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Distribution of *Candida* species in oral candidiasis patients: Association between sites of isolation, ability to form biofilm, and susceptibility to antifungal drugs

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ABSTRACT

Background: The oral cavity is a complex structure. Differences in oral mucosa surfaces are keratinized epithelium (KE) lined on the gingival, palate, and tongue surface, while non-keratinized epithelium (NKE) lined on the buccal surface and lips. In denture wearer, denture surface is also exposed in the oral cavity. Clinical manifestations of oral candidiasis vary depending on the type of infection. The ability to form biofilm which is the virulent factor of *Candida* spp. may affects by these mucosa and abiotic surfaces and leading to drug resistant strains.

Objectives: To compare the distribution of *Candida* spp. by site of infection, its ability to form biofilm, and susceptibility to antifungal agents.

Materials and methods: The samples were collected from lesions in the oral cavity by using the imprint culture technique, and yeast species were identified by conventional and PCR methods. Biofilm formation was measured by crystal violet (CV) assay. Susceptibility to amphotericin B and azoles was performed in according to CLSI guideline (M27 A3).

Results: One hundred and fifty-two isolates were identified from 99 patients. A majority of isolates were 50% isolated from KE surface (gingiva, palate, and tongue), followed by 34.9% from NKE surface (buccal mucosa and lip), and 15.1% from surface of denture. *Candida albican*s was the most common species (80.9%) frequently isolated from the tongue and buccal surface, followed by *C. tropicalis* (7.2%) frequently isolated from the tongue and palate, and *C. glabrata* (5.3%) was frequently isolated from dentures. In consideration to site of infections, yeast isolated from denture surface showed a significant lower biofilm production compared to the NKE surface (p=0.029). The percentage of drug resistant strains in *Candida* spp. isolated from denture was 17.4%, NKE surface 14.6% and KE surface 8.1%.

Conclusion: This data indicate that site of infection; KE and NKE surfaces in the oral cavity had not affected to biofilm formation of *Candida* spp., except in denture wearer. Drug resistant in clinical isolates involved in high biofilm former strains and the species *C. glabrata*.

Introduction

Candida infection of the oral cavity (oral candidiasis) can occur either as an opportunistic fungal infection or an infection in healthy individuals that impact the quality of life. This infection is common among the elderly, immunocompromised hosts, denture wearers, and even infants.[1, 2] Distribution of *Candida albican*s in the oral cavity was different in children of different nationalities[3] and in kidney transplant recipients of different geographic regions.[4] In denture-wearers, the numbers of *Candida* spp. were high on the tongue and

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palate, followed by buccal mocusoa.[5] The most frequently isolated species is *C. albicans*, followed by non-albicans *Candida* spp. (NAC), such as *C. dubliniensis*, *C. tropicalis*, *C. glabrata*, and *C. krusei*.[4] Over the last few decades, the percentage of patients infected by NAC has increased from around 10% to 60%.[6, 7] Susceptibility of these NAC species to antifungal drugs has decreased, which has correlated with an increase in use of azoles as empirical treatment and ability to form biofilm.[6, 8]

In the oral cavity of who has oral candidiasis, lesions can be found at various sites—even if it is lined with a different epithelium layer.[9] The gingival mucosa, palatal mucosa and the dorsal surface of the tongue are covered by highly keratinized epithelium (KE) whereas the inner lip, buccal mucosa and the floor of the mouth are covered by non-keratinized epithelium (NKE).[9] The manifestations include erythematous, pseudomembranous, hyperplastic, or angular cheilitis forms.[10] Denture-associated erythematous stomatitis is also a common type of oral candidiasis among denture wearers; in this stomatitis, inflammation of the oral mucous membrane occurs underneath dentures, where the areas of erythematous are observed.[10, 11]

Keratin production was purposed to involve in pathogenicity of *C. albicans* in keratinized tissue by inhibiting the transition of yeast to hyphal form.[12] This may reduce the ability of biofilm formation. Moreover, mucosal biofilm on the tongue by *C. albicans* is a complex structure composed of yeast, hyphae, commensal bacterial flora, and the host component—ketratin.[13] Most common infected area of oral candidiasis in diabetis mellitus was at the palate (KE) followed by the buccal mucosa (NKE), and tongue (KE) with psedomembranous form.[14] In patients with AIDS, the most frequent clinical manifestation was the pseudomembranous form; the common site of infection was at the tongue (KE).[2] However, there is no report in comparison of phenotypic expression of *Candida* spp. isolates from KE surface (gingiva, palate, and tongue), NKE surface (buccal mucosa and lip) and abiotic (denture) surface in the oral cavity.

This study was to investigate the distribution of yeast isolates from different mucosal surfaces; KE (gingiva, tongue, palate) and NKE (buccal mucosa, lip), and dentures surface as well as to determine the association in ability to form biofilm and drug resistance.

Materials and methods

Clinical isolates

A total of 152 clinical isolates were collected from 99 patients with oral candidiasis who attended the Dental Hospital at the Faculty of Dentistry, Chiang Mai University between July 2015 and February 2016. The samples were collected by imprint culture technique.[15] A sterilized foam pad (2.5x2.5 cm^2) was placed on a lesion in the oral cavity for 1 min and removed to place onto Sabouraud dextrose agar (SDA) plate for 6 h at 37 °C. The pad then was removed and the SDA plate was continually incubated at 37 °C for 24 to 48 hr. For the denture stomatitis cases, samples were collected from palatal mucosa or gingiva and the fitting surface of dentures if it was available. In non-denture wearers, samples were collected from wherever lesions were shown (gingiva, palate, tongue, buccal surface, and lip). Isolated yeast cells were stored in glycerol at -80 °C until used.

Identification of *Candida* spp.

All clinical isolates were identified by standard mycological methods and colour of colonies on CHORMAgar™ *Candida*. Cell morphology and chlamydospore formation were determined on rice agar at 25 °C after 24 to 48 hr. Ability to assimilate sugars[16] and utilization of urea were assessed. Pellicle in Sabouraud dextrose broth (SDB) was determined after 24 hr. *C. dubliniensis* was distinguished from *C. albicans* by PCR.[17]

DNA extraction

Genomic DNA (gDNA) was extracted by phenol/chloroform extraction method. A yeast pellet from overnight culture was suspended in lysis buffer (2% TritonX-100, 1%SDS, 100mM NaCl, 10mM Tris-HCl, 1 mM EDTA) and phenol/chloroform/isoamyl alcohol (25:24:1), and then was boiled at 100 °C for 5 min.[18] The gDNA in the supernatant was precipitated by 100% ethanol and 3 M sodium acetate. After centrifugation, the gDNA was reconstituted in sterile water and stored at -20 °C before being used.

PCR amplification

The internal transcribed spacer 2 (ITS2) region of *C. albicans* and *C. dubliniensis* ribosomal DNA was amplified with fungal-specific primers UNI2 (5'-TTCTTTTCCTCCGCTTATTG-3') and either Calb (5'-AGCTGCCGCCAGAGGTCTAA-3') or Cdub (5'-CTCAAACCCCTAGGGTTTGG-3').[17] PCR reaction was performed in a 20 µL reaction volume, consisting of 0.8x PCR buffer (200 mM $(NH_4)_2SO_4$, 750 mM Tris-HCl, pH 8.8), 3.5 mM $MgCl_2$, dNTP mixture (0.2 mM each), 0.55 µM UNI2, 0.15 µM Calb or 0.4 µM Cdub, 1 U Taq DNA polymerase and 500 ng gDNA. The remaining volumes consisted of sterilie water. PCR was performed in a thermal cycler (Sensoquest, Germany) under the following cycling conditions: initial period of denaturation at 94 °C for 10 min, 40 cycles of denaturation at 94 °C for 15 sec, annealing at 57 °C for 30 sec, extension at 65 °C for 45 sec, and final extension at 65 °C for 10 min. Ten microlitres of amplification product were separated on 2% agarose gel with 100 V and stained with ethidium bromide. The DNA fragments were visualized by a digital imaging system (Syngene, UK). The sizes of the DNA fragment by using primers UNI2 and Calb were 446 bp, and by using primers UNI2 and Cdub were 217 bp showed in Figure 1.

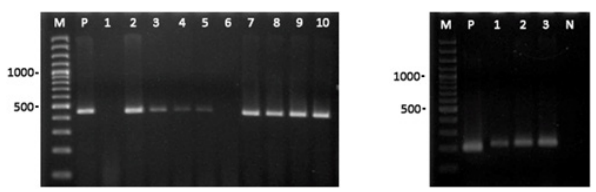

Figure 1. *Gel electrophoresis of PCR products from primers UNI2 and Calb (A), lane 2-5, 7-10 are C. albicans, lane 1 and 6 are not C. albicans and subject to do PCR with primers UNI2 and Cdub (B), lane 1-3 are C. dubliniensis. Lane M is DNA ladder, P is positive control (C. albicans ATCC 90028 and C. dubliniensis), N is negative control.*

Biofilm formation and biomass quantification

One hundred and forty-eight of *Candida* spp. was investigated the biofilm formation. The overnight culture of yeast cells in SDB was washed twice with PBS, and cells were suspended into RPMI-MOPS (RPMI1640 with 2% glucose and 0.165 M MOPS, pH 7) to McFarland No.3 (approx. $1x10^7$ cells/mL). One hundred microlitres of suspension was added into a 96-well plate and incubated at 37 °C at 75 rpm for 90 min. Non-adhering cells were washed once with 150 µL PBS and 100 µL RPMI-MOPS was added. The plate was incubated at 37°C at 75 rpm for 48 hr. Crystal violet (CV) assay was used to determine cell biomass. CV solution (0.5% [W/V]) was added into each well; after that, it was washed twice with PBS and incubated for 45 min at room temperature. The CV solution was discarded and wells were washed thrice with 200 µL sterile water, then 150 µL absolute ethanol was added into each well and left to stand for 45 min. The dissolved CV was transferred to a new flat 96-well plate and measured at OD_{595}. Biofilm producers were classified as high-, moderate-, or low-biofilm formers (HBF, MBF, or LBF) as done in a previous study.[19]

Susceptibility to antifungal drugs

Susceptibility of the yeast cells to antifungal agents (amphotericin B, AMB [BioChemica, UK], clotrimazole, CLT [Sigma, UK], fluconazole, FLC [Sigma, UK], itraconazole, ITC [Sigma, UK]) was investigated using the broth microdilution assay (CLSI M27 A3). Yeast cells ($1x10^3$ cells) were grown in RPMI-MOPS with diluted antifungal agents in a 96-well plate at 37 °C for 24 – 48 hr. The concentration of AMB and ITC ranged from 0.03-8 µg/mL, CLT ranged from 0.007-2 µg/mL, and FLC ranged from 0.5-128 µg/mL. The minimal inhibitory concentration (MIC) of azoles was determined as 50% inhibition of growth and the MIC of AMB was determined as 100% inhibition of growth compared to no drug control. The susceptibility breakpoints for FLC and ITC were followed by CLSI. Since there is no breakpoint for AMB and CLT, we use the breakpoint from the literature at MIC>1 µg/mL for AMB and >0.5 µg/mL for CLT.[20, 21]

Statistical analysis

The first analysis was distribution normality assessment using histogram evaluation. Because of the abnormal distribution, Mann-Whitney U test was used for the comparison between groups. P-values of less than 0.05 were considered to indicate statistical significance. All statistical analysis was performed using STATA™ version 10.1 software (Statacorp, College Station, TX).

Ethics statement

This study was approved by the Medical Ethics Committee of the Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand.

Results

Patients' characteristics

A total of ninety-nine patients with oral candidiasis lesions were in this study. Patients' ages ranged from 34 to 86 years (median 62 years); 33 were male and 66 were female. One-third of the patients (11 male and 21 female) were denture wearers. The lesions were found at various sites in the oral cavity: gingiva (KE), palate (KE), tongue (KE), buccal mucosa (NKE), and inner lip (NKE) (Table 1). In the non-denture wearers, the lesions were commonly shown at the tongue (48/142, 33.8%) and buccal surface (42/142, 29.6%). The erythematous was the majority of lesions on the tongue (26/48, 54.2%), and at the buccal surface the majority of lesions were pseudomembranous (26/42, 61.9%). In denture wearers, the lesions were found at the gingiva or palate, wherever the contact surface occurred.

Table 1 Characteristics of lesions in the oral cavity of patients.

Lesions	Sites of isolation, n						
	Keratinized epithelial (KE) surfaces			Non-keratinized epithelial (NKE) surfaces		Abiotic surface	Total number (%)
	Gingiva	Palate	Tongue	Buccal mucosa	Lips	Dentures*	
Erythematous	0	1	26	7	2	0	36 (25.4)
Hyperplastic	2	2	11	6	0	0	21 (14.8)
Psedomembranous	1	0	3	26	2	0	27 (19.0)
Denture-associated stomatitis	4	13	0	0	0	22	38 (26.8)
Others**	0	1	8	8	2	1	20 (14.1)
Total number (%)	7 (4.9)	17 (12.0)	48 (33.8)	42 (29.6)	6 (4.2)	22 (15.5)	142 (100)

**In cases of those who had denture-associated stomatitis, samples were collected from lesions and the fitting surface of dentures, if the dentures were available. **Others included mild anaemia, burning sensation, sores, dryness and non-identified.*

Distribution of isolated yeast

A total of 152 yeast isolated from 99 patients were identified by phenotypic methods. The differentiation between *C. dubliniensis* and *C. albicans* was done by PCR with specific primers (Figure 1). Yeast species distribution by sites of isolation was shown in Table 2. *C. albicans* was the most common species (80.9%) isolated from KE, NKE and dentures surface. The NAC included *C. tropicalis* (7.2%), *C. glabrata* (5.3%), *C. krusei* (2%), and *C. dubliniensis* (2%). It was observed that 75% (6 of 8) of the *C. glabrata* strains were isolated from denture-associated stomatitis which from gingival (1 isolate), palate (1 isolate), and from complete dentures (4 isolates).

Table 2 Distribution of yeast species isolated from the oral cavity of patients.

Yeast species, n (%)	Sites of isolation, n					
	Keratinized epithelial (KE) surfaces			Non-keratinized epithelial (NKE) surfaces		Abiotic surface
	Gingiva	Palate	Tongue	Buccal surface	Lip	Dentures*
C. albicans, 123 (80.9)	5	13	44	41	4	16
C. dubliniensis, 3 (2.0)	0	0	1	0	0	2
C. tropicalis, 11 (7.2)	1	3	3	2	1	1
C. glabrata, 8 (5.3)	1	1	0	2	0	4
C. krusei, 3 (2.0)	0	1	1	0	1	0
Trichosporon spp., 4 (2.6)	0	1	1	2	0	0
Total, 152 (100)	7 (4.6)	19 (12.5)	50 (32.9)	47 (30.9)	6 (4.0)	23 (15.1)

**In cases of those who had denture-associated stomatitis, samples were collected from lesions and the fitting surface of dentures, if dentures were available.*
***Others included mild anaemia, burning sensation, sores, dryness and non-identified.*

In this study, mixed *Candida* spp. were observed in 11 cases (11%) (Table 3). It is interesting that seven cases carried different *Candida* spp. which were isolated from different sites within the oral cavity. Additionally, different species of *Candida* were also isolated from the same site observed in four patients. The common species were *C. albicans* and *C. glabrata*. We observed that 6 of 8 of *C. glabrata* strains were mixed colonization with other *Candida* spp.

