The Thai Journal of Veterinary Medicine

Volume 46
Issue 2 *June, 2016*

Article 17

6-1-2016

Quantitative Method for Detecting Vibrio parahaemolyticus Using Bio-Theta DOXTM System

Shouichi Tanno

Naoki Fukui

Yukihiro Utaka

Saburo Ohkawa

Yukio Morita

See next page for additional authors

Quantitative Method for Detecting Vibrio parahaemolyticus Using Bio-Theta DOXTM System

Authors

Shouichi Tanno, Naoki Fukui, Yukihiro Utaka, Saburo Ohkawa, Yukio Morita, and Sumalee Boonmar

Short Communication

Quantitative Method for Detecting *Vibrio parahaemolyticus* Using Bio-Theta DOX™ System

Shouichi Tanno[1] Naoki Fukui[1] Yukihiro Utaka[1] Saburo Ohkawa[1]

Yukio Morita[2] Sumalee Boonmar[2*]

Abstract

This is the first report on the quantitative method for detecting *Vibrio parahaemolyticus* using the Bio-Theta DOX™ system. Seven *V. parahaemolyticus* strains and 23 non-*V. parahaemolyticus* strains were assessed. The rate of positive detection of *Vibrio* in samples of the 7 *V. parahaemolyticus* strains at high concentration (10^5 cfu/ml), medium concentration (10^3 cfu/ml), and low concentration (10 cfu/ml) was 100%. All 7 *V. parahaemolyticus* strains displayed good linear calibration curves for detection time relative to bacterial count ($r > 0.993$). The mean negative rate for the high-concentration (10^6 cfu/ml) non-*V. parahaemolyticus* samples was 65.2% (15/23) and that for the medium-concentration (10^3 cfu/ml) samples was 82.6% (19/23). Positive results for detection of the bacteria were obtained for some samples among the 7 *V. alginolyticus* strains and 1 *V. harveyi* strain tested; however, the detection times for these species were longer than that for *V. parahaemolyticus*. A method for measuring total *Vibrio* counts using the DOX system has previously been developed. Although further detailed field experiments are needed, regular measurement of total *Vibrio* and *V. parahaemolyticus* counts in shrimp farm pond water should initially help prevent EMS/AHPNS disease in these shrimp farming systems.

Keywords: bacteria-detecting system, DOX™ system, early precautions, *Vibrio parahaemolyticus*

[1]*Daikin Industries, Ltd., 1-1, Nishi-Hitotsuya Settsu, Osaka 566-8585, Japan*
[2]*Tokyo Kasei University, 1-18-1 Kaga, Itabashi-ku, Tokyo 173-8602, Japan*
***Correspondence**: *sumaleeka12@gmail.com*

Introduction

Early mortality syndrome (EMS) and acute hepatopancreatic necrosis syndrome (AHPNS) in cultured shrimp have likely been responsible for infections in shrimp aquaculture operations in Asian countries including Thailand, Vietnam, and China. The outbreak of EMS/AHPNS in cultured shrimp is a serious economic problem for shrimp exporting countries including Thailand. According to statistical data of the Thai Shrimp Association, production volume of cultured shrimp in 2010 was a maximum of approximately 640,000 ton. However, the volume in 2013 decreased to about 250,000 ton. Although the production volume has increased, the outbreak of the disease is still a primary concern.

The causative organism for these diseases is *V. parahaemolyticus,* which has consistently been isolated from EMS/AHPNS-infected shrimp (*Litopenaeus vannamei*) (Tran et al., 2013). *V. harveyi* was reported as the causative agent of vibriosis in tiger prawn (*Penaeus monodon*), kuruma prawn (*Penaeus japonicus*), and pearl oyster (*Pinctada maxima*) (Pass et al., 1987; Lavilla-Pitago et al., 1990; De la Pena et al., 1993). *V. alginolyticus* inhabits the aquatic environment, and the organism is easy to isolate from sea fish and seawater in tropical areas (Farmer et al., 2005). Mass mortalities in shrimp hatcheries and culture ponds in Asian countries have been reported (Sunaryanto and Mariyam, 1986; Baticados et al., 1991). The total *Vibrio* count in a typical shrimp farm pond is around 10^2-10^4 cfu/ml (Kaneko and Colwell, 1973; Paclibare et al., 2002; Thakur et al., 2004; Gopal et al., 2005). A total *V. parahaemolyticus* count of 10^6 cfu/ml in immersion challenge tests can result in the development of EMS/AHPNS in shrimp (Tran et al., 2013). Although the epidemiology of EMS/AHPNS has not been completely clarified, shrimp farmers who have monitored total *Vibrio* count and *V. parahaemolyticus* count in shrimp farming pond water and have found the count to increase are able to immediately take measures such as using antibacterial products. Thus, the development of a rapid and easy on-site method for measuring the count might be a useful mean for preventing shrimp from being infected with EMS/AHPNS or other *Vibrio* species.

