

FIRST REPORT OF ISOLATION OF *VIBRIO PARAHAEMOLYTICUS* FROM *CHRYSOMYA MEGACEPHALA* (DIPTERA: CALLIPHORIDAE) IN TAK PROVINCE, THAILAND

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Abstract. Foodborne illness is a major public health problem caused by various pathogens, one such being *Vibrio parahaemolyticus* producing gastroenteritis. There is no previous report on whether the pathogen is transmitted by blow fly *Chrysomya megacephala*, the most predominant species of Thailand and a mechanical vector of various pathogens. Prevalence of *Vibrio* spp-carrying *C. megacephala* and genetic relationship among *Vibrio* isolates from blow flies, food and water samples were determined in three sites of Mae Sot District, Tak Province, Thailand (December 2016). Only *V. parahaemolyticus* was detected, with a prevalence of 7% in *C. megacephala* and in three seafood samples but none in water samples. All *V. parahaemolyticus* isolates ($n = 10$) were resistant to ampicillin and streptomycin, and one isolate from *C. megacephala* (14%) carried *tdh*, encoding a thermostable hemolysin. Enterobacteria repetitive intergenic consensus-PCR revealed four different amplicon size profiles, with one profile present in five strains from *C. megacephala* and three from seafood samples, all obtained at the same market. This is the first report of *C. megacephala* as a mechanical vector of *V. parahaemolyticus* in Tak Province, Thailand.

Keywords: *Chrysomya megacephala*, *Vibrio parahaemolyticus*, ERIC-PCR, mechanical vector, Thailand

INTRODUCTION

Foodborne illness constitutes a major public health problem causing substantial morbidity and mortality in

people of all ages, especially in children under five years of age (Lamberti *et al*, 2012). Transmission of these illnesses is usually by a fecal-oral-route, mainly through consumption of food or drink contaminated with fecal pathogens, *eg* *Escherichia coli*, *Salmonella* spp, *Shigella* spp, *Yersinia* spp, and *Vibrio* spp (CDC, 2010; WHO, 2015).

Pathogenic *Vibrio* bacteria inhabit marine and coastal environments the world over and are recognized as seafood-

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borne bacteria (Su and Liu, 2007). *Vibrio* infection in USA increases 75% from 2006-2008 to 2013 (CDC, 2013). In Asia, with the largest global proportion of cholera cases, morbidity and mortality increase every year from 1989 to 2017 (WHO, 2018). The largest recorded cholera outbreak occurred in Yemen in late 2016, with more than 1.4 million cases (Dureab *et al*, 2019). In Thailand, periodic outbreaks of foodborne illness related to *Vibrio* spp have occurred over the years: an outbreak in northeastern Thailand in March 2015 involved consumption of raw chicken blood curd, *V. parahaemolyticus* being the etiologic agent (THAI NIH, 2014); sporadic outbreaks of infection, both nosocomial and food-related, were reported in Mae Sot District, Tak Province, a busy transit area located at the Thai-Myanmar border (Swaddiwudhipong *et al*, 2008; Swaddiwudhipong and Peanumlom, 2010; Rattanathumsakul *et al*, 2019).

Most *Vibrio* outbreaks in the Latin American countries are linked to eating raw seafood (Ackers *et al*, 1998), in Asian countries there exist additional risk factors, such as poverty, inadequate and improper hygienic facilities and poor sanitation (WHO, 2018; Dureab *et al*, 2019).

A variety of flies, in particular blow flies, are mechanical vectors of numerous pathogens, including fecal bacteria, such as *Bacillus* spp, *E. coli* O157:H7, *Enterococcus* spp, *Salmonella* spp, *Shigella* spp, and *Staphylococcus aureus* (Sukontason *et al*, 2007; Ly *et al*, 2010; Chaiwong *et al*, 2012). The blow fly *Chrysomya megacephala* (Diptera: Calliphoridae) is recognized as an insect of medical importance worldwide (Wells, 1991) owing to the behavior of laying eggs on exposed decaying organic matter, such as in markets and shops, together with the habit of feeding on openly displayed

food in open markets, facilitating spread of fecal bacterial pathogens (Tomberlin *et al*, 2017).