Table 3 Mixed colonization of *Candida* spp.

Species	Number of patients	Site of isolations[#]
C. albicans + *C. glabrata*	2	Dentures + Gingiva (1st patient)
		Buccal surface + Lip (2nd patient)
	1	Buccal surface
C. albicans + *C. tropicalis*	1	Tongue + Palate
C. albicans + *C. krusei*	2	Tongue (1st patient)
		Lower lip (2nd patient)
C. dubliniensis + *C. glabrata*	1	Dentures at upper left + dentures at upper right
C. glabrata + *C. tropicalis*	2	Dentures + Tongue (1st patient)
		Tongue + Gingiva (2nd patient)
C. dubliniensis + *C. glabrata* + *C. krusei*	1	Tongue + Dentures + Palate
C. albicans + (*C. tropicalis* + *Trichosporon* spp.)	1	Tongue + Palate

[#]The order of Candida species is corresponding to the site of isolation.

Biofilm formation of *Candida* spp. according to sites of infection

Biomass forming ability of *Candida* spp. (n=145) was determined by CV assay. Cut-off values were established by dividing the OD_{595} value into terciles to categorized high biofilm former (HBF) (OD_{595}>1.66), moderate biofilm former (MBF) (OD_{595} 0.74-1.66), and low biofilm former (LBF) (OD_{595} <0.74).[19] In Figure 2A, the biomass production had increased in *C. tropicalis* (p=0.0092, n=11), *C. glabrata* produced a lower quantity of biomass (p=0.015, n=8), and *C. dubliniensis* (n=3) showed no significant difference in biomass production (p=0.35), in comparison to *C. albicans* (n=120). The association between biofilm formation and sites of isolation was statistically analyzed and shown in Figure 2B. No significant difference (p=0.69) was found between the biomass production of yeast isolated from KE surfaces (gingiva, palate, and tongue (n=74)) and NKE surfaces (buccal surface and inner lip (n=48)). Markedly, yeast that was isolated from denture surfaces (n=23) had a significant decrease in biomass production (p=0.029) compared to yeast isolated from NKE surfaces.

Susceptibility to antifungal drugs

Among the 120 strains of *C. albicans*, 4 (4.2%) were resistant to FLC; 2 (1.7%) were susceptible-dose dependent (SDD) to FLC; and 6 (5%) were resistant to ITC. No resistance to AMB or CLT was found. Regarding the 11 strains of *C. tropicalis*, 2 (9.1%) were resistant to AMB; 1 (9.1%) was SDD to ITC, and no resistance was found to CLT or ITC. All 8 strains of *C. glabrata* showed resistance: 1 (12.5%)

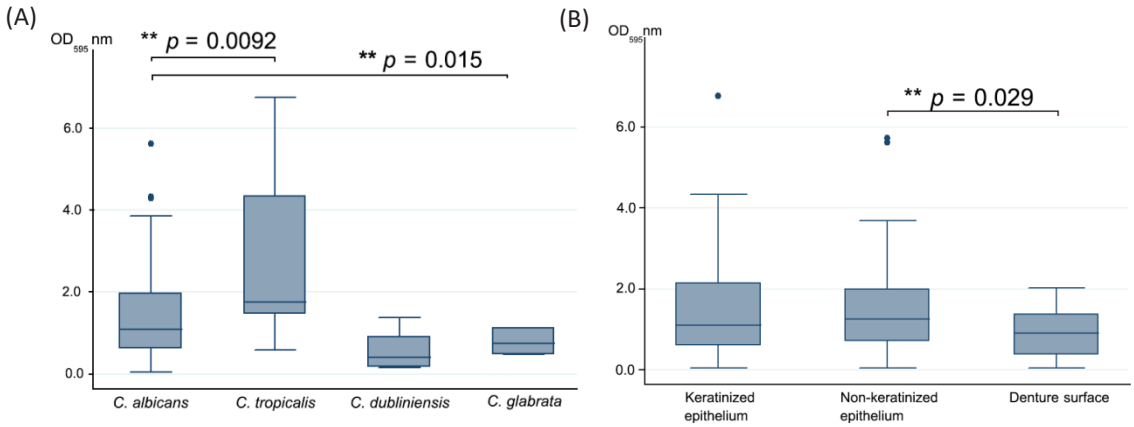

Figure 2. *Biofilm formation of Candida species at 48 hr. Production of biomass by C. albicans, C. tropicalis, C. dubliniensis and C. glabrata (A), by yeast isolated from different sites (B). *outlier data by Whisker plot, **significant difference at p<0.05.*

was resistant to AMB; 2 (25%) were resistant to FLC and 5 (62.5%) were SDD to FLC; and 7 (87.5%) were resistant to ITC. In the 3 strains of *C. krusei*, 2 (66.7%) were resistant to AMB. No drug resistance was found in *C. dubliniensis* (n=3).

In this study, 11.7% (17/145) of the *Candida* species demonstrated some resistance to the tested antifungal drugs. The distribution of resistant strains in consideration to sites of infection was different. The resistant rate of *Candida* spp. isolated from denture was 17.4% (4/23) which 3 isolates were *C. glabrata*. The rate of drug resistant in *Candida* spp. isolated from KE surface was 8.1% (6/74) which 4 isolates were HBF. The resistant rate of *Candida* spp. isolated from NKE surface was 14.6% (7/48), which 4 isolates were HBF and 2 were *C. glabrata*.

Discussion

In determination of *Candida* spp. isolated from oral candidiasis by phenotypic methods, four different *Candida* spp. were identified except *C. dubliniensis*. Furthermore, to distinguish *C. dubliniensis* from *C. albicans*, molecular analysis showed not exactly band size to *C. dubliniensis* reference strain (Fig. 1B). Therefore, those three isolates were confirmed by DNA sequencing with primer UNI2 and nucleotide blast using blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed 99-100% similarity to *C. dubliniensis* in the database (data not shown).

Our study in Northern Thai population showed that *C. albicans* was still the most common species (80.9%) with low rate of NAC among elderly (median 62 years old). This rate was not similar to the study of Muadcheingka in 2015, that oral candidiasis population was Central Thai, NAC had increased to nearly 40%.[7] The increasing of NAC from clinical specimens can cause the problem of treatment as *C. glabrata* and *C. krusei* are intrinsic resistance to azole drugs.[22, 23] This low rate of NAC in oral candidiasis in the northern Thai population is similar to prevalence of oral *Candida* carriage in Thai adolescents.[24] In this study, we observed that *C. glabrata* were common species isolated from dentures. It is consistent with the report that surface roughness affects the adhesion of organisms. *C. glabrata* had higher adhesive ability to denture acrylic surfaces than *C. albicans*.[25] This suggested that adequate cleaning of dentures is needed to remove the denture biofilm regularly. In addition, identification of the species of NAC is required for more effective therapy.

Mix colonisation of *Candida* spp. was normally reported, but sample collections were from oral rinsed or swab which could be not identified the origin of colonization.[26, 27] In this study, we found two *Candida* spp. were isolated from two sites in the oral cavity of denture wearers. In the case of *C. glabrata* isolated from the fitting surface of dentures

and *C. albicans* isolated from the surface of palatal mucosa (Table 3), it is possible that two *Candida* spp. co-localized when dentures was completely fitted in the oral. It has been reported that co-localized *C. albicans* and *C. glabrata* has a synergistic interaction and is associated with sever inflammation in denture wearers.[27, 28] Limited oxygen in between the palate and fitting surface of a complete denture could influence the virulence factors of *C. albicans*.[29] However, in the case of different *Candida* spp. isolated from other different anatomical sites (Table 3), more evidence is needed to support and investigate their specificity on different localization.

Distribution of *C. albicans* in the oral cavity, we found that the common sites of infection were at the tongue and the buccal mucosa which consistent to the report of Zahir.[5] In denture wearer *C. albicans* was common at the tongue and the palate[5] but in this study only lesions on the palate and gingiva had been investigated.

In biofilm formation study, *C. tropicalis* showed the highest biomass production, followed by *C. albicans* and *C. glabrata*. It is consistent with previous studies.[7, 19] In consideration to sites of infection, *Candida* spp. isolated from KE surface (i.e. tongue) were able to produce biofilm similar to *Candida* spp. isolated from NKE surface (i.e. buccal mucosa). It is not similar to the study of Zarkzewski that used engineered human oral epithelial mucosa (EHOM) to investigate the transition of *C. albicans* from yeast to hyphal form on keratinized (k) EHOM and non-keratinized (nk) EHOM.[12, 30] The number of *C. albicans* hyphal cells was significant higher on nkEHOM than on the kEHOM.[12] Since, the transition from yeast to hyphae is the initial process of biofilm formation of *Candida* spp. However, our results support the report of Dodd that the clinical manifestations either on tongue (KE) or on buccal mucosa (NKE) were strong indicator as severe infection.[31]

Susceptibility profile in this study showed high number of resistant strains to itraconazole, especially in *C. glabrata* compared to the other reported that showed resistant rate vary from 5-20%.[32] However, these results had been repeated. Recently epidemiological cutoff values (ECV) had been proposed to compare with CLSI cutoff values for determining the resistant strain. ECV of itraconazole for *C. glabrata* has been increased (>2μg/ml) for interpreting of resistance.[33] Therefore, the high number of itraconazole resistance may not thoroughly association in clinical treatment, as well as, clotrimazole is empirical treatment of oral candidiasis in this Dental hospital.

In this study, the local environment may affect to the drug resistant strains. The highest rate of drug resistance was strains isolated from dentures and associated to species of *C. glabrata*. In contrast, the drug resistant strains isolated from KE and NKE surface were majority of HBF strains. Therefore, awareness of treatment by azole drug should be considered on NAC and HBF strains. To determine the characteristic of HBF strains could be an indirect method to predict the outcome of treatment.

Conclusion

C. albicans was the most frequent species isolated from each site of infection. Frequency of NAC was not altered in this population. There was no association of ability to form biofilm and drug resistance with *Candida* spp. isolated from KE and NKE surface. Drug resistant strains involved in species *C. glabrata* and HBF of *C. albicans* and *C. tropicalis* strains. Finally, we proposed that phenotypic expressions of *Candida* spp. isolated from KE and NKE surface were similar except *Candida* spp. isolated from dentures.

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References

[1] Sedghizadeh PP, Mahabady S, Allen CM. Opportunistic oral infections. Dent Clin North Am. 2017; 61(2): 389-400.

[2] Gabler IG, Barbosa AC, Velela RR, Lyon S, Rosa CA. Incidence and anatomic localization of oral candidiasis in patients with AIDS hospitalized in a public hospital in Belo Horizonte, MG, Brazil. J Appl Oral Sci. 2008; 16(4): 247-50.

[3] Wu N, Lin J, Wu L, Zhao J. Distribution of *Candida albicans* in the oral cavity of children aged 3-5 years of Uygur and Han nationality and their genotype in caries-active groups. Genet Mol Res. 2015; 14(1): 748-57.

[4] da Silva-Rocha WP, Lemos VL, Svidizisnki TI, Milan EP, Chaves GM. *Candida* species distribution, genotyping and virulence factors of *Candida albicans* isolated from the oral cavity of kidney transplant recipients of two geographic regions of Brazil. BMC Oral Health. 2014; 14: 20.

[5] Zahir RA, Himratul-Aznita WH. Distribution of *Candida* in the oral cavity and its differentiation based on the internally transcribed spacer (ITS) regions of rDNA. Yeast. 2013; 30(1): 13-23.

[6] Bassetti M, Righi E, Costa A, Fasce R, Molinari MP, Rosso R, et al. Epidemiological trends in nosocomial candidemia in intensive care. BMC Infect Dis. 2006; 6: 21.

[7] Muadcheingka T, Tantivitayakul P. Distribution of *Candida albicans* and non-albicans *Candida* species in oral candidiasis patients: Correlation between cell surface hydrophobicity and biofilm forming activities. Arch Oral Biol. 2015; 60(6): 894-901.

[8] Ramage G, Williams C. The clinical importance of fungal biofilms. Adv Appl Microbiol. 2013; 84: 27-83.

[9] Squier CA, Kremer MJ. Biology of oral mucosa and esophagus. J Natl Cancer Inst Monogr. 2001; 29: 7-15.

[10] Singh A, Verma R, Murari A, Agrawal A. Oral candidiasis: An overview. J Oral Maxillofac Pathol. 2014; 18(Suppl 1): S81-5.

[11] Ramage G, Tomsett K, Wickes BL, Lopez-Ribot JL, Redding SW. Denture stomatitis: a role for *Candida* biofilms. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2004; 98(1): 53-9.

[12] Zarkzewski A, Rouabhia M. Engineered keratinized oral mucosa decreased *Candida albicans* transition through the production of keratins10, 14, 16, and 19 by oral epithelial cells. The Open Mycol J. 2007; 1: 1-8.

[13] Dongari-Bagtzoglou A, Kashleva H, Dwivedi P, Diaz P, Vasilakos J. Characterization of mucosal *Candida albicans* biofilms. PLoS One. 2009; 4(11): e7967.

[14] Al-Maskari AY, Al-Maskari MY, Al-Sudairy S. Oral manifestations and complications of diabetes mellitus: A review. Sultan Qaboos Univ Med J. 2011; 11(2): 179-86.

[15] Pongsiriwet S, Chaimano S, Kittikomton R. A comparative study of exfolative cytology and culture in diagnosis of Candidiasis in denture stomatitis patients. CM Dent J. 2001; 22(1): 67-72.

[16] Preechasuth K, Kabchan P, Khumwan C. Use of an Oxidation-Fermentation medium for identification of clinical yeast isolates. J Med Tech Assoc Thailand. 2007;35(3):2105-14.

[17] Carvalho A, Costa-De-Oliveira S, Martins ML, Pina-Vaz C, Rodrigues AG, Ludovico P, et al. Multiplex PCR identification of eight clinically relevant *Candida* species. Med Mycol. 2007; 45(7): 619-27.

[18] daSilval GA, BernardiI TL, SchakerI PDC, Valente MMP. Rapid yeast DNA extraction by boiling and freeze-thawing without using chemical reagents and DNA purification. Braz Arch Biol Technol. 2012; 55(2): 319-27.

[19] Marcos-Zambrano LJ, Escribano P, Bouza E, Guinea J. Production of biofilm by *Candida* and non-*Candida* spp. isolates causing fungemia: comparison of biomass production and metabolic activity and development of cut-off points. Int J Med Microbiol. 2014; 304(8): 1192-8.

[20] Pfaller MA, Espinel-Ingroff A, Canton E, Castanheira M, Cuenca-Estrella M, Diekema DJ, et al. Wild-type MIC distributions and epidemiological cutoff values for amphotericin B, flucytosine, and itraconazole and *Candida* spp. as determined by CLSI broth microdilution. J Clin Microbiol. 2012; 50(6): 2040-6.

[21] Pelletier R, Peter J, Antin C, Gonzalez C, Wood L, Walsh TJ. Emergence of resistance of *Candida albicans* to clotrimazole in human immunodeficiency virus-infected children: *in vitro* and clinical correlations. J Clin Microbiol. 2000; 38(4): 1563-8.

[22] Marichal P, Vanden Bossche H, Odds FC, Nobels G, Warnock DW, Timmerman V, et al. Molecular biological characterization of an azole-resistant *Candida glabrata* isolate. Antimicrob Agents Chemother. 1997; 41(10): 2229-37.

