Short Communication

Susceptibility of *Clostridium difficile* Isolated from Healthy Captive Asian Elephants to Metronidazole and Vancomycin

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Abstract

Susceptibility to metronidazole and vancomycin, drugs of choice for *Clostridium difficile* infection, of 15 *C. difficile* isolates from 6 healthy Asian elephants was determined. All of the isolates belonged to only 1 ribotype pattern and carried both toxin A and B genes. The Minimal inhibitory concentration range of metronidazole and vancomycin, drugs of choice for treatment of *C. difficile* infection, was 0.125-4.0 µg/ml and 0.125-2.0 µg/ml, respectively. Moreover, MIC_{50} and MIC_{90} for metronidazole were 0.75 and 1.5 µg/ml while vancomycin was 1.0 and 2.0 µg/ml. There was no evidence of resistance to these antimicrobials. These results might be a preliminary data for further study of animal *C. difficile*.

Keywords: captive Asian elephant, *Clostridium difficile*, metronidazole, MIC, vancomycin

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บทคัดย่อ

ความไวรับของเชื้อ *Clostridium difficile* ที่คัดแยกได้จากช้างเลี้ยงเอเชียต่อยาเมทโทรนิดาโซลและแวนโคมัยซิน

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การศึกษานี้เป็นการศึกษาความไวรับของเชื้อ *C. difficile* จำนวน 15 สายพันธุ์ จากช้างเลี้ยงเอเชียจำนวน 6 เชือกต่อยาเมทโทรนิดาโซลและแวนโคมัยซิน ซึ่งเป็นยาตัวเลือกสำหรับโรคติดเชื้อ *C. difficile* จากการศึกษาพบว่าแบคทีเรีย *C. difficile* ทุกสายพันธุ์มีรูปแบบของไรโบทัยป์เดียวกัน และทุกสายพันธุ์มีทั้งยีนของท็อกซินเอและบีตามลำดับ ค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งการเจริญของเชื้อ (Minimal inhibitory concentration) ต่อยาเมทโทรนิดาโซลและแวนโคมัยซินซึ่งเป็นยาต้านจุลชีพที่ใช้ในการรักษาโรคติดเชื้อแบคทีเรีย *C. difficile* อยู่ในช่วง 0.125-4.0 ไมโครกรัมต่อมิลลิลิตร และ 0.125-2.0 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ นอกจากนั้นค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งการเจริญของเชื้อร้อยละ 50 และ 90 (MIC_{50} และ MIC_{90}) ต่อยาเมทโทรนิดาโซลเท่ากับ 0.75 และ 1.5 ไมโครกรัมต่อมิลลิลิตร สำหรับยาแวนโคมัยซินเท่ากับ 1.0 และ 2.0 ไมโครกรัมต่อมิลลิลิตร ทั้งนี้ไม่พบการดื้อต่อยาต้านจุลชีพทั้งสองชนิดนี้ ผลการทดลองที่ได้รับจะเป็นข้อมูลเบื้องต้นในการศึกษาเชื้อ *C. difficile* จากสัตว์ต่อไป

คำสำคัญ: ค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งการเจริญของเชื้อ ช้างเลี้ยงเอเชีย เมทโทรนิดาโซล แวนโคมัยซิน *Clostridium difficile*

