit ileal loop and Ussing chambers: evidence for the presence of additional secretogen. J. Med. Microbiol. **48**: 51-57.

8. Morita M, M. Ohnishi M, E. Arakawa, N. A. Bhuiyan, S. Nusrin, M. Alam, A. K. Siddique, F. Quadri, H. Izumiya, G. B. Nair, and H. Watanabe. 2008. Development and validation of a mismatch amplification mutation PCR assay to monitor the

dissemination of an emerging variant of *Vibrio cholerae* O1 biotype El Tor. Microbiol. Immunol, **52**: 314-317.

9. Nair, G. B., S. M. Faruque, N. A. Bhuiyan, M. Kamruzzaman, A. K. Siddiue, D. A. Sack. 2002. New variants of *Vibrio cholerae* O1 biotype El Tor with attributes of the classical biotype from hospitalized patients with acute diarrhea in Bangladesh. J. Clin. Microbiol. **40**: 3296-3299.

10. Nair, G. B., F. Qadri, J. Holmgren, A. M. Svennerholm. A. Safa, N. A. Bhuiyan, Q. S. Ahmad, S. M. Faruque, A. S. Faruque, Y. Takeda, and D. A. Sack. 2006. Cholera due to altered El Tor strains of *Vibrio cholerae* O1 in Bangladesh. J. Clin. Microbiol. **44**: 4211-4213.

11. Nguyen, B. M., J. H. Lee, N. T. Cuong, S. Y. Choi, N. T. Hien, D. D. Anh, H. R. Lee, M. Ansaruzzaman, H. P. Endtz, J. Chun, A. L. Lopez, C. Czerkinsky, J. D. Clemens, and D. W. Kim. 2009. Cholera outbreaks caused by an altered *Vibrio cholerae* O1 El Tor biotype strain producing classical cholera toxin B in Vietnam in 2007 to 2008. J. Clin. Microiol. **47:** 1568-1571.

12. Oku Y., Y. Uesaka, T. Hirayama, and Y. Takeda. 1988. Development of a highly sensitive bead-ELISA to detect bacterial protein toxins. Microbiol. Immunol. **32**: 807-816.

13. Raychoudhuri, A., A. K. Mukhopadhyay, R. K. Nandy, T. Ramamurthy, R. K. Nandy, Y. Takeda, and G. B. Nair. 2008. Biotyping of *Vibrio cholerae* O1: Time to redefine the scheme. Ind. J. Med. Res. **128:** 695-698.

14. Raychoudhuri, A., T., Patra, K. Ghosh, T. Ramamurthy, R. K. Nandy, Y. Takeda, G.B. Nair, and A. K. Mukhopadhyay. 2009. Classical *ctxB* in *Vibrio cholerae* O1, Kolkata, India. Emerg. Infect. Dis. **15**: 131-132.

15. Safa, A., J. Sultana, P. D. Cam, J. C. Mwansa, and R. Y. C. Kong. 2008. *Vibrio*

cholerae O1 hybrid El Tor strains, Asia and Africa. Emerg. Infect. Dis. **14**: 987-988.

16. Siddique, A. K., G. B. Nair, M. Alam, D. A. Sack, A. Huq, A. Nizam, I. M. Longini, Jr., F. Qadri, S. M. Faruque, R. R. Colwell, S. Ahmed, A. Iqbal, N. A. Bhuiyan, and R. B. Sack. 2010. El Tor cholera with severe disease: a new threat to Asia and beyond. Epidemiol. Infect. **138:** 347-352.

17. Sithivong, N., H. Izumiya, K. Munnalath, T. Phouthavane, K. Chomlasak, L. Sisavath, A. Vongdouangchanh, P. Vongprachanh, H. Watanabe, and M. Ohnishi. 2010. Cholera outbreak, Laos, 2007. Emerg. Infect. Dis. **16**: 745-746.

18. Taneja, N., A. Mishra, G. Sangar, G. Singh, and M. Sharma. 2009. Outbreaks caused by new variants of *Vibrio cholerae* O1 El Tor, India. Emerg. Infect. Dis. **15:** 352-354.