[23] Katiyar SK, Edlind TD. Identification and expression of multidrug resistance-related ABC transporter genes in *Candida krusei*. Med Mycol. 2001; 39(1) :109-16.

[24] Santiwongkarn P, Kachonboon S, Thanyasrisung P, Matangkasombut O. Prevalence of oral *Candida* carriage in Thai adolescents. J Investig Clin Dent. 2012; 3(1): 51-5.

[25] Luo G, Samaranayake LP. *Candida glabrata*, an emerging fungal pathogen, exhibits superior relative cell surface hydrophobicity and adhesion to denture acrylic surfaces compared with *Candida albicans*. APMIS. 2002; 110(9): 601-10.

[26] Kilic K, Koc AN, Tekinsen FF, Yildiz P, Kilic D, Zararsiz G, et al. Assessment of *Candida* species colonization and denture-related stomatitis in bar- and locator-retained overdentures. J Oral Implantol. 2014; 40(5): 549-56.

[27] Coco BJ, Bagg J, Cross LJ, Jose A, Cross J, Ramage G. Mixed *Candida albicans* and *Candida glabrata* populations associated with the pathogenesis of denture stomatitis. Oral Microbiol Immunol. 2008; 23(5): 377-83.

[28] Silva S, Henriques M, Hayes A, Oliveira R, Azeredo J, Williams DW. *Candida glabrata* and *Candida albicans* co-infection of an *in vitro* oral epithelium. J Oral Pathol Med. 2011; 40(5): 421-7.

[29] Sardi JC, Duque C, Hofling JF, Goncalves RB. Genetic and phenotypic evaluation of *Candida albicans* strains isolated from subgingival biofilm of diabetic patients with chronic periodontitis. Med Mycol. 2012; 50(5): 467-75.

[30] Rouabhia M, Deslauriers N. Production and characterization of an *in vitro* engineered human oral mucosa. Biochem Cell Biol. 2002; 80(2): 189-95.

[31] Dodd CL, Greenspan D, Katz MH, Westenhouse JL, Feigal DW, Greenspan JS. Oral candidiasis in HIV infection: pseudomembranous and erythematous candidiasis show similar rates of progression to AIDS. AIDS. 1991; 5(11): 1339-43.

[32] Vale-Silva LA, Sanglard D. Tipping the balance both ways: drug resistance and virulence in *Candida glabrata*. FEMS Yeast Res. 2015; 15(4): fov025.

[33] Ben-Ami R, Hilerowicz Y, Novikov A, Giladi M. The impact of new epidemiological cutoff values on *Candida glabrata* resistance rates and concordance between testing methods. Diagn Microbiol Infect Dis. 2014; 79(2): 209-13.

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Different effects of palmitic and oleic acid on LPS induced nitric oxide production and its association with intracellular lipid accumulation

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ABSTRACT

Background: Total free fatty acids (FFAs) levels were elevated in blood circulation of obese, T2DM as well as patients with cardiovascular events. Among structural differences of FFAs found in plasma, almost 60% were palmitic acid (PA) and oleic acid (OA). In previous vitro studies, PA was the most potent lipotoxin that caused apoptosis in various cells. On the other hand, OA tended to be stored as non-toxic neutral lipid droplets inside the cells. These indicated that different structures of fatty acids had different effects in cellular metabolism. Thus, this study aimed to characterize ability of palmitic acid and oleic acid in mitigating lipopolysaccharide (LPS) induced inflammation in macrophages and to investigate how lipid droplets (LD) loaded macrophages responded to LPS.

Materials and Methods: RAW 264.7 macrophages cytotoxicity of PA and OA after a two-day incubation were analyzed by MTT assay. The ability of inflammatory protection was investigated by incubating the cells with non-toxic concentration of fatty acids for 24 hr and followed by incubating the cells with 0.5 μg/mL LPS for another 24 hr. Cell supernatants were collected and nitric oxide concentrations were assayed by griess reaction. Lipid droplets formation was assessed by determining cellular triglyceride and neutral lipid oil red O staining.

Results: PA showed higher lipotoxic activity compared to oleic acid at the same concentration. OA at 200 μM mitigated LPS induced nitric oxide production in parallel with LD accumulation in macrophages, whilst PA at its non-toxic concentration (50μM) was unable to diminish inflammation and did not alter lipid accumulation.

Conclusion: Lipid loaded macrophages mediated by OA mitigated LPS induced inflammation. The association between anti-inflammation and LD formation should be further investigated.

Introduction

The underlying mechanism of metabolic disease development is complicate, but evidently involves with metabolic and inflammatory response of monocytes/macrophages, endothelial cells, muscle cells and adipocytes.[1-3] It has been suggested that free fatty acids (FFAs) could be a key linkage contributing to the cross-talk between those cells. For example, culture media taken from macrophages pre-treated with saturated fatty acid, palmitic acid, directly inhibited glucose uptake in muscle cells.[4] FFAs can be classified into 3 major groups depending on their chemical structures, unsaturated, monounsaturated and polysaturated fatty acid.[5] Total FFAs level was elevated in the blood circulation of obese, T2DM as well as patients with cardiovascular events.[6,7] Among structural different of FFAs found in plasma, palmitic acid (PA) and oleic acid (OA) represented almost 60%.[6] Accumulating data also indicated the different effects of PA and OA on cell metabolism. In vitro studies, saturated fatty acid, PA, was the most potent lipotoxin that caused apoptosis in various cells.[8-10] On the other hand, monounsaturated fatty acid, OA, tended to be stored as non-toxic neutral lipid droplets (LD) inside the cells.[10] In addition,

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there is firm belief that some fatty acids such as polyunsaturated fatty acid, oleic acid, which is abundant in Mediterranean food is health beneficial in metabolic diseases.[11] This suggested that different structures of fatty acids possessed different effects in cell metabolism.

Recent discoveries in lipid droplets biological function notified that LD was not just the simple energy storage in cells but it was a powerful factor contributing to several metabolic diseases.[12,13] For example, high fat diet induced fatty acid accumulation in liver cells, leading to steatosis. In hepatic cells culture, OA showed more steatogenic effect than PA.[14] and these was possibly because hepatocytes more tolerated under higher concentration of OA treatment than PA. In atherosclerosis, increasing LD formation in macrophages likely decelerated atherosclerotic progression since free fatty acid was toxic to both macrophages and endothelial cells. However, when persistent accumulation overwhelmed cellular triglyceride expenditure, LD could further continue tissue inflammation and fatty streak formation. According to our knowledge, how LD loaded macrophages response to its typical activator is not well defined.

Therefore, this study aimed to characterize ability of PA and OA loaded macrophage in response to LPS induced inflammation. Furthermore, this study also investigated cellular triglyceride and cholesterol after exposure of those fatty acids. As expected, our results indicated that saturated fatty acid, PA, possessed lipotoxic activity compared to monounsaturated fatty acid, OA, at the same concentration. OA also induced lipid accumulation in macrophage and mitigated LPS induced inflammation. Thus, under chronic inflammation condition, replacement of high PA with OA might help to attenuate inflammatory state. On the other hand, under healthy condition, OA replacement possibly affects the immune function.

Materials and methods

Cell Culture

The mouse macrophage cell line, RAW 264.7, was kindly provided by Assoc. Prof. Dr. Siriwan Ongchai. The cells were maintained in Dulbecco's modified Eagle's medium Dulbecco's modified Eagle's medium (DMEM) supplement with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (10,000 units penicillin/streptomycin) at 37 °C in 5% CO_2 incubator.

Free fatty acid preparations and treatments

FFA preparations and treatments were performed using the procedures previously described with slightly modification.[15] In brief, FFAs were dissolved in 0.1% NaOH in DMSO at 55 °C to prepare the 500 mM stock concentration then the stock concentrations were dissolved in DMSO for the 50 mM working stock concentration preparation and stored at -20 °C.

The 50 mM stock concentration were added to 10% FBS DMEM culture media to prepare the final FFA treatment concentrations at 50 and 200 μM. FFAs at 50 and 200 μM concentrations were be complexed with approximately 0.25% albumin in media at the FFA/albumin molar ratio at 1.3 and 5.3 respectively.

Cytotoxicity of free fatty acids

The cells were seeded into 96-well plate at a concentration of $5x10^4$ cells/well overnight and exposed to PA and OA at various concentrations for 24 hr. After free fatty acids incubation, cell viability was tested by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Twenty μL of 5 mg/mL MTT dye was added into each well. After 4 hr incubation at 37 °C in 5% CO_2 incubator, cell supernatant was discarded and 200 μL of DMSO was added to dissolve the MTT formazan product. The absorbance was assessed at 570 nm. The cell viability was represented as percentage using 0.4% DMSO as vehicle control.

Effect of free fatty acids on LPS induced inflammation

After the cells were treated with various concentrations of free fatty acids for 24 hr, 0.5 μg/mL of LPS was added to induce inflammation for 24 hr. Culture supernatants were collected and immediately determined NO level.

Nitric oxide determination

Nitric oxide (NO) in culture supernatants were measured by using Griess reagent. One hundred μL of culture supernatants were transferred into 96-well plate. Then, the same volume of Greiss reagent was also added to the plate in order to measuring NO. Sodium nitrite at the concentrations of 0-100 μM was used as standard. The absorbance was read at 562 nm.

Effect of free fatty acids on cellular lipid droplet accumulation

Macrophages were seeded at 3.5 x 10^6 cells density in petri dishes for overnight. After that the cells were exposed to 50 μM PA and 200 μM OA for 24 hr. Then, the cells were 3 time washes with ice cold sterile PBS.

To determine cellular triglyceride content, cellular proteins were collected in ice cold PBS by using cell scrapper. Collected proteins were sonicated at 40 °C, 100% amplitude, for 30 second and kept in -20 °C until use. Ten μL of sonicated cellular protein in PBS was added into 96-well plate. Intracellular triglyceride was quantified by enzymatic assay according to the manufacturer instruction. The absorbance was assessed at 550 nm. Cellular proteins were also quantified (Pierce™ BCA protein assay kit.)

For quantitative neutral lipid content in macrophages, the cells were fixed with 10% formalin for 30 min, after that the cells were washed 2 times with sterile water. Four mL of 60% Isopropanol were added into the petri dish and incubated for 1 min, then discarded Isopropanol. Three mL of 300 mg oil red O in 60% Isopropanol were added and incubated for 30 min. Then, the cells were washed with sterile water for 6 times. One mL of absolute isopropanol was added to dissolve the lipid staining. The absorbance was assessed at 520 nm.

Statistical analysis

Data are shown as mean±SD of 3 independent experiments. The data set was tested for normality by Shapiro-Wilk test and then differences between groups were assessed by one way ANOVA. $p<0.05$ was taken as significance.

Results

Palmitic acid was more toxic than oleic acid to Macrophages RAW 264.7

Mouse macrophages, RAW264.7, were exposed to increasing concentration of PA and OA for 24 hr. As expected, PA showed the higher toxicity compared to OA at the same concentration with IC50 of 293±9.6 μM, whereas, IC_{50} of OA was more than 400 μM (Figure 1). Non-cytotoxic concentrations of each FAs, PA at 50 μM and OA at 200 μM, which caused the cell survival more than 80%, were used in all the next experiments.

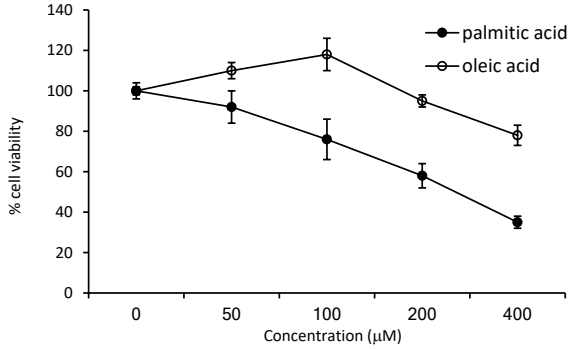

Figure 1. *Palmitic (PA) and oleic acid (OA) lipotoxicity. RAW 246.7 macrophages were incubated with 0-400 μM PA (close circle) or OA (open circle) for 48 hr. Cell viabilities were determined by MTT assay. The toxicity study was performed in triplicates from three independent experiments. Data are average of percentage of cell viability ±SD.*

Oleic acid induced neutral lipid accumulation

PA at the concentration of 50 μM did not alter intracellular lipid storage when determined by quantitative oil red O staining (Figure 2A) as well as TG assay (Figure 2B). On the other hand, the cells incubated with OA at the concentration of 200 μM increased the absorbance of neutral lipid staining approximately 25% compared to vehicle control (Figure 2C) and the increased of TG accumulation in the cells was also observed (control =573.5±96.7, OA =866.7±44.6 μg TG/mg protein) (Figure 2D). There were no interfering effects of reducing property of both PA and OA on TG assay when each of the treatment media containing FAs were directly incubated with TG reagent (OD of control =0.086±0.003 , PA 0.087±0.002 = and OA =0.093±0.002)

Oleic acid mitigated LPS induced NO production

To investigate whether different structures of FAs effect the LPS induced NO production, the cells were incubated with PA and OA for 24 hr, then followed by exposed the cells with 0.5 μg/mL LPS. Incubation the cells with PA and OA alone did not increase NO production compared to the control, suggesting that both PA and OA at their non-toxic concentrations did not induce inflammation.

When the cells exposed to PA for 24 hr and then further co-incubated with LPS for the next 24 hr, there was no difference in NO concentration compared to the cells that exposed to LPS alone (Figure 3A). The cells pre-incubated with OA for 24 hr before being co-exposed with LPS showed the reduction of NO level compared to LPS control group (Figure 3B), suggesting that the increasing LD in macrophage had ability to mitigate inflammation.