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Introduction

Clostridium difficile is a gram-positive anaerobic bacterium with sporulated bacilli which is recognized as a causative agent of a severe colon disease called pseudomembranous colitis in human. In addition, *C. difficile* is known to be the first cause of nosocomial diarrhea (Kelly and Lamont, 1998) or diarrhea associated with antimicrobial usage. Predominant virulent factors of this bacterium are two producing toxins A and B. Both are potent toxins and cause extensive colonic inflammation and epithelial tissue damage in the infected host (Carter et al., 2012). Diagnosis of *C. difficile* is generally performed by fecal culture with selective media in anaerobic condition and detection of its toxins (Delmée, 2001; Delmée et al., 2005). Bojesen et al. (2006) showed the first case report of elephant *C. difficile* infection in Denmark. Post-mortem necropsy and laboratory diagnosis confirmed overgrowing of *C. difficile* in gastrointestinal (GI) tract of elephant induced by Sulforaphane-like substance in broccoli even though the bacterium was recognized as a normal flora (Songer, 1996; Sthitmatee and Boonmar, 2011). Our previous study isolated 15 strains of C. difficile from 6 healthy elephants and concluded that *C. difficile* was a normal microbe in GI tract of healthy elephants (Sthitmatee and Boonmar, 2011). Moreover, the previous study in Austrian suggested that animal reservoirs were possible sources, via direct contact with food or meat products, of *C. difficile* infection in human (Indra et al., 2009). In Thailand, captive elephants are for shows and forest sightseeing. Thus, there are many possibilities for human especially mahouts and tourists to direct expose to *C. difficile*. However, there is still scarce therapeutic information on animal *C. difficile* isolates, only in pigs (Post and Songer, 2004). Therefore, the objectives of the present study were (1) to obtain the therapeutic information on disease caused by *C. difficile* strains isolated from elephant source, determining the antimicrobial susceptibility of these 15 isolates to metronidazole (MTZ) and vancomycin (VAN), which are the drugs of choice for the treatment of *C. difficile* infection in human (Johnson, 2009; Leffler and Lamont, 2009) and (2) to characterize the isolates by detection of toxin genes and ribotyping. The advantage of this study is preliminary information for further investigation of this bacterium in animals.

Materials and Methods

Bacterial culture: Bacteria were anaerobically cultured at 37^{0}C for 48 hours on cycloserine cefoxitin fructose agar (CCFA) supplemented with egg yolk (Oxoid, Basingstoke, UK), 500 µg/ml cycloserine (Oxoid) and 6 µg/ml cefoxitin (Oxoid) as described previously (Post and Songer, 2004). The anaerobic condition was composed of 5% Hydrogen, 5% Carbon dioxide and 90% Nitrogen, and was conducted by Bactron X-2 Anaerobic Chamber (Engineered Production System, Orange Country, CA). Positive colonies which appeared grayish, opaque and had typical horse manure odor were selected. Then, a

single colony was picked and incubated in tryptic soy broth (TSB: Merck, Darmstadt, Germany) for DNA preparation.

DNA preparation and molecular characterization of the strains: Bacterial suspension was subjected to prepare chromosomal DNA with High Pure PCR template preparation Kit (Roche, Mannheim, Germany). Toxin gene typing was performed by multiplex PCR for identification of genes *tpi*, *tcd*A and *tcd*B as described previously (Lemee et al., 2004). The PCR ribotyping was employed by the protocol reported previously (Keel et al., 2007).

MIC: MIC was adapted and modified based on agar dilution method as described by a revised standard method of the Japanese Society of Antimicrobials for Animals in 2003. Briefly, metronidazole (MTZ: Sigma-Aldrich, St. Louis, MO, USA) and vancomycin (VAN: Sigma-Aldrich) were prepared with 2-fold dilution before adding into culture agar and pouring into sterile culture plates as described previously (Zheng et al., 2007). Agar medium used was 5% horse blood Brucella agar (Oxoid) supplemented with hemin (Acros Organics, Geel, Belgium) and vitamin K (Acros Organics). Subsequently, single colony was grown in TSB at 37^0C in anaerobic chamber until bacterial suspension reached McFarland Standard No. 1.5. Then, the plates were inoculated in anaerobic condition at 37^0C for 48 hours. The lowest concentration of an antimicrobial, which completely inhibited bacterial growth, was considered to be the endpoint and the concentration was regarded as the MIC. The resistance breakpoint values of VAN was ≥4 µg/ml and MTZ was >16 µg/ml, respectively (Zheng et al., 2007). Moreover, *Bacteroides fragilis* (ATCC 25285) and *B. thetaiotaomicron* (ATCC 29741) were included as controls.

Results and Discussion

Six elephants were raised at 2 different camps. There were four elephants in camp A, one male (21 years old) and three females (17, 22 and 25 years old) and, one male (18 years old) and one female (27 years old) in camp B. All captive elephants were fed on bananas, wild sugarcanes (*Saccharum spontaneum*) and additional commercial concentrate feeds during daytime. There were also abundance of water and plants such as grass, bamboo leaves, and wild sugarcanes in the area where the elephants were held. Moreover, elephant health management was under supervision of veterinarians. According to elephants' individual health records, there was no evidence of antimicrobial usage six month prior to the experiment.