The full genome sequence of *V. parahaemolyticus* isolated from EMS/AHPNS-infected shrimp in Thailand was previously reported (Kondo et al., 2014). A quantitative method for detecting *Vibrio* species using the Bio-Theta DOX™ system has been developed in our previous study (Tanno et al., 2014). Moreover, a method for specifically detecting *V. parahaemolyticus* using the DOX system has subsequently been developed, and the present study appears to be the first report on a *V. parahaemolyticus*-specific detection method.

Materials and Methods

Isolates: Seven *V. parahaemolyticus* strains and 23 non-*V. parahaemolyticus* bacterial strains were analyzed in the present study; all of these strains are listed in Table 1. Eleven bacterial strains were obtained from the American Type Culture Collection; seven strains were obtained from the Research Institute for Microbial Diseases, Osaka University, Japan; and one strain was obtained from the Japan Collection of Microorganisms, Japan. The other 11 strains were isolated from shrimp farm ponds (4 strains), seawater (3 strains), human patients (2 strains), shrimp (*Litopenaeus vannamei*) samples (1 strain), and oyster (*Crassostrea gigas*) samples (1 strain).

DOX system examination of the isolates: All *Vibrio* strains used in the present study were streaked on trypticase soy agar (Nissui, Tokyo, Japan) with 1.5% NaCl and the non-*Vibrio* strains were streaked on trypticase soy agar alone. All plates were incubated at 37°C for 16-20 h under aerobic conditions. The growing *V. parahaemolyticus* strains were diluted to approximately 10^5 cfu/ml (high concentration), 10^3 cfu/ml (medium concentration), and 10^1 cfu/ml (low concentration). Dilutions were prepared in a custom dilution medium consisting of 1.25 ml of Solution A (pH 7.2 containing 34 g calcium dihydrogen phosphate and 10.2 g sodium hydroxide brought to a total volume of 1,000 ml with distilled water) mixed with 1000 ml of 1.5% sodium chloride (NaCl) solution. The non-*V. parahaemolyticus* strains were diluted to about 10^6 cfu/ml (high concentration) or 10^3 cfu/ml (medium concentration) in Solution A without added NaCl. Bacterial concentrations were initially measured by determining optical density values at 660 nm. Subsequently, each custom test organism dilution (1 ml) was combined with 1 ml DOX *V. parahaemolyticus* media and inoculated into the DOX coliform cassette and shaken by hand for 30 s. The cassette was placed in the sample port of the DOX system and left for 24 h. When the DOX system revealed positive results, total measurement time was recorded. All samples were examined in triplicate.

Results and Discussion

Quantitative analysis of V. parahaemolyticus strains: The results of the high-, medium-, and low-concentration *V. parahaemolyticus* samples detected by the DOX system are shown in Table 2. The mean positive rate for the high- (10^5 cfu/ml), medium- (10^3 cfu/ml), and low-concentration (10 cfu/ml) *Vibrio* samples was 100%. The detection time for positive *V. parahaemolyticus* samples was 214.3-311.3 min for the high-concentration samples, 352.0-527.7 min for the medium-concentration samples, and 514.3-710.3 min for the low-concentration samples. The rate of change of the *V. parahaemolyticus* samples during the detection period was 0.7-4.2% for the high-concentration samples, 0.3-6.6 % for the medium-concentration samples, and 0.4-3.9% for the low-concentration samples. A linear calibration curve between detection times and bacterial counts was observed for all *V. parahaemolyticus* strains. The correlation coefficient (r) between detection times and bacterial counts for all the *V. parahaemolyticus* strains was >0.993.