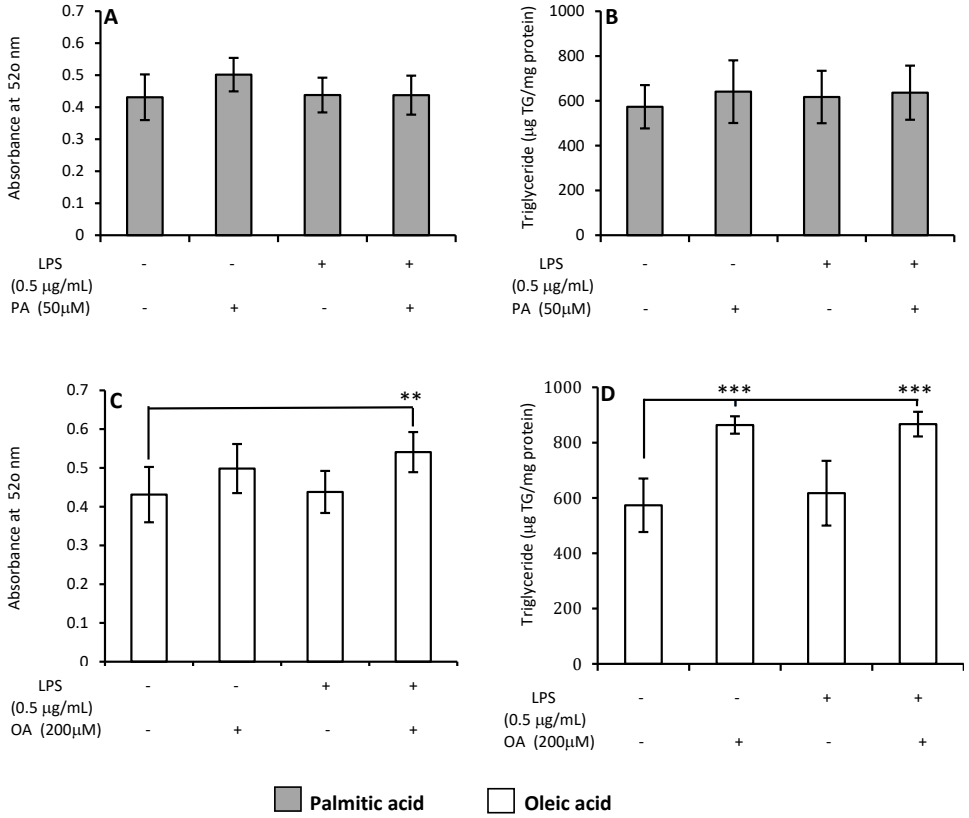

Figure 2. *Effects of Palmitic acid (PA, A and B) and Oleic acid (OA, C and D) on intracellular lipid contents. Macrophages, RAW 264.7, were treated with 50 μM PA and 200 μM OA for 24 hr. Intracellular lipid contents were assessed by quantitative Oil Red O staining (A and C) and Triglyceride accumulation (B and D). Cellular protein levels were also determined and there was no significantly different between each experimental condition. The difference of lipid contents were assessed by one way ANOVA test (** p<0.01; *** p<0.001).*

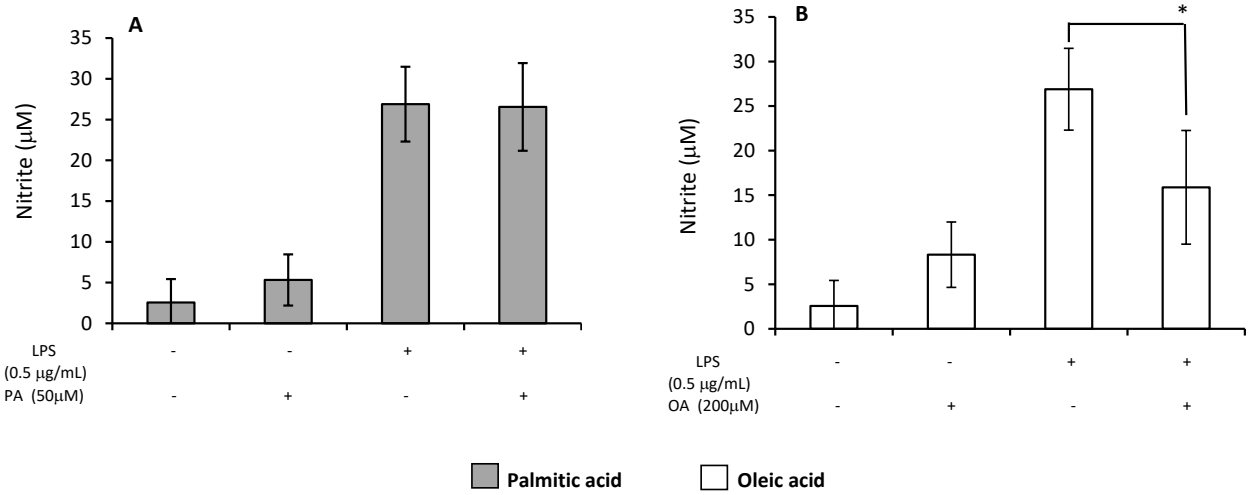

Figure 3. *Effects of Palmitic acid (PA, A) and Oleic acid (OA, B) on LPS induced NO production. Macrophages, RAW 264.7, were treated with 50 μM PA and 200 μM OA for 24 hr. Then the cells were exposed to 0.5 μg/mL of LPS for another 24 hr. No levels in cell supernatants were immediately measured. The difference of NO were assessed by one way ANOVA test (* p<0.05).*

Discussion

Replacing dietary oil containing high ratio of saturated fatty acid with unsaturated fatty acid in order to cardiovascular disease prevention are widely recommended by health care professional.[16] However, numerous data reported the contradict effects of long term consumption of PUFAs. Some studies showed that high PUFAs diets decreased risk of metabolic dysregulation, on the other hand, some studies showed the disagreement results.[17] These controversy effects could be explained by the study designs which the majority of articles reported about the association between SAFs, MUFAs, PUFAs and metabolic abnormalities were long term observational cohort studies which many confounding factors, such as total energy controlling, were difficult to manage. Thus, studies of the individual fatty acids on cellular activities were important in order to providing more information regarding their real effects. Our study focused on the effect of PA and OA which are the main fatty acids found in macrophages in diabetes patients.[18]

There are accumulating data affirmed that PA was lipotoxic in various cells.[19-21] Our results also reaffirmed that PA (C16:0) caused cell death in mouse macrophages, whilst monounsaturated fatty acid, OA (C18:1) showed less toxicity.

Previous studies also indicated that combination treatment of PA with OA prevented the hepatocyte.[19] and embryonic fibroblasts from apoptosis, suggesting the protective role of OA in saturated fatty acids induced lipotoxicity.[18,22] Interestingly, those effects seemed to associate with LD formation. Our study observed that cells treated with PA alone did not alter intracellular triglyceride content, whereas OA increased intracellular triglyceride at their non-toxic concentration. Listenberger LL and his colleagues showed that PA at its apoptotic induced concentration (700 μM) did not change triglyceride content in Chinese hamster ovary cells. They suggested that PA dominantly underwent the generation of reactive oxygen species and ceramide which consequently activated the caspase activation rather than triglyceride synthesis.[21] Unlike PA, OA activated PPARα as well as diacylglycerol acyltransferase (DGAT) activity which accelerated triglyceride production in cells.[21,23] However, recent study reported that knockdown DGAT neither enhanced PA induced apoptosis nor inhibited the protective role of OA against PA.[24] Thus, mechanism of OA involved in lipotoxic prevention is still unclear.

Apart from the association between LD formation and lipotoxicity, LD induction also affected several cellular metabolisms. Over-expression of DGAT in adipocytes caused induction of intracellular triglyceride accumulation in mouse adipocytes and the metabolic abnormalities such as hyperglycemia and inhibition of insulin signaling in hepatocytes were also observed.[25] Induction of Intracellular triglycerides in skeletal muscles of zinc transporter 7 knockout mice caused mitochondria enlargement and initiated mitochondria dysfunction.[26] However, according to our knowledge, how the accumulated intracellular triglyceride affected macrophage activity was unwell addressed. In our study, as expected, we found that OA induced triglyceride accumulation in macrophages at its non-toxic concentration. Thus, we further challenged the lipid loaded macrophages with its typical instigator, LPS, and the levels of nitric oxide production were observed as a marker of inflammatory response. We found that both PA and OA at their non-toxic concentration did not increase NO levels. Treatment the cells with PA for 24 hr did not induced intracellular triglyceride accumulation and when PA treated macrophage was further exposed to LPS, NO level was not altered, compared to LPS treatment in vehicle control macrophages. Whilst, the association between the inflammatory diminishing effect and induction of cellular triglyceride accumulation was observed in OA treatment. Cellular triglyceride levels were increased when macrophages were incubated with OA. Challenging the high intracellular triglyceride macrophages induced by OA with LPS showed the reduction of NO in culture media, indicating the anti-inflammatory activity of OA.

Recent study showed the anti-inflammatory effects of OA in adipocytes derived from human subjects by lowering TNFα and IL-6 in non-obese subjects compared with the

cells taken from obese subjects. It also showed the induction of adiponectin after 24 hr OA treatment.[27] In macrophages, there was an experiment which macrophages, J774 cell line, were both incubated with pre-and post LPS exposure. They found that the combination treatment of OA and PA at the FFA/albumin ratio of 2:1 after LPS exposure worsened the inflammatory state by increasing TNFα. Interestingly, the 5 days combination treatment prior to LPS exposure reduced TNFα mRNA level compared to LPS alone.[28] However, they did not mention about the lipid accumulation in the cells. Our study was conducted to study the individual effect of each free fatty acid in response to LPS. We emphasized that OA had ability to mitigate LPS induced inflammation by reducing NO production. The association between anti-inflammation and LD formation should be further investigated.

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Disclosures

None

References

[1] Agabiti-Rosei C, Paini A, De Ciuceis C, Withers S, Greenstein A, Heagerty AM, et al. Modulation of Vascular Reactivity by Perivascular Adipose Tissue (PVAT). Curr Hypertens Rep. 2018; 20(5): 44.

[2] Abranches MV, Oliveira FC, Conceição LL, Peluzio MD. Obesity and diabetes: the link between adipose tissue dysfunction and glucose homeostasis. Nutr Res Rev. 2015; 28(2): 121-32.

[3] Engin AB. Adipocyte-Macrophage Cross-Talk in Obesity. Adv Exp Med Biol. 2017; 960: 327-43.

[4] Samokhvalov V, Bilan PJ, Schertzer JD, Antonescu CN, Klip A. Palmitate- and lipopolysaccharide-activated macrophages evoke contrasting insulin responses in muscle cells. Am J Physiol Endocrinol Metab. 2009; 296(1): E37-46.

[5] Ratnayake WM, Galli C. Fat and fatty acid terminology, methods of analysis and fat digestion and metabolism: a background review paper. Ann Nutr Metab. 2009; 55(1-3): 8-43.

[6] Wang L, Folsom AR, Zheng ZJ, Pankow JS, Eckfeldt JH, Investigators AS. Plasma fatty acid composition and incidence of diabetes in middle-aged adults: the Atherosclerosis Risk in Communities (ARIC) Study. Am J Clin Nutr. 2003; 78(1): 91-8.

[7] Vessby B. Dietary fat, fatty acid composition in plasma and the metabolic syndrome. Curr Opin Lipidol. 2003;14(1):15-9.

[8] Moravcová A, Červinková Z, Kučera O, Mezera V, Rychtrmoc D, Lotková H. The effect of oleic and palmitic acid on induction of steatosis and cytotoxicity on rat hepatocytes in primary culture. Physiol Res. 2015;64 Suppl 5: S627-36.

[9] Martins de Lima T, Cury-Boaventura MF, Giannocco G, Nunes MT, Curi R. Comparative toxicity of fatty acids on a macrophage cell line (J774). Clin Sci (Lond). 2006; 111(5): 307-17.

[10] Cnop M, Hannaert JC, Hoorens A, Eizirik DL, Pipeleers DG. Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. Diabetes. 2001; 50(8): 1771-7.

[11] Palomer X, Pizarro-Delgado J, Barroso E, Vázquez-Carrera M. Palmitic and Oleic Acid: The Yin and Yang of Fatty Acids in Type 2 Diabetes Mellitus. Trends Endocrinol Metab. 2018; 29(3): 178-90.

[12] Welte MA, Gould AP. Lipid droplet functions beyond energy storage. Biochim Biophys Acta. 2017; 1862(10 Pt B): 1260-72.

[13] Xu S, Zhang X, Liu P. Lipid droplet proteins and metabolic diseases. Biochim Biophys Acta. 2018; 1864(5 Pt B): 1968-83.

[14] Ricchi M, Odoardi MR, Carulli L, Anzivino C, Ballestri S, Pinetti A, et al. Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. J Gastroenterol Hepatol. 2009; 24(5): 830-40.

[15] Laine PS, Schwartz EA, Wang Y, Zhang WY, Karnik SK, Musi N, et al. Palmitic acid induces IP-10 expression in human macrophages via NF-kappaB activation. Biochem Biophys Res Commun. 2007; 358(1): 150-5.

[16] Szostak-Wegierek D, Kłosiewicz-Latoszek L, Szostak WB, Cybulska B. The role of dietary fats for preventing cardiovascular disease. A review. Rocz Panstw Zakl Hig. 2013; 64(4): 263-9.

[17] Fattore E, Massa E. Dietary fats and cardiovascular health: a summary of the scientific evidence and current debate. Int J Food Sci Nutr. 2018: 1-12.

[18] Senanayake S, Brownrigg LM, Panicker V, Croft KD, Joyce DA, Steer JH, et al. Monocyte-derived macrophages from men and women with Type 2 diabetes mellitus differ in fatty acid composition compared with non-diabetic controls. Diabetes Res Clin Pract. 2007; 75(3): 292-300.

[19] Chen X, Li L, Liu X, Luo R, Liao G, Liu J, et al. Oleic acid protects saturated fatty acid mediated lipotoxicity in hepatocytes and rat of non-alcoholic steato-hepatitis. Life Sci. 2018; 203: 291-304.

[20] Tumova J, Malisova L, Andel M, Trnka J. Protective Effect of Unsaturated Fatty Acids on Palmitic Acid-Induced Toxicity in Skeletal Muscle Cells is not Mediated by PPARδ Activation. Lipids. 2015; 50(10): 955-64.

[21] Borradaile NM, Han X, Harp JD, Gale SE, Ory DS, Schaffer JE. Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death. J Lipid Res. 2006; 47(12): 2726-37.

[22] Listenberger LL, Han X, Lewis SE, Cases S, Farese RV, Ory DS, et al. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. Proc Natl Acad Sci U S A. 2003; 100(6): 3077-82.

[23] Nolan CJ, Larter CZ. Lipotoxicity: why do saturated fatty acids cause and monounsaturates protect against it? J Gastroenterol Hepatol. 2009; 24(5): 703-6.

[24] Leamy AK, Hasenour CM, Egnatchik RA, Trenary IA, Yao CH, Patti GJ, et al. Knockdown of triglyceride synthesis does not enhance palmitate lipotoxicity or prevent oleate-mediated rescue in rat hepatocytes. Biochim Biophys Acta. 2016; 1861(9 Pt A): 1005-14.

[25] Chen N, Liu L, Zhang Y, Ginsberg HN, Yu YH. Whole-body insulin resistance in the absence of obesity in FVB mice with overexpression of Dgat1 in adipose tissue. Diabetes. 2005; 54(12): 3379-86.

[26] Huang L, Tepaamorndech S, Kirschke CP, Newman JW, Keyes WR, Pedersen TL, et al. Aberrant fatty acid metabolism in skeletal muscle contributes to insulin resistance in zinc transporter 7 (J Biol Chem. 2018; 293(20): 7549-63.

[27] Rodriguez-Pacheco F, Gutierrez-Repiso C, Garcia-Serrano S, Alaminos-Castillo MA, Ho-Plagaro A, Valdes S, et al. The pro-/anti-inflammatory effects of different fatty acids on visceral adipocytes are partially mediated by GPR120. Eur J Nutr. 2017; 56(4): 1743-52.

[28] Kheder RK, Hobkirk J, Stover CM. In vitro Modulation of the LPS-Induced Proinflammatory Profile of Hepatocytes and Macrophages- Approaches for Intervention in Obesity? Front Cell Dev Biol. 2016; 4: 61.

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Comparison of dynamics of HIV-1 coreceptor usage in a long-term antiretroviral treatment adolescent by genotypic and phenotypic assays

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ABSTRACT

Background: Based on coreceptor usage, HIV-1 variants can be classified as R5, X4, and dual/mixed viruses. Currently, the determination of HIV-1 coreceptor usage can be performed by both phenotypic and genotypic assays. Although, the accessibility, simple, and low cost makes those genotypic assays a more feasible alternative to phenotypic assays, but they are not always accurate. Here, we discussed the coreceptor usage obtained by both assays in HIV-infected patient who acquired HIV-1 CRF01_AE and received antiretroviral therapy for at least 10 years.