All the isolates carried both toxin A and B genes and the ribotyping analyses indicated that there was only 1 pattern among 15 isolates. MIC range of MTZ was 0.125-4.0 µg/ml while VAN was 0.125-2.0 µg/ml. Moreover, MIC_{50} and MIC_{90} for MTZ were 0.75 and 1.5 µg/ml while MIC_{50} and MIC_{90} for VAN were 1.0 and 2.0 µg/ml, respectively. In addition, there was no evidence of resistance to these antimicrobials.

Clostridium spp. is recognized as enteric pathogens in human, domestic animals and also wildlife (Songer, 1996; Magdesian et al., 2002). *C. difficile* caused pseudomembranous colitis in human, hemorrhagic necrotizing enterocolitis in foals (Magdesian et al., 2002), fatal enterocolitis in Asian elephants (Bojesen et al., 2006), and neonatal enteritis in piglet (Post and Songer, 2004; Avbersek et al., 2009). However, *C. difficile* could be isolated from the environment including healthy animal species (Medina-Torres et al., 2011) by control of the normal flora in GI tract. Disturbances to the normal flora in GI tract affect microbial growth and eventually cause disease in animals; for example, the over feeding broccoli in Danish elephants leading to overgrowth of *C. difficile* and resulting in fatal enterocolitis (Bojesen et al., 2006). *C. difficile* produces two types of exotoxins; A (TcdA: enterotoxin) and B (TcdB: cytotoxin) (Carter et al., 2012). These two toxins react to cell cytoskeleton by damaging cell tight junction and cause epithelium cell erosion. *C. difficile* infection in animals caused moderate to severe mesocolonic edema and pasty-to-watery yellowish colonic contents in 1-7 day-old piglets (Songer, 2004) and diarrhea calves (Hammitt et al., 2008). There is still no evidence of human infected with *C. difficile* genetically related to elephant *C. difficile*, however, mahouts and tourists have a higher risk of exposure to this bacterium. Moreover, the results of Indra et al. (2009) suggested that animal reservoirs are possible sources, via food, of human *C. difficile* infection. Therefore, molecular epidemiology among human strains and elephant strains need to be investigated.

MTZ and VAN are the drugs of choice for disease caused by *C. difficile* in human (Post and Songer, 2004). Ranges of MIC for MTZ and VAN have been reported (Wong et al., 1999; Aspevall et al., 2006). MIC of *C. difficile* strains to MTZ and VAN from Swedish University Hospital were 0.032-1.0 and 0.5-2.0 µg/ml (Aspevall et al., 2006) while in Hong Kong they ranged from 0.094-1.5 and 0.125-2.0 µg/ml, respectively (Wong et al., 1999). As our results, MIC range from elephant isolates were close to all human isolates. Moreover, MIC_{90} of MTZ and VAN in elephant isolates was similar to MIC_{90} from human isolates (Huang et al., 2009). However, resistance to antimicrobials in *C. difficile* varied between countries (Huang et al., 2009). Most isolates are still susceptible to VAN and MTZ but decrease in sensitivity is emerging. Huang et al. (2009) suggested that resistant mechanisms of *C. difficile* are similar to the Gram-positive bacterium but trends and mechanisms were still required for further study.

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References

Aspevall O, Lundburg A, Burman LG, Akerlund T and Svenungsson B 2006. Antimicrobial susceptibility pattern of *Clostridium difficile* and its relation to PCR ribotype in Swedish University Hospital. Antimicrob Agents Chemother. 50: 1890-1892.

Avbersek J, Janezic S, Pate M, Rupnik M, Zidaric V, Logar K, Vengust M, Zemljic M, Pirs T and Ocepek M 2009. Diversity of *Clostridium difficile* in pigs and other animals in Slovenia. Anaerobe 15: 252-255.

Bojesen AM, Olsen KEP and Bertelsen MF 2006. Fatal enterocolitis in Asian elephants (*Elephas maximus*) caused by *Clostridium difficile*. Vet Microbiol. 116: 329-335.