Quantitative analysis of non-V. parahaemolyticus strains: For the 23 non-*V. parahaemolyticus* strain samples, the mean negative rate for the high-concentration (10^6 cfu/ml) samples was 65.2% (15/23) and that for the medium-concentration (10^3 cfu/ml)

samples was 82.6% (19/23). The results for the 8 positive non-*V. parahaemolyticus* samples detected by the DOX system are shown in Table 3. Using high-concentration samples, 5 strains of *V. alginolyticus* (Strains No. 8, 10, 11, 13, and 14), and 1 strain of *V. harveyi* (Strain No. 18) showed positive for each of 3 replicates. Two of 3 replicate samples of *V. alginolyticus* (Strain No. 9) and 1 of 3 replicate samples of *V. alginolyticus* (Strain No. 12) were positive. The detection times for *V. alginolyticus* and *V. harveyi* were 644.0-1102.0 min, and 308.0 min, respectively. At the intermediate concentration, all 3 replicate samples of *V. harveyi* (Strain No. 18) and 1 of 3 samples of *V. alginolyticus* (Strains No. 8, 13, 14) were positive. The detection times for *V. harveyi* and *V. alginolyticus* were 693.0 min, and 882.0-1125.0 min, respectively.

Table 1 *Vibrio parahaemolyticus* and other strains examined in this study

Strain No.	Species	Strain or source
1	*V.parahaemolyticus*	ATCC[a)] 27969
2	*V.parahaemolyticus*	RIMD[b)] 2212197
3	*V.parahaemolyticus*	Indonesian sea water
4	*V.parahaemolyticus*	Indonesian pond water for prawn culturing
5	*V.parahaemolyticus*	Shrimp
6	*V.parahaemolyticus*	Clinical
7	*V.parahaemolyticus*	Clinical
8	*V.alginolyticus*	ATCC 17749
9	*V.alginolyticus*	Indonesian pond water for prawn culturing
10	*V.alginolyticus*	Indonesian sea water
11	*V.alginolyticus*	Indonesian sea water
12	*V.alginolyticus*	Indonesian pond water for prawn culturing
13	*V.alginolyticus*	Fresh oyster
14	*V.alginolyticus*	Indonesian pond water for prawn culturing
15	*V.cholerar* non-O:1	RIMD 2203259
16	*V.fluvialis*	RIMD 2220001
17	*V.Furnissii*	RIMD 2223001
18	*V.harveyi*	RIMD 2224001
19	*V.mimicus*	RIMD 2218001
20	*V.vulnificus*	RIMD 2219031
21	*Aeromonas hydrophila*	JCM[c)] 1027
22	*Citrobacter freundii*	ATCC 8090
23	*Enterobacter cloacae*	ATCC 13047
24	*Enterococcus faecalis*	ATCC 29212
25	*Escherichia coli*	ATCC 25922
26	*Klebsiella pneumoniae*	ATCC 13883
27	*Proteus mirabills*	ATCC 29906
28	*Pseudomonas aeruginosa*	ATCC 27853
29	*Staphylococcus aureus*	ATCC 25923
30	*Salmonella* Typhimurium	ATCC 14028

a) ATCC: American Type Culture Collection.
b) RIMD: Research Institute for Microbial Diseases, Osaka University, Japan.
c) JCM: Japan Collection of Microorganisms.

Table 2 Results for high-, intermediate-, and low-concentration *V. parahaemolyticus* samples analyzed using the DOX system

Strain No.	High concentration	Intermediate concentration	Low concentration	Correlation coefficient (r)
1	5.21[a)] 311.3 (0.7%) [b)]	3.21 527.7 (1.2%)	1.21 710.3 (0.4%)	y = -99.75x + 836.86 0.9986
2	5.67 214.3 (0.7%)	3.67 352.0 (0.3%)	1.67 514.3 (1.9%)	y = -75x + 635.21 0.9981
3	5.57 280.0 (3.7%)	3.57 465.0 (0.9%)	1.57 674.0 (3.6%)	y = -98.5x + 824.58 0.9963
4	5.61 302.0 (4.2%)	3.61 465.3 (3.4%)	1.61 618.0 (3.9%)	y = -79x + 747.1 0.9931
5	5.74 253.7 (3.9%)	3.74 450.0 (3.5%)	1.74 650.7 (2.8%)	y = -99.25x + 822.2 0.9971
6	5.94 234.3 (1.3%)	3.94 412.7 (2.1%)	1.94 591.7 (3.6%)	y = -89.333x + 765.26 0.9971
7	5.85 251.0 (0.8%)	3.85 468.0 (6.6%)	1.85 652.0 (3.1%)	y = -100.25x + 843.46 0.9933

a) Log cfu/ml of sample.
b) Mean detection period (min) and rate of change (%) for triplicate measurements per sample.