19. World Health Organization. 1987. Manual for Laboratory Investigations of Acute Enteric Infections.

20. World Health Organization. 2008. Cholera 2007. Weekly Epidem. Rec. **83:** 269-284.

Legend to Figures

Fig. 1. A result of Western blotting of the culture supernatant of a representative strain of El Tor variant biotype. Lane 1 and 6: 100 ng of the purified classical CT; Lane 2 and 7: 100 ng of the purified El Tor CT; Lane 3 and 8: sample of El Tor variant strain AM157; Lane 4 and 9; sample of El Tor strain N16961; Lane 5 and 10; sample of classical strain L362. Panel A: results with the monoclonal antibody against classical CTB; Panel B: results with the monoclonal antibody against El Tor CTB.

Fig. 2. Amount of CT produced by various biotypes of *V. cholerae* O1. Each circle represents an average of 4 determinations.

263 Table 1. *V. cholerae* O1 strains used.

El Tor variant[a] AM157 (1996)[b], 06-049 (2006), IDH60 (2007), BD200 (2002), 06-098 (2006), CRC220 (2000), AM168 (1996), DO2669 (1998), NLC96 (1999), CRC17 (2000), AM352 (1997), NLC41 (1999), NLC49 (1999), D26942 (1998), SC32 (2003), G27875 (2001), IDH32 (2007), SC216 (2003), NLC8 (1999)
El Tor[a] N16961, V100, V114, V113, VC60, M14716, V7, VC64, V54, V24, V32
Classical[a] L362, GP15, GP8, GP148, GP147, 569B, GP145

264 [a]Strains used are listed in the order of the CT production (from high to low).

265 [b]The year of the isolation is in parenthesis.

266

267 Table 2. Comparison of the amount of CT produced by strains of various biotypes of *V.*
268 *cholerae* O1[a].

Culture medium	CT concentration (ng/ml/OD_{600})		
	El Tor variant	El Tor	Classical
AKI	2044.1 ± 966.8	91.3 ± 104.6	1664.4 ± 782.0
Syncase	81.3 ± 147.2	4.5 ± 3.7[b]	114.7 ± 188.8

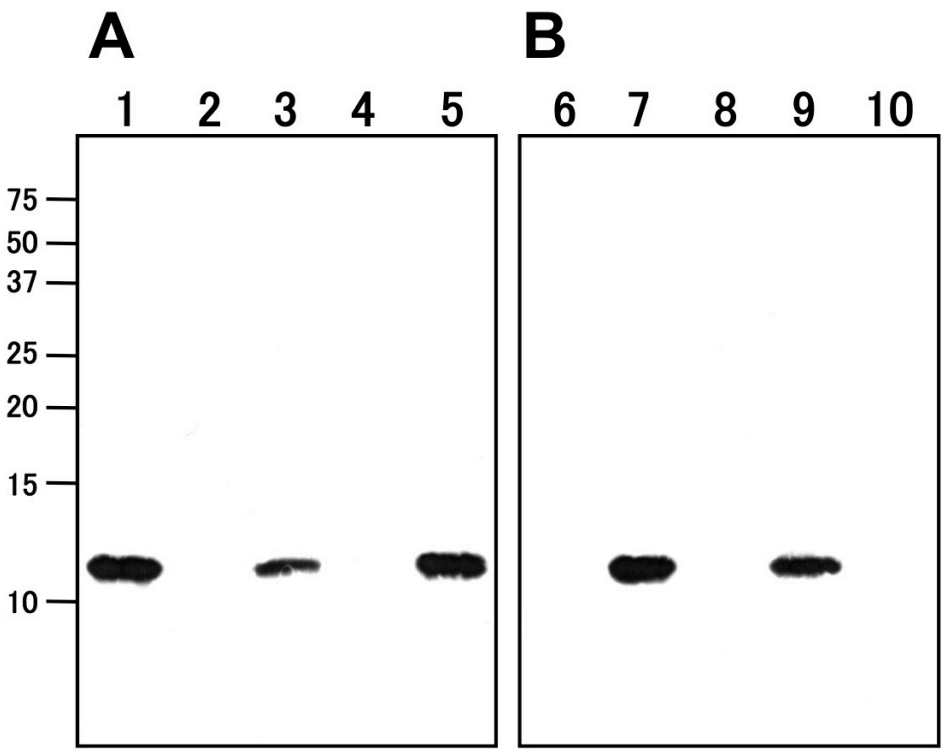
269 [a] Strains examined were as listed in Table 1 unless indicated.
270 [b] Only 5 strain of El Tor biotype (N16961, V113, VC64, VC60, V24) grew in Syncase
271 medium cultured at 37ºC with shaking.
272