Objectives: To determine the HIV-1 coreceptor usage by both genotypic and phenotypic assays at different three times in long-term antiretroviral treatment adolescent.

Materials and methods: The remained RNA was collected at different three times to determine the HIV-1 coreceptor usage by both phenotypic and genotypic assays. Firstly, HIV-1 V3 region was amplified, sequenced, and then V3 amino acid sequences were used as templates for prediction of coreceptor usage by genotypic predictors. Secondly, the entire gp160 envelope fragment was amplified from the same remained RNA to produce env-pseudotyped virus. The viruses were tested for coreceptor usage using U373.R5 and U373.X4 cells, and viral entry was assessed with luciferase activity measurement.

Results: From all time points at which coreceptor usage was determined, the genotypic results showed that the coreceptor usage trend to be more X4 phenotype using genotypic predictors, but it contrasted with phenotypic result which only voted to R5 phenotype. Although, the genotypic results showed the evolution of V3 amino acid sequences but it still not sufficient for coreceptor changed when confirmed with phenotypic assay. The presence of positively charged amino acid in V3 sequences causes a high net charge which can lead to mis-prediction by genotypic predictors.

Conclusion: This finding suggested that the predictions are not always accurate; a false prediction of X4 variants may lead to unnecessarily precluding patients who could have benefited from receiving CCR5 inhibitors whereas a false prediction of R5 variants may lead to the reemergence of X4-strains under CCR5 inhibitors pressure. Thus, the utilization of genotypic predictors should be carefully considered.

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Introduction

This case was of a HIV-infected patient who acquired HIV-1 subtype CRF01_AE and received antiretroviral therapy for at least 10 years. He was born in 1998, and his HIV testing was positive in 2004. He has been virologically and immunologically followed up since then. This study was approved by ethical committees from the Faculty of Associated Medical Sciences, Chiang Mai University (Reference No. 008EXP/56). The HIV-1 viral loads and number of CD4 cell counts were observed during 2004-2015 as shown in Figure 1. The HIV-1 viral load was stable as 3-4 $\log_{10}$copies/mL between 2009 and 2011. In 2012, he had experienced a virological breakthrough when the viral load had reached a peak (6 $\log_{10}$copies/mL) together with a rapid fall in CD4 cell counts. However, in 2013, his viral load could only be detected as 1.53 $\log_{10}$copies/mL whereas the patients CD4 cell counts were increasing. His viral load increased to 4.34 $\log_{10}$copies/mL in 2015 whereas CD4 cell counts had slightly increased from 2014 to 2015.

This patient was promptly started on antiretroviral treatment that consisted of stavudine, lamivudine, and nevirapine during 2004-2009. During this time, the numbers of CD4 cell counts were only observed. In October 2009, he received HIV-1 drug resistance testing at our faculty for the first time. At this time, he had evidence of resistance to NRTIs and NNRTIs, but no evidence of resistance to PIs. Then, he started to receive indinavir and kaletra that consisted of lopinavir and ritonavir. In December 2010, he received HIV-1 drug resistance testing for the second time. The result showed no evidence of resistance to NRTIs and NNRTIs but he had evidence of resistance for PIs. However, he continued to receive the drug the same as previously until July 2012. At this time, he had experienced a virological breakthrough when the viral load had reached a peak (6 $\log_{10}$copies/mL) together with a rapid fall in CD4 cell counts (76 cells/mm^3). After testing the HIV-1 drug resistance, he showed no evidence of resistance for NRTIs and NNRTIs but still resistant to PIs. He has been continually followed up and treated with drug regimens consisting of lamivudine, stavudine, and kaletra.

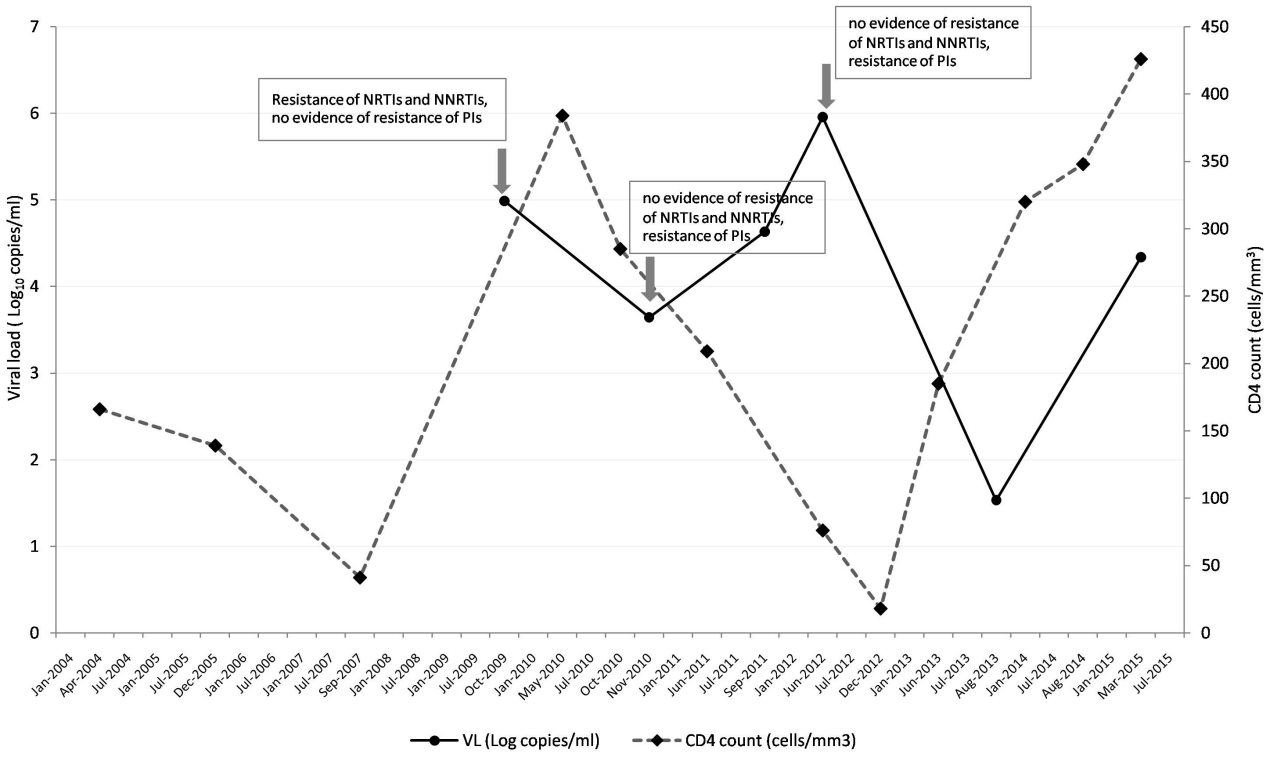

Figure 1. *Evolution of CD4 cell counts and HIV-1 RNA viral loads over 2004-2015. CD4 cell counts results are represented in diamonds (broken line) and HIV viral load in black circles (solid line). Vertical arrows at the top indicate the time points at which coreceptor usage was determined. The drug resistance results are presented in the box for each time point.*

Based on coreceptor usage, HIV-1 variants can be classified as R5, X4, and dual or mixed viruses.[1] X4 virus generally emerges in more advanced diseases and is associated with a sharp decline of CD4 T cells.[2-3] In this regard, we hypothesized that the coreceptor usage may be involved in the clinical pathogenesis during disease progression for this case. Then, the remaining RNA from HIV-1 drug resistant testing in three time points was collected to determine the HIV-1 coreceptor usage by both phenotypic and genotypic assays. The vertical arrows at the top of Figure 1 indicate the time points at which the coreceptor usage was determined.

Firstly, the V3 region was amplified from the remaining RNA and then this amplified product (~329 bp) was cloned using the pCR® 2.1 Vector TA cloning kit (Invitrogen, USA). Plasmid DNA containing V3 inserts (15-20 clones) were purified, and then directly sequenced using Big Dye Terminator v3.1 on an ABI3130 (Applied Biosystem). The V3 amino acid

sequences were then analyzed and used as templates for the prediction of coreceptor usage by the available genotypic predictors. Several algorithms have been presented for the prediction of coreceptor usage, including the 11/25 rule which predicts CXCR4 coreceptor usage if a positively charged amino acid (Arginine[R] or Lysine[K]) is presented at a position of 11 and/or 25, and if the overall net charge of V3 region is ≥5, a sample will be labeled as X4 according to the net charge rule.[4-5] Moreover, we also determine the coreceptor usage using bioinformatics tools (Geno2Pheno$_{[coreceptor]}$ and webPSSM) that are widely used and freely available on the website.[6-7] The V3 amino acid sequences and predicted coreceptor usage at three time points using different interpretation algorithms is shown in Table 1. At the first time point, the V3 region indicated that most of the clones (16/18 clones) had identical V3 amino acid sequences and most of genotypic predictors predicted as R5 phenotype. For the second time point, the V3 amino acid sequences were heterogeneous and several clones (12 clones) had an amino acid sequence equal to the first time point. Remarkably, five clones showed a high net charge because of the presence of positively charged amino acids (Arginine [R], Lysine [K]) in their sequences that lead to the prediction of X4. At the third time point, the result demonstrated that the V3 amino acid sequences were heterogeneous and different from previous points. These sequences were predicted as X4 phenotype by genotypic predictor, particularly by Geno2Pheno. Only one clone had an amino acid sequence as same as the prior. The genotypic results suggested that V3 amino acid sequences from those three time points were different and predicted that the coreceptor usage trend would be changed to an X4 phenotype. To confirm this finding, we repeated the determination of coreceptor usage by phenotypic assay.

Secondly, the phenotypic assay was performed to determine the coreceptor usage based on *env*-pseudotyped viruses. The entire gp160 envelope fragment was amplified from the same remaining RNA that was used to sequence in the V3 region as previously described. This envelope fragment (~2600 bp) was inserted into a PCI expression vector, transformed into the competent cells, and then the plasmid containing the HIV envelope fragment was extracted for pseudovirus production. This plasmid was co-transfected into the 293T cells with a full-length of the HIV gene without the envelope and luciferase was then used as a reporter. To determine the coreceptor usage, the pseudotyped viruses were inoculated in CCR5- and CXCR4-expressing cells. Viruses were then classified as R5, X4, or dual/mixed depending on their tropism. The phenotypic assay can only be performed at the first and third time points. At the first time point, the coreceptor usage showed as CCR5-using virus (mean Log Relative Light Unit (RLU) =5) and the coreceptor usage at the third time point was still served as CCR5-using virus (mean Log RLU=5.8). These phenotypic results suggested that the coreceptor changed was not found whereas the genotypic results were mostly predicted as X4 phenotype.

Table 1 V3 amino acid sequences and coreceptor usage at three time points determined by phenotypic assay and different interpretation algorithms.

Date of sample collection	V3 amino acid sequences	clone	Phenotype	Genotypic predictors						
				11/25 rule	net charge rule	G2p 1%	G2p 2.5%	G2p 5% and G2p 10%	G2p 15% and G2p 20%	PSSM
CM244	CTRPSNNTRTSITIGPGQVFYRTGDIIGDIRKAYC									
Oct-2009	..........VH..........G......Q...	*bulk*	R5	R5	R5	R5	R5	R5	R5	R5
	..........VH..........G......Q...	16		R5	R5	R5	R5	R5	R5	R5
	.....T.....VH..........G......Q...	1		R5	R5	R5	R5	R5	R5	R5
	..........VH..........G......R...	1		R5	X4	R5	R5	R5	X4	R5
Dec-2010	..........VH..........G......Q...	11	ND	R5	R5	R5	R5	R5	R5	R5
	.I..F.K.K...H....RM....K..G.T.K..H.	3		R5	X4	R5	R5	X4	X4	X4
	..........VH.....A.....G......Q...	1		R5	R5	R5	R5	R5	R5	R5
	.I..F.K.K.N.H....RM....K..G.T.K..H.	1		R5	X4	R5	X4	X4	X4	X4
	..........VH..........S......Q...	1		R5	R5	R5	R5	R5	R5	R5
	...L.......VH..........G......Q...	1		R5	R5	R5	R5	R5	R5	R5
	.I..F.K.K...H....RM....K..G.T.K.GH.	1		R5	X4	R5	R5	X4	X4	X4
	..........VH..........G......R...	1		R5	X4	R5	R5	R5	X4	R5
Jul-2012	.S.........TRM.................Q.H.	*bulk*	R5	R5	R5	R5	R5	X4	X4	R5
	.S.........TRM.................Q.H.	13		R5	R5	R5	R5	X4	X4	R5
	.S.........TR..................Q.H.	1		R5	R5	R5	R5	X4	X4	R5
	.SG........TRM........A........Q.H.	1		R5	R5	R5	R5	X4	X4	X4
	..........TRM.................Q.H.	1		R5	R5	R5	R5	X4	X4	R5
	..........VH..........G......Q...	1		R5	R5	R5	R5	R5	R5	R5
	.....H.....VH..........G......Q...	1		R5	X4	R5	R5	R5	R5	R5
	..........GVHM.........G......Q...	1		R5	R5	R5	R5	R5	R5	R5

ND: Not done, G2p: Geno2pheno$_{[coreceptor]}$ at different false positive rate

Note: *The V3 sequence of HIV-1 CM244, used as the reference sequence, is presented at the top of column. Sequences of clones obtained at each time point are presented below with the number of clones sequenced. Changes in an amino acid are identified by a single-letter code and amino acids identical to the amino acid in the reference sequence are identified by dot. Coreceptor usage was determined using bulk phenotypic assays as well V3 sequence data interpreted with different algorithms are showed in the right.*

Discussion

Currently, the determination of HIV-1 coreceptor usage based on genotypic tools is available and widely used to predict the coreceptor usage from the V3 amino acid sequence. The accessibility, simplicity, and low cost makes those genotypic assays a more feasible alternative to phenotype assays. Several studies have determined the coreceptor usage based on V3 amino acid sequences instead of phenotype assays due to its superiority.[8-11] In fact, these tools have been developed based on the data of HIV-1 subtype B and C mainly, thus they are less accurate for non-B subtypes as previously reported.[12-14] Here, we discussed a patient who had evidence of a coreceptor usage trend showing more of an X4 phenotype using genotypic predictors, but it contrasted with phenotypic result, which only voted to R5 phenotype. Although, the genotypic results showed the evolution of V3 amino acid sequences which were quite different in the two years between the first and third time points, but it is still not sufficient for coreceptor changed when confirmed with phenotypic assay. Notably, genotypic assays typically predict the coreceptor usage as X4 if the V3 sequence contains more positively charged amino acids. The presence of these positively charged amino acids in V3 sequence causes a high net charge, which can lead to the prediction of X4 by genotypic tools. Furthermore, a previous study also reported that Geno2pheno$_{[coreceptor]}$ and WebPSSM overestimated CXCR4 usage for CRF01_AE and CRF02_AG subtype,[15] which were also found in this study. The discordant results between genotypic and phenotypic assays could be raised from the comparison of coreceptor usage predictions based on V3 amino acid sequence only to a phenotypic assay taking the whole envelope region. The other domains in gp120 V1, V2 as well as in gp41 region have also been reported to influence the coreceptor usage.[16-18] Thus the prediction based on V3 sequences only may impact the reliability of tools. The other reasons could account for discordant results between genotypic and phenotypic assay. Of note, the genotypic tools have been specifically developed based on subtype B and C mainly, thus using these tools for determining the coreceptor usage of non-B and non-C subtypes have not been turned specifically. Moreover, it may be important that when clonal sequencing is used for genotypic assay, all possible clones are genotyped, and a prediction is provided for each clone. In this phenomenon, genotypic tools would infer tropism for sequences that do not exist in the viral population, eventually leading to an overestimation of prediction, whereas the phenotypic assay only measures existing strains or dominant viral population.