Carter GP, Rood JI and Lyras D 2012. The role of toxin A and toxin B in the virulence of *Clostridium difficile*. Trends Microbiol. 20: 21-29.

Delmée M 2001. Laboratory diagnosis of *Clostridium difficile* disease. Clin Microbiol Infect. 7: 411-416.

Delmée M, Van Broeck J, Simon A, Janssens M and Avesani V 2005. Laboratory diagnosis of *Clostridium difficile*-associated diarrhea: A plea for culture. J Med Microbiol. 54: 187-191.

Hammitt MC, Bueschel D, Keel MK, Glock R, Cuneo DP, DeYoung D, Reggiardo C, Trinh HT and Songer JG 2008. A possible role for *Clostridium difficile* in the etiology of calf enteritis. Vet Microbiol. 127: 343-352.

Huang H, Weintraub A, Fang H and Nord CE 2009. Antimicrobial resistance in *Clostridium difficile*. Int J Antimicrob Agents. 34: 516-522.

Indra H, Lassnig H, Baliko N, Much P, Fiedler A, Huhulescu S and Allerberger F 2009. *Clostridium difficile*: A new zoonotic agent?. Wien Klin Wochenschr. 121: 91-95.

Johnson S 2009. Recurrent *Clostridium difficile* infection: Causality and therapeutic approaches. Int J Antimicrob Agents. 33(S1): S33-S36.

Keel K, Brazier JS, Post KW, Weese S and Songer JG 2007. Prevalence of PCR ribotypes among *Clostridium difficile* isolates from pigs, calves, and other species. J Clin Microbiol. 45: 1963-1964.

Kelly CP and Lamont JT 1998. *Clostridium difficile* infection. Ann Rev Med. 49: 375-390.

Leffler DA and Lamont JT 2009. Treatment of *Clostridium difficile*-Associated Disease. Gastroenterology 136: 1899-1912.

Lemee L, Dhalluin A, Testelin S, Mattrat MA, Maillard K, Lemeland JF and Pons JL 2004. Multiplex PCR targeting tpi (Triose Phosphate Isomerase), tcdA (Toxin A), and tcdB (Toxin B) genes for toxigenic culture of *Clostridium difficile*. J Clin Microbiol. 42: 5710-5714.

Magdesian KG, Hirsh DC, Jang SS, Hansen LM and Madigan JE 2002. Characterization of *Clostridium difficile* isolates from foals with diarrhea: 28 cases (1993-1997). J Am Vet Med Assoc. 220: 67-73.

Medina-Torres CE, Weese JS and Staempfli HR 2011. Prevalence of *Clostridium difficile* in horses. Vet Microbiol. 152: 212-215.

Post KW and Songer JG 2004. Antimicrobial susceptibility of *Clostridium difficile* isolated from neonatal pigs with enteritis. Anaerobe 10: 47-50.

Songer JG 1996. Clostridial enteric diseases of domestic animals. Clin Microbiol Rev. 9: 216-234.

Songer JG 2004. The emergence of *Clostridium difficile* as a pathogen of food animals. Anim Health Res Rev. 5: 321-326.

Sthitmatee N and Boonmar S 2011. Epidemiology of fermenter bacteria in intestine of captive Asian elephants (*Elephas maximus*). Chiang Mai: Chaing Mai University Press. 45 pp.

Stubbs SLJ, Brazier JS, O'neill GL and Duerden BI 1999. PCR targeted to the 16S-23SrRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. J Clin Microbiol. 37: 461–463.

The determination method of minimum inhibitory concentration (MIC) of antimicrobials against bacteria isolated from animals (Revised standard method of the Japanese society of antimicrobials for animals in 2003). In: Proceeding of the Japanese society of antimicrobials for animals, 26: 64-74.

Wong SS, Woo PC, Luk W and Yuen K 1999. Susceptibility testing of *Clostridium difficile* against metronidazole and vancomycin by disk diffusion and Etest. Diagn Microbiol Infect Dis. 34: 1-6.

Zheng L, Citronb DM, Genheimera CW, Sigmona SF, Carmana RJ, Lyerlya DM and Goldstein EJC 2007. Molecular characterization and antimicrobial susceptibilities of extraintestinal *Clostridium difficile* isolates. Anaerobe 13: 114–120.