C. megacephala is the most common blow fly species in Thailand (Bunchu *et al*, 2012). As yet, there is no report of prevalence and characteristics of *Vibrio* spp carried by this fly species in Mae Sot District, Tak Province, Thailand where there are frequent reports of foodborne illness, including several reports due to *Vibrio* infection (Swaddiwudhipong *et al*, 2008; Swaddiwudhipong and Peanumlom, 2010; Swaddiwudhipong *et al*, 2012; Rattanathumsakul *et al*, 2019). Here, prevalence of two foodborne illness-related *Vibrio* spp, namely, *V. cholerae* and *V. parahaemolyticus*, isolated from *C. megacephala* in Mae Sot District as well as genetic relatedness of *Vibrio* isolates from blow flies, and environmental (food and water) samples were investigated. Results from the study should provide baseline data on the role of *Vibrio* spp in sporadic outbreaks of foodborne illness in this region of the country.

MATERIALS AND METHODS

Collection of blow fly samples

Blow flies were collected outdoors using sterilized insect sweeping nets at three natural breeding sites in Mae Sot District, Tak Province, Thailand in December 2016. Collection sites were located (i) at a main seafood (Rimmoei) market close to the Thai-Myanmar border, typically busy and crowded, with plentiful discarded decaying organic matter; (ii) largest garbage dump of the district containing a mound of exposed refuse; and (iii) a wooded recreation area near Mae Kuet Creek with several vendor huts. Blow files were collected at each site on different days for a period of one hour

during 10:00 and 16:00 hours, peak capture period (Bunchu *et al*, 2012). Specimens at each collection were pooled and transported in sterilized tubes on ice to the laboratory (Shoklo Malaria Research Unit, Faculty of Tropical Medicine, Mahidol University, Tak Province) where *C. megacephala* was visually identified using a taxonomic key of family Calliphoridae in Thailand (Kurahashi and Bunchu, 2011) and randomly divided into five groups of 55 flies, from which seven fly samples were randomly selected for subsequent bacterial isolation and confirmation of fly species.

Food and water samples collection

Three types of fresh seafood samples (whole crab, crab claw and prawn) were collected from the seafood market. Three potentially contaminated water samples were collected from the garbage dump site, and one water sample from the creek of the recreation area. Each sample was placed into a sterilized plastic bag or glass bottle and transported on ice to General Microbiology Research Unit, Faculty of Medical Science, Naresuan University, Phitsanulok Province for bacterial isolation.

Bacterial isolation

From blow flies

Individual blow fly was placed in one ml of alkaline peptone water (APW) (Himedia, Mumbai, India), shaken for 30 seconds, left standing at ambient temperature for 30 minutes, then one ml aliquot was combined with 5 ml of APW and incubated at 37°C for eight hours prior to inoculation on thiosulfate-citrate-bile salts-sucrose agar (Himedia, Mumbai, India) and incubated at 37°C for 24 hours. A green or yellow colony was randomly collected from each plate and streaked on CHROMagar Vibrio (Chromagar,

Paris, France) and subjected to Gram staining, oxidase test and a battery of standard biochemical tests (Matté *et al*, 1994). *V. parahaemolyticus* ATCC 17802 and *V. cholerae* ATCC15211 were used as positive controls. Confirmation of *Vibrio* species was performed by PCR-based amplification of 16S rDNA (170 bp amplicon) and *toxR* (368 bp amplicon) (Kong *et al*, 2002; Zulkifli *et al*, 2009b). Following bacterial isolation, blow fly samples were dried at 50°C and identity of *C. megacephala* re-confirmed by examination under a stereo microscope (40 x magnification).

From fresh seafood and water samples

A piece (25 g) of each seafood sample was homogenized in 125 ml of APW and one ml aliquot was added to 9 ml of APW, incubated at 37°C for eight hours and bacteria were isolated and identified as described above.

Antibiogram profiling

Cultures were inoculated on Mueller-Hinton agar (Himedia, Mumbai, India) supplemented with 3% (w/v) of NaCl and subjected to a disc diffusion assay following CLSI (2006) guidelines using discs (Oxoid, Hampshire, UK) containing ampicillin (10 µg), ampicillin/sulbactam (10/10 µg), amikacin (30 µg), amoxicillin/clavulanic acid (20/10 µg), ceftazidime (30 µg), cefotaxime (30 µg), cephalothin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), doxycycline (30 µg), gentamicin (10 µg), imipenem (10 µg), levofloxacin (5 µg), meropenem (10 µg), penicillin G (10 units), streptomycin (10 µg), and tetracycline (30 µg). Results are reported as sensitive, intermediate and resistant.