Table 3 Results of positive non-*V. parahaemolyticus* samples obtained using the DOX system.

Strain No.	Species	High concentration 1st	2nd	3rd	Mean (Rate of change)	Intermediate concentration 1st	2nd	3rd	Mean (Rate of change)
8	*V.alginolyticus*	6.14[a)] 700 [b)]	579	689	656.0 (10.2%)	ND [c)]	ND	1125	1125.0 (-)
9	*V.alginolyticus*	7.16 1145	ND	1059	1102.0 (5.5%)	ND	ND	ND	4.16
10	*V.alginolyticus*	7.13 781	711	716	736.0 (5.3%)	ND	ND	ND	4.13
11	*V.alginolyticus*	7.19 1052	838	1000	963.3 (11.6%)	ND	ND	ND	4.19
12	*V.alginolyticus*	7.00 ND	1030	ND	1030.0 (-)	ND	ND	ND	4.00
13	*V.alginolyticus*	7.11 739	640	553	644.0 (14.5%)	1070	ND	ND	4.11 1070.0 (-)
14	*V.alginolyticus*	6.51 691	713	701	701.7 (1.6%)	ND	882	ND	3.51 882.0 (-)
18	*V.harveyi*	6.43 306	317	301	308.0 (2.7%)	667	670	742	3.43 693.0 (6.1%)

a) log cfu/mL.
b) Detection time (min) for the 1st technical replicate using high-concentration samples.
c) No detection.

Relationship between detection time and bacterial count in 7 V. parahaemolyticus strains: The relationship between detection times and bacterial counts is shown in Figure 1. Of the seven strains examined for *V. parahaemolyticus*, the multiple correlation coefficient (r^2) between detection times and bacterial counts was 0.9274.

A quantitative method for detecting *V. parahaemolyticus* using the DOX system has been developed in our previous study. Some of the *V. harveyi* and *V. alginolyticus* strains analyzed also showed positive results for detection of *V. parahaemolyticus*. A good linear calibration curve was observed between detection times and bacterial counts for all examined *V. parahaemolyticus* strains ($r \geq 0.9931$). With our DOX system, it took about 5 h to detect *V. parahaemolyticus* at high concentrations (5 Log cfu/ml), and about 12 h to detect this bacterium at low concentrations (1 Log cfu/ml). Therefore, *V. parahaemolyticus* could be detected using our DOX system within half a day.

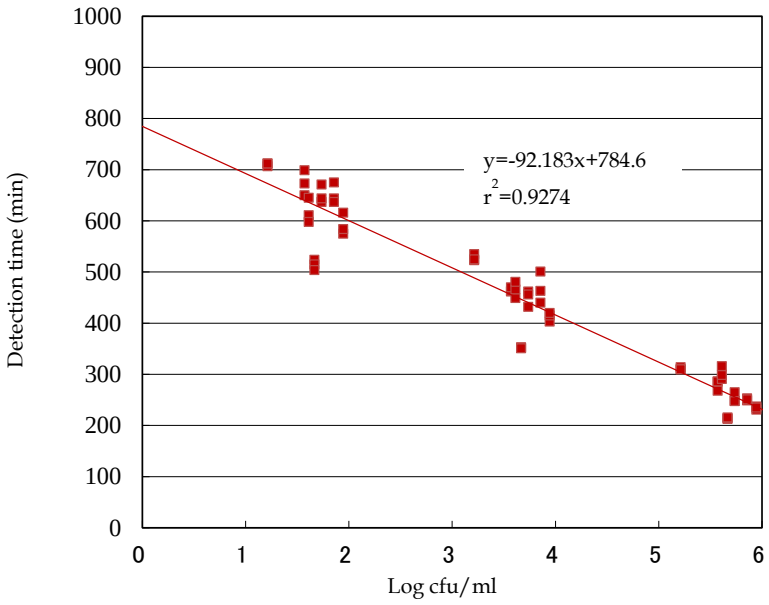

Figure 1 Relationship between detection times (min) and bacterial counts (Log cfu/ml) for 7 strains of *Vibrio parahaemolyticus* in this study