273 Table 3. Results of rabbit ileal loop test[a].

Biotype	Strain	FA (ml/cm)[a]	CFU/ml[b]	CT (ng/ml)[a]	CT (ng/CFU)
El Tor variant	NLC41	0.90 ± 0.29	1.0×10^{9}	1006	1.006×10^{-6}
El Tor	VC60	0	-[c]	-	-
Classical	L362	0.83 ± 0.38	1.6×10^{8}	17.5	1.09×10^{-7}

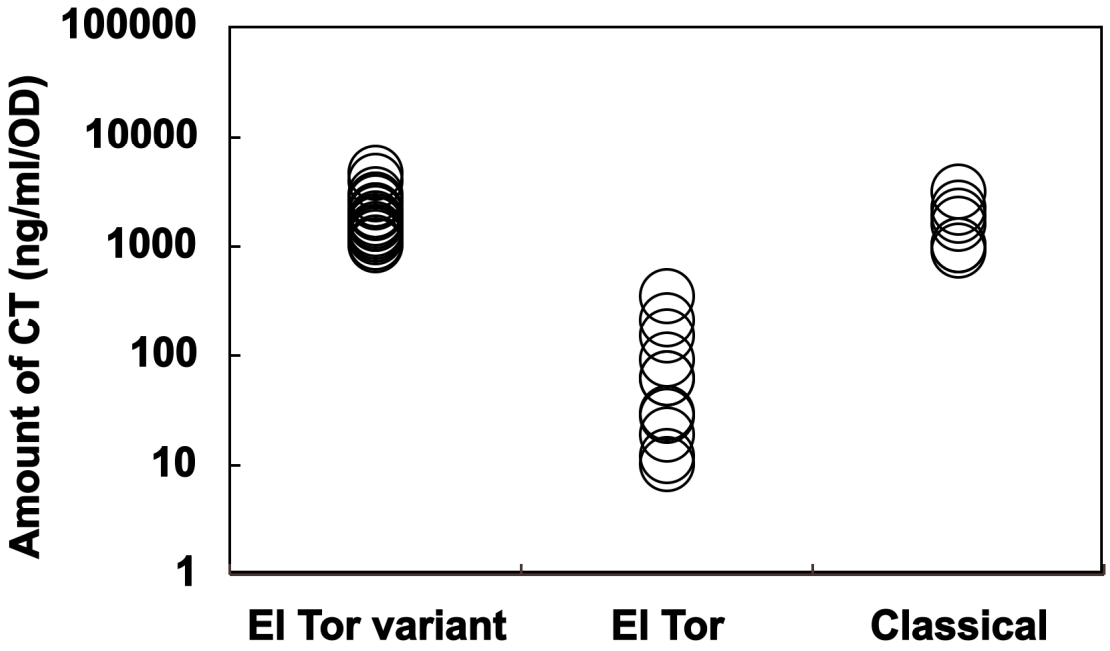
274 [a] An average of 4 determinations (2 loops each in 2 rabbits). Statystical analysis was
275 performed by Microsoft Excel 2004 for Mac.
276 [b] An average of 2 determinations (2 loops of 1 representative rabbit)
277 [c] Not applicable as no fluid accumulation occurred.
278

Fig. 1



296 Fig. 2

297


100000
10000
1000
100
10
1
Amount of CT (ng/ml/OD)
El Tor variant
El Tor
Classical