Virulence genes assay

After confirmation of *Vibrio* species by amplification of the 16S rRNA and *toxR* genes, all confirmed bacterial

isolates were tested for virulence genes. *V. parahaemolyticus* DNA was isolated using NucleoSpin Microbial DNA kit (MACHEREY- NAGEL GmbH & Co KG, Düren, Germany) according to the manufacturer's protocol. Multiplex PCR was performed for detection of *V. parahaemolyticus* *tdh* (encoding thermostable direct hemolysin) and *trh* (encoding thermostable direct hemolysin-related hemolysin) (Bej *et al*, 1999). Primers used for detecting *tdh* were *tdhF* (5'- GTAAAGGTCTCTGACTTTTGGAC -3') and *tdhR* (5'- TGGAATAGAAC-CTTCATCTTCACC -3'), and for *trh* were *trhF* (5'- TTGGCTTCGATATTTTCAGTATCT -3') and *trhR* (5'- CATAACAAACATATGCCCATTTCCG -3'). Reaction solution (20 μ l) contained 1X PCR buffer (Toyobo, Osaka, Japan), 1.5 mM MgCl₂, 0.5 μ M each primer, 0.1 mM dNTPs, 1 U *Taq* DNA polymerase (Toyobo, Osaka, Japan). Thermocycling was conducted in T100™ Thermal Cycler (Bio-Rad, Hercules, CA) as follows: 95°C for 8 minutes; 40 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute; with a final step of 72°C for 10 minutes. Amplicons (269 and 500 bp of *tdh* and *trh* fragment respectively) were separated by 1.2% agarose gel-electrophoresis and visualized by staining with ethidium bromide.

Repetitive intergenic consensus (ERIC)-PCR

ERIC-PCR was performed using the primers ERIC1 (5'-ATGTAAGCTCCTGGGGATTAC-3') and ERIC2 (5'-AAGTAAGTACTGGGGTGAGCG-3') (Versalovic *et al*, 1991). Reaction volume (20 μ l) contained 1X PCR buffer (Toyobo, Osaka, Japan), 1.5 mM MgCl₂, 0.5 μ M each primer, 0.1 mM dNTPs, 1 U *Taq* DNA polymerase (Toyobo, Osaka, Japan), 1 μ l of DNA solution, and double-distilled water to adjust to final

volume. Thermocycling was carried out as described above but using the following conditions: 95°C for 7 minutes; followed by 30 cycles of 90°C for 30 seconds, 58°C for 60 seconds and 65°C for 5 minutes; and a final step of 68°C for 16 minutes (Zulkifli *et al*, 2009a). Amplicons were separated by 1.2% agarose gel-electrophoresis at 90 volts for 45 minutes, stained with ethidium bromide and recorded using a gel documentation system (Syngene, Frederick, MD).

RESULTS

Prevalence of *Vibrio* spp. in blow flies and in seafood and water samples

C. megacephala ($n = 103$) were collected from a seafood market ($n = 34$, 33%), a garbage dump ($n = 34$, 33%) and a recreation area ($n = 35$, 34%). *V. parahaemolyticus* isolates ($n = 10$), identified by selective culture, biochemical tests and PCR-based detection of *V. parahaemolyticus*-specific 16S rDNA and *toxR* were obtained from blow flies (five isolates from seafood market (prevalence = 15%) and two from garbage dump (prevalence = 6%)) and seafood samples (three isolates (prevalence = 100%, 3/3), but none from water samples ($n = 4$). No other *Vibrio* spp were isolated from blow fly samples.

V. parahaemolyticus antibiogram profiles and presence of virulence genes

Among the 17 antibiotics tested, all ten *V. parahaemolyticus* isolates were resistant only to ampicillin and streptomycin. Multiplex PCR assay revealed only one *V. parahaemolyticus* isolate (from blow fly collected at the seafood market) carried *tdh* (data not shown).