The detection times for *V. alginolyticus*, *V. harveyi*, and *V. parahaemolyticus* at medium concentrations (3-4 Log cfu/ml) using the DOX system were from 13-19 h, 12 h, and 6-9 h, respectively. Detecting *V. parahaemolyticus* in EMS/AHPNS-monitoring shrimp farming ponds will be feasible because of the sensitivity of the DOX system for detection of *V. parahaemolyticus*, *V. harveyi*, and *V. alginolyticus*. The DOX system provides rapid results within half a day, and requires no special techniques for measurement. In addition, upon *Vibrio*-positive results from the DOX system, *V. parahaemolyticus* could then be isolated on conventional *V. parahaemolyticus* isolation agars such as TCBS agar using the reagents in the DOX cassette.

Tanno et al. (2014) have previously developed methods to measure total *Vibrio* counts using the DOX system. The present report describes the measurement of *V. parahaemolyticus* counts using the DOX system. Based on the results of the present laboratory analysis, the DOX system can be used to detect *V. parahaemolyticus* contamination in environmental water samples in a relatively short time (within 12 h). Although further detailed field experiments are needed, regular measurement of total *Vibrio* or *V. parahaemolyticus* counts in shrimp farm pond water is an initial step towards preventing EMS/AHPNS disease.

References

Baticados MCL, Lavilla-Pitago CR, Cruz-Lazierda ER, De La Pena LD and Sunaz NA 1991. Studies on the chemical control of luminous bacteria *V.harveyi* and *V. splendidus* isolated from diseased *P. monodon* larvae and rearing water. Dis. Aquat. Org. 9: 133-139.

De La Pena LD, Tamaki KT, Momoyama TN and Muroga K 1993. Characteristic of causative bacterium of vibriosis in kuruma prawn *Penaeus Japanicus.* Aquaculture. 115: 1-12.

Farmer III JJ, Janda J M, Brenner F W, Cameron D N and Birkhead Karen M 2005. Genus I Vibrio. In. Bergey's manual of systematic bacteriology, 2nd ed Volume Two, DJ Brenner, NR Krieg, JT Staley and GM Garrity (eds) Berlin : Springer 494-546.

Gopal S, Otta SK, Kumar S, Karunasagar I, Nishibuchi M and Karunasagar I 2005. The occurrence of *Vibrio* species in tropical shrimp culture environments; implications for food safety. Int J Food Microbiol. 15:102:151-159.

Kaneko T and Colwell RR 1973. Ecology of *Vibrio parahaemolyticus* in Chesapeake bay. J Bacteriol. 113: 24-32.

Kondo H, Tinwongger S, Proespraiwong P, Mavichak R, Unajak S, Nozaki R and Hirono I 2014. Draft Genome Sequences of Six Strains of *Vibrio parahaemolyticus* Isolated from Early Mortality Syndrome/Acute Hepatopancreatic Necrosis Disease Shrimp in Thailand. Genome Announc. 2(2): e00221-14.

Lavilla-Pitago CR, Baticados MCL, Cruz-Lacierda ER and De la Pena LD 1990. Occurrence of luminous bacterial disease of *Penaeus monodon* larvae in the Philippines. Aquaculture 91: 1-13

Paclibare JO, Usero RC, Somga JR and Visitacion RN 2002. Integration of finfish in shrimp (*Penaeus monodon*) culture: an effective disease prevention strategy. In. Disease Control in Fish and Shrimp Aquaculture in Southeast Asia - Diagnosis and Husbandry Techniques: Proceedings of the SEAFDEC-OIE Seminar – Workshop on Disease Control in Fish and Shrimp Aquaculture in Southeast Asia - Diagnosis and Husbandry Techniques. Y Inui and E R Cruz-Lacierda (Eds.), Iloilo, Philippines: SEAFDEC Aquaculture Department 151-171.

Pass DA, Dybdahl R and Mannion MM 1987. Investigation into the causes of mortality of the pearl oyster, *Pinctada maxima* (Jamson), in western Australia. Aquaculture. 65: 149-169.

Sunaryanto A and Mariyam A 1986. Occurence of a pathogenic bacteria causing luminescence in penaeid larvae in Indonesian hatcheries. Bull. Brackish Water Aqua. Devl. Centre. 8: 64-70.