This finding suggested that genotypic tools are not always accurate for predicting HIV-1 coreceptor usage and the phenotypic assay is still a reference method, which is the most accurate method for the determination of HIV coreceptor usage, thus the utilization of genotypic predictors should be carefully considered. A false prediction of X4 variants may lead to unnecessarily precluding patients who could have benefited from receiving CCR5 inhibitors whereas a false prediction of R5 variants may lead to the reemergence of X4-strains under CCR5 inhibitors pressure. Further studies need to improve the prediction tools when non-B subtypes are involved or new subtype-specific rules should be developed to accurately predict the coreceptor usage and avoid the exclusion of patients who need to access the coreceptor inhibitors.

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References

[1] Berger EA, Doms RW, Fenyo EM, Korber BT, Littman DR, Moore JP, et al. A new classification for HIV-1. Nature. 1998; 391(6664): 240.

[2] Koot M, Keet IP, Vos AH, de Goede RE, Roos MT, Coutinho RA, et al. Prognostic value of HIV-1 syncytium-inducing phenotype for rate of CD4+ cell depletion and progression to AIDS. Ann Intern Med. 1993; 118(9): 681-8.

[3] Richman DD, Bozzette SA. The impact of the syncytium-inducing phenotype of human immunodeficiency virus on disease progression. J Infect Dis. 1994; 169(5): 968-74.

[4] De Jong JJ, De Ronde A, Keulen W, Tersmette M, Goudsmit J. Minimal requirements for the human immunodeficiency virus type 1 V3 domain to support the syncytium-inducing phenotype: analysis by single amino acid substitution. J Virol. 1992; 66(11): 6777-80.

[5] Fouchier RA, Groenink M, Kootstra NA, Tersmette M, Huisman HG, Miedema F, et al. Phenotype-associated sequence variation in the third variable domain of the human immunodeficiency virus type 1 gp120 molecule. J Virol. 1992; 66(5): 3183-7.

[6] Jensen MA, Li FS, van 't Wout AB, Nickle DC, Shriner D, He HX, et al. Improved coreceptor usage prediction and genotypic monitoring of R5-to-X4 transition by motif analysis of human immunodeficiency virus type 1 env V3 loop sequences. J Virol. 2003; 77(24): 13376-88.

[7] Lengauer T, Sander O, Sierra S, Thielen A, Kaiser R. Bioinformatics prediction of HIV coreceptor usage. Nat Biotechnol. 2007; 25(12): 1407-10.

[8] Ng KY, Chew KK, Kaur P, Kwan JY, Khong WX, Lin L, et al. High prevalence of CXCR4 usage among treatment-naive CRF01_AE and CRF51_01B-infected HIV-1 subjects in Singapore. BMC Infect Dis. 2013; 13: 90.

[9] To SW, Chen JH, Wong KH, Chan KC, Chen Z, Yam WC. Determination of the high prevalence of Dual/Mixed- or X4-tropism among HIV type 1 CRF01_AE in Hong Kong by genotyping and phenotyping methods. AIDS Res Hum Retroviruses. 2013; 29(8): 1123-8.

[10] Phuphuakrat A, Phawattanakul S, Pasomsub E, Kiertiburanakul S, Chantratita W, Sungkanuparph S. Coreceptor tropism determined by genotypic assay in HIV-1 circulating in Thailand, where CRF01_AE predominates. HIV Med. 2014; 15(5): 269-75.

[11] Neogi U, Sahoo PN, Arumugam K, Sonnerborg A, De Costa A, Shet A. Higher prevalence of predicted X4-tropic strains in perinatally infected older children with HIV-1 subtype C in India. J Acquir Immune Defic Syndr. 2012; 59(4): 347-53.

[12] Raymond S, Delobel P, Chaix ML, Cazabat M, Encinas S, Bruel P, et al. Genotypic prediction of HIV-1 subtype D tropism. Retrovirology. 2011; 8: 56.

[13] Raymond S, Delobel P, Mavigner M, Cazabat M, Souyris C, Encinas S, et al. Genotypic prediction of human immunodeficiency virus type 1 CRF02-AG tropism. J Clin Microbiol. 2009; 47(7): 2292-4.

[14] Raymond S, Delobel P, Rogez S, Encinas S, Bruel P, Pasquier C, et al. Genotypic Prediction of HIV-1 CRF01-AE Tropism. J Clin Microbiol. 2013; 51(2): 564-70.

[15] Mulinge M, Lemaire M, Servais JY, Rybicki A, Struck D, da Silva ES, et al. HIV-1 tropism determination using a phenotypic Env recombinant viral assay highlights overestimation of CXCR4-usage by genotypic prediction algorithms for CRRF01_AE and CRF02_AG. PLoS One. 2013; 8(5): e60566.

[16] Dimonte S, Mercurio F, Svicher V, D'Arrigo R, Perno CF, Ceccherini-Silberstein F. Selected amino acid mutations in HIV-1 B subtype gp41 are associated with specific gp120v(3) signatures in the regulation of co-receptor usage. Retrovirology. 2011; 8: 33.

[17] Labrosse B, Treboute C, Brelot A, Alizon M. Cooperation of the V1/V2 and V3 domains of human immunodeficiency virus type 1 gp120 for interaction with the CXCR4 receptor. J Virol. 2001; 75(12): 5457-64.

[18] Thielen A, Sichtig N, Kaiser R, Lam J, Harrigan PR, Lengauer T. Improved prediction of HIV-1 coreceptor usage with sequence information from the second hypervariable loop of gp120. J Infect Dis. 2010; 202(9): 1435-43.

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Effects of crude medicinal Thai flower extracts on cytotoxicity and FMS-like tyrosine kinase 3 protein expression in EoL-1 leukemic cell line

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ABSTRACT

Background: Leukemia is one cause of death worldwide. Nowadays, natural therapies such as the use of medicinal plants may reduce chemotherapy side effects of leukemia. Accordingly, the anticancer properties of medicinal plants have been recognized for centuries. This study focuses on Thai flowers from traditional drug recipes including phikun (*Mimusops elengi* Linn.), boonark (*Mesua ferrea* Linn.), ketawa (*Gardenia jasminoides* J.Ellis), and sarapi (*Mammea siamensis* (Miq.) T. Anders) for cytotoxicity and leukemia suppression. Fms-like tyrosine kinase 3 (FLT3) is a prognostic marker for acute myeloblastic leukemia (AML) and leukemic cell proliferation. Thus, FLT3 is a reliable marker for minimal residual disease (MRD) assessment in leukemia patients.

Objectives: To investigate the cytotoxicity of crude ethanolic extracts from four Thai flowers and to determine their effects on FLT3 protein expression in EoL-1 cells.

Materials and methods: Phikun, boonark, ketawa, and sarapi flowers were extracted by using 95% ethanol. An MTT assay was performed to evaluate cytotoxicity of each crude ethanolic flower extract. The effective crude ethanolic flower extract was further determined its inhibitory effect on FLT3 protein expression by Western blot analysis. Total cell number was determined by the trypan blue exclusion method.

Results: Crude boonark ethanolic extract demonstrated the strongest cytotoxic activity with the inhibitory concentration at 50% (IC_{50}) values of 62.5±3.9 µg/mL. Moreover, it could decrease the total cell number and FLT3 protein expression by a time- and dose-dependent manner.

Conclusion: The crude boonark ethanolic extract inhibited cell proliferation *via* the suppression of FLT3 expression. It could be suggested that crude boonark ethanolic extract is a promising approach for new anti-leukemic drug candidates.

Introduction

Leukemia is a group of blood diseases characterized by diversity of chromosomal and molecular changes. It was characterized by the hematopoietic progenitor cells losing their ability to differentiate normally and responding to normal regulators of proliferation. Some alterations of protein levels provided useful molecular biomarkers which have been evaluated in leukemia patients.[1,2] Previous studies showed that overexpression of Fms-like tyrosine kinase 3 (FLT3) are associated with leukemogenesis.[3-6]

FLT3 belongs to the type III receptor tyrosine kinase and has an important role in hematopoietic progenitor cell

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proliferation. It is a prognostic marker for acute myeloblastic leukemia (AML).[7,8] Ligand-FLT3 binding promotes receptor dimerization, subsequent signaling and phosphorylation of multiple cytoplasmic proteins as well as the activation of several downstream signaling pathways, such as Ras/Raf, MAPK, and PI3 kinase cascades.[7,9] Previous study showed that upregulation of FLT3 and its ligand by leukemic cells creates an autocrine signaling loop which stimulates proliferation of EoL-1 cell line.[10] Furthermore, high levels of wild-type FLT3 have been reported for blast cells in 20-25% of AML patients without FLT3 mutations. This may be considered to represent an attractive therapeutic target in AML.

Nowadays, chemotherapy is a generally effective treatment and is widely used for leukemia treatment. To avoid the severe side effects of drug chemotherapy, plants having anticancer activity are an alternative for cancer treatment. South-East Asian countries like Malaysia, Thailand, and Borneo (Indonesia) have a long history of using medicinal plant that offer considerable pharmaceutical potential. Lee and Houghton found that the South-East Asian region, owing to the vast bio-diversity of its flora, holds great promise for the discovery of novel biologically-active compounds.[11] According to the World Health Organization (WHO), most populations in the world still rely on traditional medicines for their psychological and physical health requirements. People living in rural areas from their personal experience know that these traditional remedies are valuable source of natural products to maintain human health.[12] Medicinal plants have long been used as a traditional source of healing in Thailand.[13] In the Thai folk medicine, five Thai flowers including mali (jasmine; *Jasminum sambac* (L.) Aiton), phikun (*Mimusops elengi* Linn.), boonark (*Mesua ferrea* Linn.), sarapi (*Mammea siamensis* (Miq.) T. Anders), and bualuang (sacred lotus or pink lotus-lily; *Nelumbo nucifera* Gaertn), have been used as a drug recipe.[14] This recipe has been used for heart tonic modulation. Phikun,[15] boonark,[16] and sarapi[17] were selected for the study to determine biological activities. Ketawa (*Gardenia jasminoides* J.Ellis)[18] was selected for the study because it also has been used in Thai folk medicine (skin disease treatment). The inhibitory effects of sarapi flower extract on FLT3, WT, and Bcr/Abl proteins expressions in leukemic cell lines have been previously reported.[17,19] In this study, the properties of crude ethanolic extracts from the Thai flowers of pikun, boonark, ketawa, and sarapi were investigated on FLT3 protein expressions in EoL-1 cell line.

Materials and methods

Cell culture and condition

Eosinophilic leukemic (EoL-1) cells were used as human leukemic cell line model in this study. Cells were purchased from RIKEN BRC Cell Bank, Japan (RCB0641) and cultured in RPMI 1640 medium (GIBCO™, Thermo Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (Capricon, Ebsdorfergrund, Germany), 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin (GIBCO™, Thermo Fisher Scientific, MA, USA) at 37°C in a humidified incubator with 5% CO_2.

Plant material and preparation

The Thai flowers were used in this study were phikun, boonark, ketawa, and sarapi. The flowers were collected in the Chiang Mai Province of Thailand in 2014. The flowers were dried in a hot-air oven before being extracted by ethanol and were blended into a dehydrated powdered form. One hundred grams of each dried flower powder was macerated with 1,000 mL of 95% ethanol for 48 hr at room temperature. The liquid extracts were collected from three times of maceration, filtered, and then the solvent was removed under vacuum using a rotary evaporator at 45°C. Crude ethanolic flower extracts were obtained after complete removal of the solvent. The extracts were further dried in evaporator. The four flowers crude ethanolic extracts were kept in the refrigerator at -20°C until used and suspended in DMSO to prepare the stock solution (25,000 µg/mL).

MTT assay

Cytotoxicity of the crude ethanolic flower extracts, phikun, boonark, ketawa, and sarapi were determined by MTT assay. EoL-1 cells were seeded (5.0×10^4 cells/well) in 96-well plates and incubated overnight at 37°C with 5% CO_2. Cells were then treated with four extracts (0-100 µg/mL) for 48 hr. A complete medium with DMSO was used as a vehicle control (VC). After incubation, 15 µL of MTT dye (Sigma-Aldrich, St Louis, MO, USA) solution (5 mg/mL) was added to each well and the plate was then incubated at 37°C for another 4 hr. Formazan crystal products were dissolved in 200 µL of DMSO, and the absorbance was measured at 578 nm by an AccuReader™ microplate reader (Metertech Inc, Taipei, Taiwan) with reference blank at 630 nm. The percentage of cell viability was calculated as following formula.

$$\% \text{ cells viability} = \frac{(\text{OD average of tested well} \times 100)}{\text{OD average of vehicle control}}$$

The average of cell viability obtained from triplicate experiments was plotted on a graph. The inhibitory concentration at 50% growth (IC_{50}) value was presented as the lowest concentration that decreases cell growth by 50%, whereas the IC_{20} value was determined as a non-cytotoxic dose and used for protein expression analysis.

Protein extraction and Western blotting

After treatment, leukemic cells were harvested, and total viable cell numbers were counted using 0.4% trypan blue. Thereafter, the total protein from the treated cells was harvested and extracted by using RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate ($C_{24}H_{39}NaO_4$), and 0.1% SDS). Protein concentration was measured by the Folin-Lowry method. FLT3 protein was separated by 7.5% SDS polyacrylamide gel and detected using rabbit polyclonal anti-FLT3 antibody (1:1,000 in blocking buffer, Invitrogen™, Carlsbad, CA, USA). GAPDH (Santa Cruz Biotechnology, CA, USA) was used as a loading control with a dilution of 1:1,000. HRP-conjugated goat anti-rabbit IgG (1:20,000) was used as a secondary antibody. Protein of interest was detected by Luminata™ Forte Western HRP substrate

(Millipore Corporation, Billerica, MA, USA). The protein band was quantified by a scan densitometer and Quantity One software, version 4.6.3 (Bio-Rad laboratories, Hercules, CA, USA). Density values of each FLT3 band were normalized to the GAPDH band.

Statistical analysis

Average of triplicate experiments and standard deviation (SD) were used for quantification. Levels of target protein expression were compared to vehicle control. The results were shown as mean±SD. Differences between means of each experiment were analyzed by One-way analysis of variance (One-way ANOVA). Statistic significances were considered at $p<0.05$, $p<0.01$, and $p<0.001$.

Results

Preparation of crude ethanolic extract from phikun, boonark, ketawa, and sarapi

One hundred grams of dried powder from each flower material was extracted by ethanol (without preservatives). The percentage yield recovery (% yield) of the four flowers crude ethanolic extracts (phikun, boonark, ketawa, and sarapi) were 20.66, 25.15, 27.16, and 29.50%, respectively.