Genetic relatedness of *V. parahaemolyticus* isolates

Genetic relatedness of the ten *V.*

parahaemolyticus strains was examined by ERIC-PCR, which revealed (by visual inspection) four different amplicon size patterns, VW-1 - VW-4: VW-1 associated with four strains from blow flies collected at the seafood market and three strains from seafood samples from the same market, VW-2 with a strain from blow flies collected at the same seafood market, VW-3 with a strain from blow flies collected at the garbage dump, and VW-4 with a strain from blow flies collected at the same garbage dump (Fig 1). The results indicated *V. parahaemolyticus*-contaminated seafood were from the blow flies, which (probably) did not carry the

Vibrio strains from the garbage dump (located 8 km from the market location).

DISCUSSION

The study is the first to identify blow fly *C. megacephala* collected at a seafood market and a rubbish dump in Mae Sot District, Tak Province as a mechanical vector of *V. parahaemolyticus*; no other *Vibrio* spp were detected from 103 flies collected. In Thailand, *C. megacephala* has been reported to carry several species of bacterial pathogens (Sukontason *et al*, 2007; Chaiwong *et al*, 2012). However there has been no previous report of isolation and

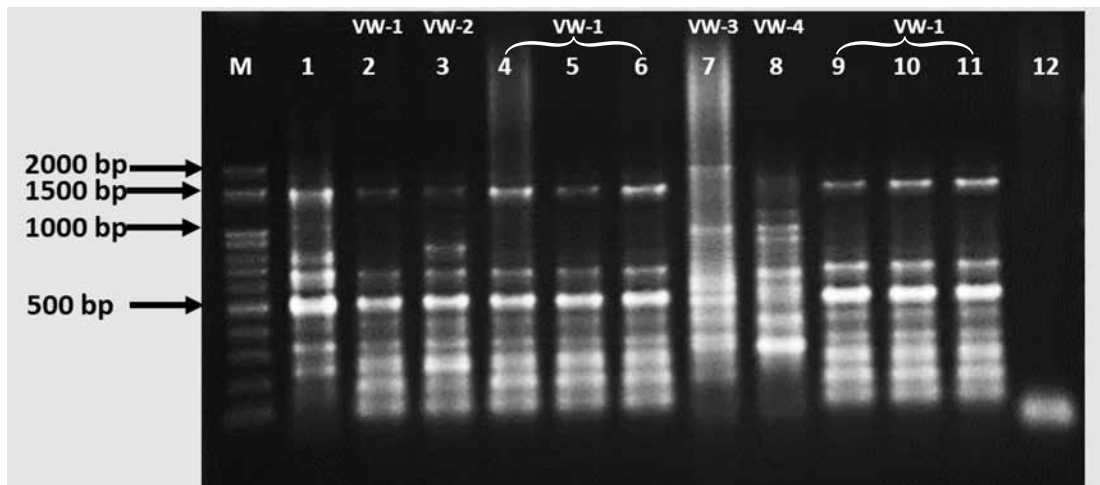


Fig 1-Enterobacteria repetitive intergenic consensus (ERIC)-PCR profiles of *Vibrio parahaemolyticus* strains isolated from blow fly *Chrysomya megacephala* specimens and seafood samples in Mae Sot District, Tak Province, Thailand (December 2016).

V. parahaemolyticus DNA was amplified using primers ERIC1 5'-ATGTAAGCTC CTGGGGATTAC-3' and ERIC2 5'-AAGTAAGTGACTGGGGTGAGCG-3' (Versalovic *et al*, 1991), separated by 1.2% agarose gel-electrophoresis and stained with ethidium bromide.

Lane M: 100 bp size markers; Lane 1: *V. parahaemolyticus* ATCC 17802; Lane 2: VA-1; Lane 3: VA-2; Lane 4: VA-3; Lane 5: VA-4; Lane 6: VA-5; Lane 7: VB-1; Lane 8: VB-2; Lane 9: VS-1; Lane 10: VS-2; Lane 11: VS-3; Lane 12: negative control.

VA: *V. parahaemolyticus* strain from blow flies collected at a seafood market; VB: *V. parahaemolyticus* strain from blow flies collected at a garbage dump; VS: *V. parahaemolyticus* strain from seafood sample; VW-1 - VW-4: ERIC-PCR profile types.

identification of *Vibrio* spp in or on blow flies, although *V. cholerae* is associated with many species of arthropods including fruit fly (Blow *et al*, 2005). Housefly *Musca domestica* was reported a mechanical vector of *V. cholerae* and involved in the dissemination of *V. cholerae* in Delhi, India (Fotedar, 2001). Insects may also serve as vectors or reservoirs of *V. cholerae* (Broza and Halpern, 2001; Halpern *et al*, 2003; Halpern *et al*, 2004).