Tanno S, Fukui N, Utaka Y, Ohkawa S, Morita Y and Boonmar S 2014. Quantitative method for detecting *Vibrio* species using Bio-Theta DOX™ system. Thai J Vet Med. 44: 541-546.

Thakur AB, Vaidya RB and Suryawanshi SA 2004. *Vibrio abundance* in modified extensive culture ponds of *Penaeus monodon*. Indian J Fish. 51: 147-152.

Tran L, Nunan L, Redman RM, Mohney LL, Pantoja CR, Fitzsimmons K and Lightner DV 2013. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Dis Aquat Org. 105: 45-55.

บทคัดย่อ

การตรวจหาปริมาณเชื้อ *Vibrio parahaemolyticus* โดยใช้ระบบ Bio-Theta DOX™

โชอิชิ แทนโน[1] นาโอกิ ฟูกุย[1] ยูกิฮิโร อูตากะ[1] ซาบูโร โอกาวะ[1] ยูกิโอ โมริต้า[2] สุมาลี บุญมา[2*]

รายงานนี้เป็นรายงานครั้งแรกของการตรวจหาปริมาณเชื้อ *Vibrio parahaemolyticus* โดยใช้ระบบ Bio-Theta DOX ™ โดยทดลองกับเชื้อ *Vibrio parahaemolyticus* จำนวน 7 ตัวอย่าง และตัวอย่างที่ไม่ใช่เชื้อ *Vibrio parahaemolyticus* (non-Vibrio parahaemolyticus) จำนวน 23 ตัวอย่าง ระบบนี้สามารถตรวจหาปริมาณความเข้มข้นสูงสุด (10^5 cfu/ml) ความเข้มข้นระดับกลาง (10^3 cfu/ml) และความเข้มข้นระดับต่ำ (10 cfu/ml) จากเชื้อ *Vibrio parahaemolyticus* ทั้ง 7 ตัวอย่าง โดยมีอัตราการตรวจพบเป็นร้อยละ 100 ความสัมพันธ์ของเวลาที่ใช้ในการตรวจพบและจำนวนเชื้อเป็นสัดส่วนที่ดีคือ เป็น linear calibration curve (r> 0.993) นอกจากนี้ระบบนี้ยังสามารถตรวจหาอัตราผลลบของเชื้อที่มีปริมาณความเข้มข้นสูงสุด (10^6 cfu/ml) และความเข้มข้นระดับกลาง (10^3 cfu/ml) ในตัวอย่างที่ไม่ใช่เชื้อ *Vibrio parahaemolyticus* ทั้ง 23 ตัวอย่าง ในอัตราส่วนร้อยละ 65.2 (15/23) และร้อยละ 82.6 (19/23) ตามลำดับ และสามารถตรวจพบ *Vibio alginolyticus* จำนวน 7 ตัวอย่าง และ *V. harveyi* จำนวน 1 ตัวอย่าง อย่างไรก็ดี เวลาที่ใช้ในการตรวจพบนานกว่าการตรวจหา *V. parahaemolyticus* เราได้ทำการพัฒนาวิธีการตรวจหาเชื้อ Vibrio อย่างเดียวมาแล้ว และยังคงต้องทำการศึกษาต่อไปในภาคสนาม เพื่อให้ได้วิธีการที่เหมาะสมในการตรวจหาเชื้อ Vibrio และ *V. parahaemolyticus* ในฟาร์มเพาะเลี้ยงกุ้ง ระบบนี้สามารถนำไปใช้ในการป้องกันและควบคุมโรค EMS/AHPNS ในฟาร์มเพาะเลี้ยงกุ้ง

คำสำคัญ: ระบบการหาปริมาณเชื้อแบคทีเรีย ระบบ DOX™ การป้องกันล่วงหน้า เชื้อ *V. parahaemolyticus*

[1]ไดกิน 1-1, นิชิฮิโตซุยา เซ็ทซู โอซากา 566-8585, ประเทศญี่ปุ่น

[2]มหาวิทยาลัยโตเกียวเคซาย 1-18-1 กากะ อิตาบาชิคุ โตเกียว ประเทศญี่ปุ่น 173-8602

****ผู้รับผิดชอบบทความ** E-mail: sumaleeka12@gmail.com*