Cytotoxicity of crude ethanolic extract from phikun, boonark, ketawa, and sarapi on EoL-1 cell line

The inhibitory concentration at 50% (IC_{50}) values of crude ethanolic flower extracts of phikun, boonark, ketawa, and sarapi on EoL-1 cells were >100, 62.5±3.9, >100, and >100 µg/mL, respectively (Figure 1).

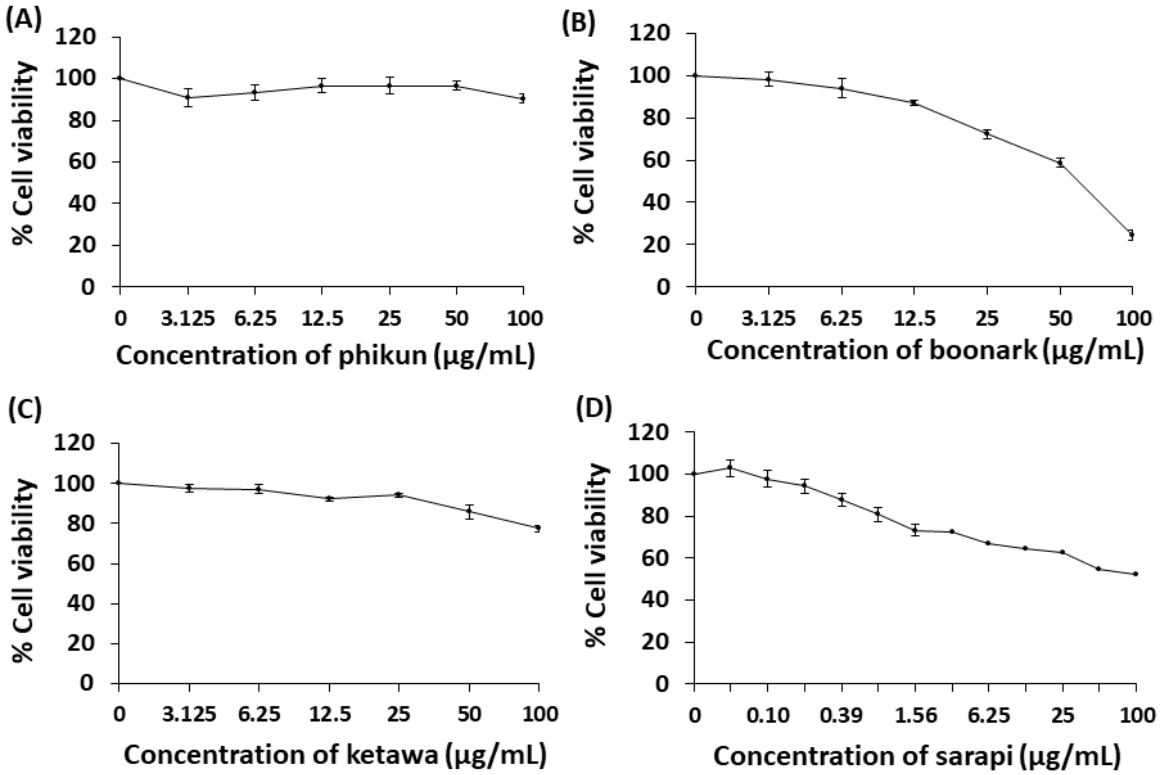

Figure 1. *Cytotoxicity of crude ethanolic extract from phikun, boonark, ketawa, and sarapi in EoL-1 cell line. EoL-1 cells (5×10^4 cells/mL) were cultured in the presence of various concentrations of crude ethanolic extract from (A) phikun, (B) boonark, (C) ketawa, and (D) sarapi (0-100 µg/mL) for 48 hr. The cell viability was determined by MTT assay. Each point presents the mean value±SD of three times independent experiments performed in triplicate.*

Crude ethanolic extract from phikun, boonark, ketawa, and sarapi suppressed FLT3 expression in EoL-1 cell line

When examining the effect of crude ethanolic extracts from phikun, boonark, ketawa, and sarapi on FLT3 protein expression, their IC_{20} values (100, 18.6, 85.4, and 0.9 µg/mL, respectively) were used. *n*-Hexane extract of sarapi (Hex-sarapi) with concentration of 0.07 µg/mL was used as a positive control.[17] EoL-1 cells were examined with those of Hex-sarapi and crude ethanolic extracts on FLT3 protein expression. After treatment of EoL-1 cells with four crude ethanolic extracts for 48 hr, the crude ethanolic extract from boonark at 18.6 µg/mL showed the most effective suppression against FLT3 expression by 58.4±3.7% ($p<0.01$) when compared to the vehicle control (Figure 2A), Hex-saraphi, phikun, ketawa, and sarapi could suppress FLT3 protein expression by 6.4±1.8, 15.4±2.1, 2.8±0.7, and 35.3±4.9%, respectively. The total cell numbers were significantly decreased after treatment with positive control (Hex-saraphi) and crude ethanolic flower extract from phikun, boonark, ketawa, and sarapi with the values of 29.0±3.5, 42.9±4.0, 64.3±1.5, 10.0±6.4, and 47.7±2.6%, respectively, when compared to the vehicle control (Figure 2B). The percentages of dead cells were in the range of 0-0.1% of that determined by trypan blue exclusion method.

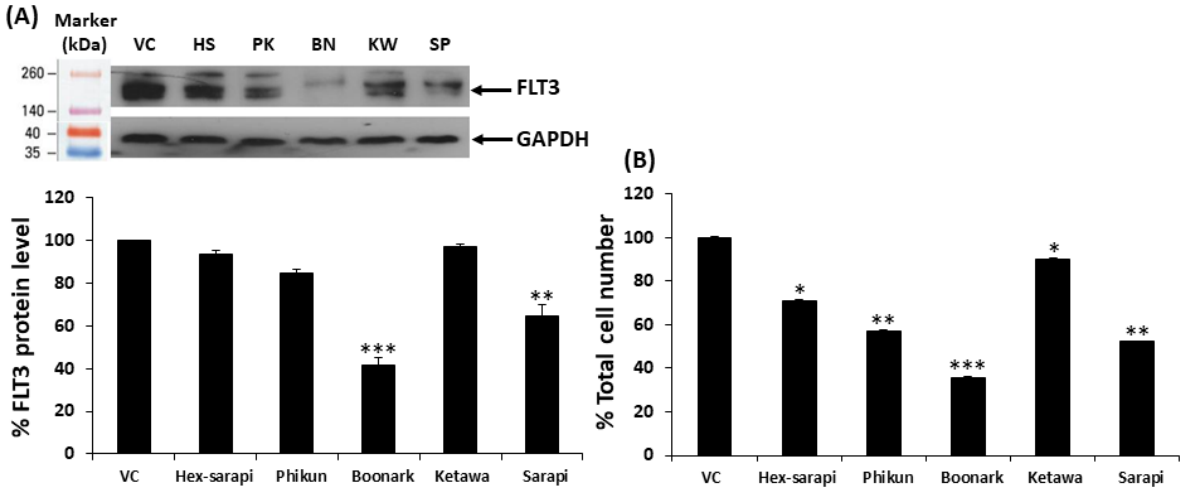

Figure 2. *Effect of crude ethanolic extracts from phikun (PK), boonark (BN), ketawa (KW), and sarapi (SP) on FLT3 protein expressions in EoL-1 cells. (A) Level of FLT3 protein expression after treatments were assessed by Western blotting; GAPDH was used as the loading control. Hex-sarapi (HS) was represented as positive control. (B) Total cell numbers of EoL-1 cells after treatment with Hex-sarapi and four flower crude ethanolic extracts were determined by trypan blue exclusion method. Data points were mean values±SD of three independent experiments. Asterisks (*), double asterisks (**) and triple asterisks (***) denote values that were significantly different from the vehicle control (VC) at p<0.05, p<0.01, and p<0.001, respectively.*

Effect of different time points of crude boonark ethanolic extract on FLT3 protein level in EoL-1 cells

To examine the effect of time period of the most effective crude ethanolic flower extract on FLT3 protein expression in EoL-1 cells, the cells were treated with crude boonark ethanolic extract with the concentration of 18.6 µg/mL (IC_{20} value) for 24, 48, and 72 hr. FLT3 protein levels after treatment with crude boonark ethanolic extract were decreased by 4.0±1.9, 49.9±4.5 (p<0.01), and 88.1±3.0% (p<0.001), respectively, when compared to the vehicle control (Figure 3A). The total cell numbers after treated with crude boonark ethanolic extract from were decreased by 8.3±1.3 (p<0.05), 45.9±6.3 (p<0.01), and 53.9±3.3% (p<0.001), respectively when compared to the vehicle control (Figure 3B).

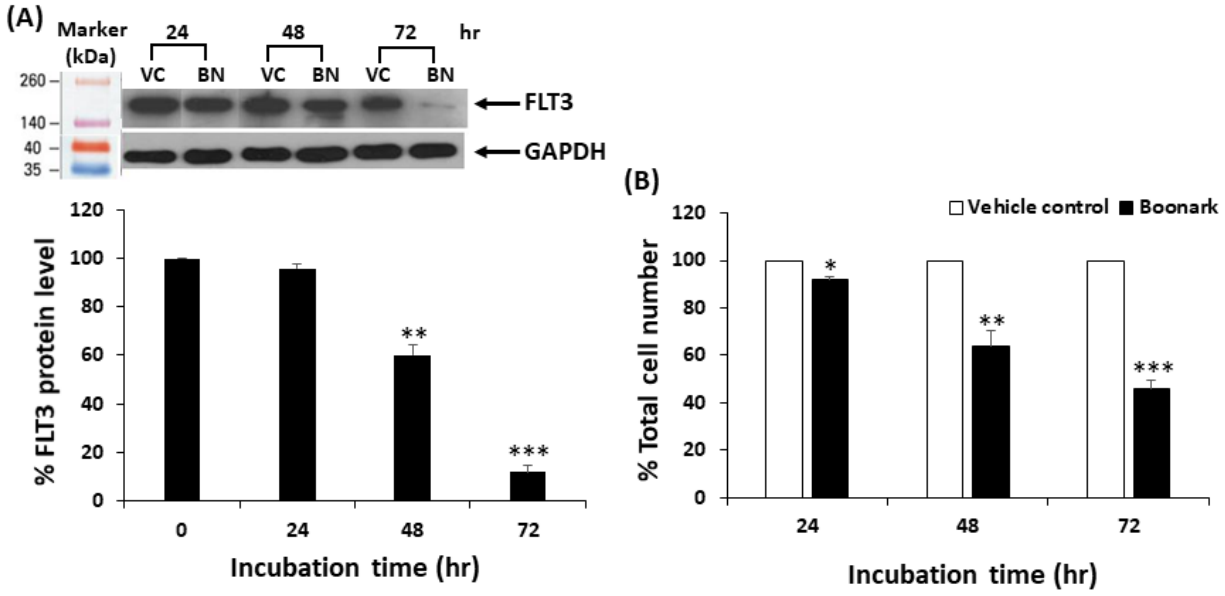

Figure 3. *Effect of incubation time period of crude boonark ethanolic extract on FLT3 protein expressions in EoL-1 cells. (A) Level of FLT3 protein expression after treatments were assessed by Western blotting; GAPDH was used as the loading control. (B) Total cell numbers of EoL-1 cells after treatment with crude boonark ethanolic extract (BN) were determined by trypan blue exclusion method. Data points were mean values±SD of three independent experiments. Asterisks (*), double asterisks (**) and triple asterisks (***) denote values that were significantly different from the vehicle control (VC) at p<0.05, p<0.01, and p<0.001, respectively*

Effect of different concentrations of crude boonark ethanolic extract in EoL-1 cells

To examine the effect of concentrations of crude boonark ethanolic extract on FLT3 protein expression in EoL-1 cells, cells were treated with medium containing DMSO (vehicle control) and various non-cytotoxic doses of crude boonark ethanolic extract (12, 15, and 18 µg/mL) for 48 hr. The protein levels of FLT3 were decreased by 14.8±1.6 (p<0.05), 48.4±0.5 (p<0.01), and 61.4±1.1% (p<0.001) in response to concentrations of 12, 15, and 18 µg/mL, respectively when compared to the vehicle control (Figure 4A). The total cell numbers were decreased by 30.5±5.0 (p<0.01), 59.2±3.8 (p<0.001), and 66.6±4.9% (p<0.001) in response to concentrations of 12, 15, and 18 µg/mL of crude boonark ethanolic extract when compared to the vehicle control (Figure 4B).

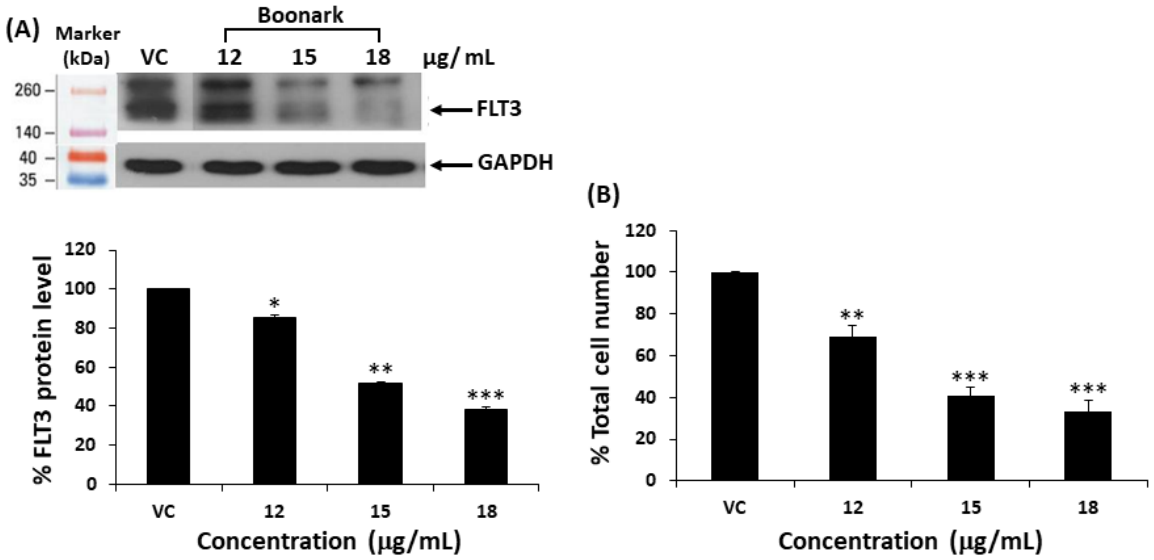

Figure 4. *Effect of dose of crude boonark ethanolic extract on FLT3 protein expressions in EoL-1 cells. (A) Level of FLT3 protein expression after boonark (BN) treatments were assessed by Western blotting; GAPDH was used as the loading control. (B) Total cell numbers of EoL-1 cells after treatment with crude boonark ethanolic extract were determined by trypan blue exclusion method. Data points were mean values±SD of three independent experiments. Asterisks (*), double asterisks (**), and triple asterisks (***) denote values that were significantly different from the vehicle control (VC) at p<0.05, p<0.01 and p<0.001, respectively.*

Discussion

This study is the first report of Thai recipe flower extracts on FLT3 protein expression in leukemic cells. FLT3 is a receptor tyrosine kinase involved in hematopoietic cell proliferation, differentiation, and apoptosis.[20] Moreover, FLT3 has been defined as a type III of receptor tyrosine kinases, and it plays an important role in leukemogenesis.[3-6] In this study, crude ethanolic extracts from three out of five flowers in Thai traditional flower recipes (mali (jasmine), phikun, boonark, sarapi, bualuang)[14] were tested. They have been used for a long time as a Thai traditional medicine. The cytotoxic effect of four crude ethanolic extracts from phikun, boonark, ketawa, and sarapi in EoL-1 cells were examined by MTT assay. Crude boonark ethanolic extracts showed the best cytotoxicity in EoL-1 cells, whereas crude ethanolic extracts from phikun, ketawa, and sarapi did not show cytotoxicity (IC_{50} >100 µg/mL) in EoL-1 cells, however cytotoxicity of sarapi trended to decrease by a dose-dependent manner (Figure 1D). Sangkaruk et al. previously reported that crude sarapi ethanolic extract had cytotoxic effects on EoL-1 cells with IC_{50} values of 5.5 µg/mL.[17] The results were reported in different IC_{50} values when compared to the results in this study. It is possible that the sarapi flowers were collected in different location, plant, and harvesting time.[21,22] Moreover, a cell line in different time of cell culture might show in different IC_{50} values.