V. parahaemolyticus was identified using both phenotypic and genotypic assays as phenotypic tests alone are not definitive for distinguishing among *Vibrio* spp (Matté *et al*, 1994). Amplification of *V. parahaemolyticus*-specific 16S rDNA and *toxR* were employed as they have been previously used successfully in identifying *V. parahaemolyticus* in both environmental and clinical samples (Kong *et al*, 2002; Zulkifli *et al*, 2009b).

Only one *V. parahaemolyticus* specimen (out of five) from blow flies collected at a seafood market carried hemolysin-encoding *tdh*, which is present in approximately 10% of clinical strains and is absent in the majority of environmental strains (Nishibuchi and Kaper, 1995; Rojas *et al*, 2011). This is a worrisome observation, but due to the small sample size, needs to be confirmed. The absence of *trh* among the *V. parahaemolyticus* isolates collected is not unusual as *trh*-positive strains are more common in colder climate while *tdh*-positive strains with warmer weather (Rodriguez-Castro *et al*, 2010).

Vibrio spp, both environmental and clinical isolates, are usually sensitive to most antibiotics (Elmahdi *et al*, 2016). All ten *V. parahaemolyticus* strains were resistant to ampicillin and streptomycin among 17 antibiotics tested, consistent with previous reports (Elmahdi *et al*,

2016; Lopatek *et al*, 2018; Park *et al*, 2018). Although antibiotic treatment is not usually needed for *Vibrio* gastroenteritis, tetracycline or ciprofloxacin are prescribed in cases of prolonged diarrhea caused by *V. parahaemolyticus* (Wong *et al*, 2015).

ERIC-PCR has been previously reported to be most discriminating method for subtyping *V. parahaemolyticus* strains, followed by ribotyping and pulsed-field gel-electrophoresis (Marshall *et al*, 1999). In the present study, ERIC-PCR profiling revealed similarity in *V. parahaemolyticus* strains present in blow flies and seafood samples from the same market. It is unclear if the blow flies were transmitting *V. parahaemolyticus* from one contaminated food sample to another or brought the microbe from another location to the market. Further studies will be needed to settle this issue. Nevertheless, a comprehensive blow fly management measures should be implemented, in particular the elimination of potential breeding sites both in the market and surrounding areas, especially at the seafood market.

The flight range of *C. megacephala* is approximately 2-3 km/day (Badenhorst and Villet, 2018) and areas with highest risk of *V. parahaemolyticus* infection carried by blow flies from the seafood market should therefore be within this distance. This could explain why no *Vibrio* isolates were found on *C. megacephala* collected from the wooded recreation area near a creek, which is located 10 km distant from the seafood market. However, it is conceivable *V. parahaemolyticus* could be found in this area as it inhabits both freshwater and saltwater locations worldwide (Yang *et al*, 2019).

The weakness of this study is the small number of *V. parahaemolyticus* specimens, both from blow flies and food

samples, to allow any firm conclusions to be made from the data. In addition, blow flies were collected during a winter season while isolation of *Vibrio* spp would be higher in summer when there is a higher abundance of flies (Ngoen-Klan *et al*, 2011). Thus, surveillance of prevalence of pathogens on blow flies should be conducted out throughout the year.

In summary, this study is the first report of blow fly *Chrysomya megacephala* as a mechanical vector of *Vibrio parahaemolyticus*. Survey of three sites (seafood market, rubbish dump and a wooded recreational area) in Mae Sot District, Tak Province in December 2016 showed a total prevalence of 7%. Of the 10 *V. parahaemolyticus* strains isolated, seven were from blow flies (two and five strains from blow flies collected at seafood market and rubbish dump respectively) and three from seafood samples at the same market. All strains were resistant to ampicillin and streptomycin, and one strain carried a hemolysin gene. Enterobacteria repetitive intergenic consensus-PCR profiling exhibited four amplicon size patterns, with one predominant pattern present in strains from blow flies and seafood samples at the same market. These findings highlight the urgent need for implementation of control measures of flies in food markets as well as improvement in elimination of blow fly breeding sites in Tak Province and elsewhere in the country.

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