According to the effects of four crude flower extracts on FLT3 protein expressions by Western blotting in EoL-1 cells at non-cytotoxic doses (IC_{20} values), boonark flower extract (18.6 µg/mL) was the most effective extract to decrease the FLT3 protein level by 58.4±3.7% without affecting cell death suggesting that boonark flower extract suppressed EoL-1 cell proliferation. FLT3 protein was previously reported to be inhibited by Hex-sarapi (1.0 µg/mL) flower extract by 21.1±6.3%17 while Hex-sarapi (0.07 µg/mL) in this study could inhibit FLT3 protein by 6.4±1.8%. Boonark flower extract showed better inhibitory effect than Hex-sarapi flower extract (0.07 µg/mL) for 2.8-fold when compared to that result from Sangkaruk et al.[17] Next, the most effective crude ethanolic extracts were used for further experiments. As shown in the cytotoxicity test of four flowers crude ethanolic extracts, the crude boonark ethanolic extract had also showed the strongest cytotoxicity on EoL-1 cells. It decreased FLT3 protein levels and inhibited EoL-1 cell proliferation by a time- and dose-dependent manner when compared to the vehicle control and did not alter cell viability. Thus, the results from these experiments exhibited that crude boonark ethanolic extract could inhibit cell proliferation and downregulate the target FLT3 protein levels at non-cytotoxic doses. Boonark (*M. ferrea* L.) belongs to the family Clusiaceae (Guttiferea). The plant has shown various pharmacological activities including anti-neoplastic, immunomodulatory, anti-oxidant, anti-inflammatory activities, etc.[23] Flowers and stamens of boonark have

reported the various constituents such as glycosides, coumarin, flavonoids, xanthones, triglycerides, and resins.[12] The crude extract of *M. ferrea* L. showed a strong cytotoxic activity toward T lymphocyte leukemia cells and weak antimicrobial activities against *Staphylococcus aureus*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*.[24]

This is the first report that revealed the effect of crude boonark ethanolic extract have the inhibitory mechanisms *via* FLT3 protein expression in EoL-1 cells. However, the target molecule of crude boonark ethanolic extract to inhibit *FLT3* gene expression is unclear because FLT3 involved in three main signal transduction networks, including phosphatidylinositol-3-kinase (PI3K), MAP kinase, and Jak-STAT pathways. Activation of these pathways is critical for leukemic cell proliferation and survival.[20] Furthermore, the active compounds of boonark extract are of interest for further study.

Conclusion

The crude boonark ethanolic extract possessed anti-proliferation of EoL-1 cells. Furthermore, crude boonark ethanolic extract decreased FLT3 protein level in both time- and dose-dependent manner. This is the first report of the inhibitory effects of crude boonark ethanolic extract on FLT3 protein expression in leukemic cells. Therefore, an active compound from the fraction will be further investigated in the future. This result indicates that crude boonark ethanolic extract used in traditional Thai medicine may be useful as an alternative therapeutic agent in human acute myeloblastic leukemic cells. The study has provided a basis for the future study of crude boonark ethanolic extract to confirm its effect on leukemia treatment.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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References

[1] Marcucci G, Mrozek K, Bloomfield CD. Molecular heterogeneity and prognostic biomarkers in adults with acute myeloid leukemia and normal cytogenetics. Curr Opin Hematol 2005; 12(1): 68-75.

[2] Sakamoto Y, Mariya Y, Sasaki S, Teshiromori R, Oshikiri T, Segawa M, et al. WT1 mRNA Level in Peripheral Blood Is a Sensitive Biomarker for Monitoring Minimal Residual Disease in Acute Myeloid Leukemia. Tohoku J Exp Med 2009; 219(2): 169-76.

[3] Stirewalt DL, Radich JP. The role of FLT3 in haematopoietic malignancies. Nat Rev Cancer 2003; 3: 650-65.

[4] Fathi AT, Arowojolu O, Swinnen I, Sato T, Rajkhowa T, Small D, et al. A potential therapeutic target for FLT3-ITD AML: PIM1 kinase. Leukemia Res 2012; 36(2): 224-31.

[5] Weisberg E, Boulton C, Kelly LM, Manley P, Fabbro D, Meyer T, et al. Inhibition of mutant FLT3 receptors in leukemia cells by the small molecule tyrosine kinase inhibitor PKC412. Cancer Cell 2002; 1(5): 433-43.

[6] Zarrinkar PP, Gunawardane RN, Cramer MD, Gardner MF, Brigham D, Belli B, et al. AC220 is a uniquely potent and selective inhibitor of FLT3 for the treatment of acute myeloid leukemia (AML). Blood 2009; 114(14): 2984-92.

[7] Choudhary C, Muller-Tidow C, Berdel WE, Serve H. Signal transduction of oncogenic Flt3. Int J Hematol 2005; 82(2): 93-9.

[8] Chu SH, Small D. Mechanisms of resistance to FLT3 inhibitors. Drug Resist Updat 2009; 12(1-2): 8-16.

[9] Choudhary C, Schwable J, Brandts C, Tickenbrock L, Sargin B, Kindler T, et al. AML-associated Flt3 kinase domain mutations show signal transduction differences compared with Flt3 ITD mutations. Blood 2005; 106(1): 265-73.

[10] Zheng R, Levis M, Piloto O, Brown P, Baldwin BR, Gorin NC, et al. FLT3 ligand causes autocrine signaling in acute myeloid leukemia cells. Blood 2004; 103(1): 267-74.

[11] Lee CC, Houghton P. Cytotoxicity of plants from Malaysia and Thailand used traditionally to treat cancer. J Ethnopharmacol 2005; 100(3): 237-43.

[12] Alakh S, Hemalatha S, Sairam K. Phytopharmacological review of Mesua ferrea Linn. Int J Phytopharmacol 2014; 5(1): 6-14.

[13] Cheeptham N, Towers GHN. Light-mediated activities of some Thai medicinal plant teas. Fitoterapia 2002; 73(7-8): 651-62.

[14] Poonpaiboonrote N. Study of immunomodulatory effect of water extracts of five-flower remedy and its compositions on macrophages [Thesis (M.Sc.)]: Chulalongkorn University; 2014.

[15] Gami B, Pathak S, Parabia M. Ethnobotanical, phytochemical and pharmacological review of Mimusops elengi Linn. Asian Pac J Trop Biomed 2012; 2(9): 743-8.

[16] Chahar MK, DS SK, Geetha L, Lokesh T, Manohara K. Mesua ferrea L.: a review of the medical evidence for its phytochemistry and pharmacological actions. Afr J Pharm Pharmacol 2013; 7(6): 211-9.

[17] Sangkaruk R, Tima S, Rungrojsakul M, Chiampanichayakul S, Anuchapreeda S. Effects of Saraphi (Mammea siamensis) flower extracts on cell proliferation and Fms-like tyrosine kinase 3 expression in leukemic EoL-1 cell line. Bull Chiang Mai Assoc Med Sci 2016; 49(2): 286-93.

[18] Chaichana J, Niwatananum W, Vejabhikul S, Somna S, Chansakaow S. Volatile constituents and biological activities of Gardenia jasminoides. J Health Res 2009; 23(3): 141-5.

[19] Sangkaruk R, Rungrojsakul M, Tima S, Anuchapreeda S. Effect of Thai saraphi flower extracts on WT1 and BCR/ABL protein expression in leukemic cell lines. Afr J Tradit Complement Altern Med 2017; 14(2): 16-24.

[20] Grafone T, Palmisano M, Nicci C, Storti S. An overview on the role of FLT3-tyrosine kinase receptor in acute myeloid leukemia: biology and treatment. Oncol Rev 2012; 6(1): 64-74.

[21] Saha S, Monroe A, Day MR. Growth, yield, plant quality and nutrition of basil (Ocimum basilicum L.) under soilless agricultural systems. Ann Agric Sci 2016; 61(2): 181-6.

[22] Ncube B, Finnie J, Van Staden J. Quality from the field: the impact of environmental factors as quality determinants in medicinal plants. S Afr J Bot 2012; 82: 11-20.

[23] Sharma A, Sharma S, Parashar B. Mesua ferrae linn:-A Review of the Indian Medical Herb. Sys Rev Pharm 2017; 8(1): 19-23.

[24] Nordin K, Ahmad F, Taufiq-Yap Y, Ali AM. Volatile Components of Methanol Extract from the Flower of Malaysian Musea Ferrea Linn. Orient J Chem 2004; 20: 69-72.

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Comparison the effect of two analysis methods of brain volume: Absolute brain volume and brain volume normalized with intracranial volume in methamphetamine abusers

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ABSTRACT

Introduction: Magnetic Resonance Imaging (MRI) documented abnormal brain structure in methamphetamine abusers with inconsisting results. It is likely that this discrepency could be from different analysis methods for example absolute volume analysis method and intracranial volume(ICV) normalization.

Objectives: To compare the effect of analysis method between absolute volume analysis and normalized volume with ICV analysis among methamphetamine abusers (MA) and healthy controls (HC) groups.

Materials and methods: Ten MAs and 14 HCs with gender and age matched were recruited. MRI of brain were acquired on 1.5 Tesla MR Scanner (Achieva, Philips, Netherland). Axial T1-weighted images with 3D FFE pulse sequence were used for MRI acquisition with the following data acquisition parameters: TE/TR =4.6/20 ms, FOV = 24 cm, 256x128 imaging matrix and 120 slices. Measurement of brain volume were performed with Freesurfer (FS) version 5.3. Absolute volume and normalized volume with ICV between HCs and MAs groups were compared. Pearson correlation was used to find the correlation of the brain volume between the two methods.

Results: In absolute volume analysis method, we observed consistent significant larger brain volumes in HCs compared to MAs. A positive correlation in most parts of brains was observed between absolute volume analysis method and ICV in HCs and MAs group. In contrast, negative correlation in most parts f brains was observed between ICV normalized volume method and ICV between HC and MA. The different of correlation for each brain region between HCs and MAs could be associated with the effect of methamphetamine.

Conclusion: The difference of the two analysis methods give similar results but varied in different brain regions. The choice of analysis method should be carefully selected in brain imaging study.

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Introduction

Human brain is considered a vital organ of human body. Its primary functions include controlling and commanding muscle coordination of movement, seeing, hearing, speaking, thinking process, memory, emotion and all kinds of feeling. Brain structure abnormalities could be fetal. These abnormalities can occur from various causes such as head injury,[1,2] some infection,[3,4] and poison or addictive substance receiving.[5] There are several methods for brain structure analysis such as the absolute and intracranial corrected volume.[6] In previous studies, Magnetic resonance imaging (MRI) has been widely used,[7-9] together with software programs to evaluate brain volume.[10,11] The method is noninvasive, no side effect and can be done repeatedly. The analysis software that widely used for brain volume evaluation include Statistical Parametric Mapping (SPM) and Freesurfer (FS). Previous studies revealed that FS software package give more accurate results than SPM. [12,13] A study of brain volume can indicate brain atrophy or changes in brain structure at the macroscopic level and increase understanding if changes occur in a certain area of the brain resulting in treatment or prevent any potential abnormality of the brain in the future.[14]

Studies of brain volume in methamphetamine users showed some conflict results. Some reports showed an increase in size of the caudate nucleus, putamen and globus pallidus,[8,15,16] in drug abusers while other reports indicated a decrease in the brain volume.[17] The inconsistency results may come from different analysis techniques or methods. Some researchers used absolute volume obtained from a software program for analysis,[8,16,18-20] while the others used the brain volume normalized with intracranial volume.[7, 17] Consequently, this study aimed to find a correlation between the 2 analysis methods; with the absolute volume and the analysis with the brain volume normalized with intracranial volume. The results obtained from the 2 methods were compared between groups of methamphetamine abuser and healthy control.

Materials and methods

Study Participants

Twenty-four participants aged between 19 -35 years old were recruited. All are required to pass inclusion and exclusion criteria approved by Internal Review Board (IRB) of the Research Institute for Health Sciences (RIHES), Chiang Mai University. Participants were divided into 2 groups: 10 methamphetamine abuser group (MAs) and 14 healthy control group (HCs) (Table 1).

Inclusion criteria for MAs included: 1) passing the evaluation of Diagnostic and Statistical Manual of Mental Disorder IV (DSM-IV), 2) having history of using methamphetamine regularly at least 12 months and the latest usage was not over than 1 week before participating in the research and the methamphetamine was the main addictive substance, and 3) having negative Human Immunodeficiency Virus (HIV), Hepatitis B, and Hepatitis C Virus tests.

Inclusion criteria for the HCs were: 1) no history of drug abuse, 2) healthy and no regularly medicines for treatment, 3) no history of addictive substance and methamphetamine confirming with negative urine test, and 4) negative HIV, Hepatitis B Virus, and Hepatitis C Virus tests.

Exclusion criteria were those who had history of mental illness regardless of addictive substance use, depression or schizophrenia, history of head injury, claustrophobia, having devices or implants in bodies such as cardiac peacemaker, aneurysm clips, and ear implant. All the subjects were recruited from Chiang Mai Thanyarak Hospital, Chiang Mai Province, and within the community areas.

Table 1 Demographics information of the subjects.

	Healthy Control (HCs) (n=14)	Methamphetamine Abuser (MAs) (n=10)
Mean age (SD)	22.701(3.361)	24.50(4.696)
Intracranial volume (ICV)		
- Max	1718.443 cm^2	1505.800 cm^2
- Min	1383.234 cm^2	1301.250 cm^2
- Mean	1507.501 cm^2	1415.246 cm^2
Gender (females)	14(0)	9(1)
Education (grade)	Secondary school	Secondary school
Race (%asian)	100%	100%

Image acquisition

Philips Achieva 1.5 tesla, Netherlands. A quadrature channel head coil was employed. Axial Images were acquired and covered from the vertex of the skull to the lowest part of the cerebellum. Pulse sequence used was 3D FFE with the scanning parameters similar to that of British Imaging Research Network (BIRN) as shown in Table 2.

Table 2 Magnetic resonance imaging acquisition parameters.

Parameters	
Pulse sequence	Three-Dimensional Fast Field Echo (3D FFE)
Plane	Axial
Repetition Time (TR)	20 ms.
Echo Time (TE)	4.6 ms.
Flip angle	30°
Voxel size	0.94 x 0.94 x 1 mm^3
Field of view (FOV)	240 x 240 mm^2

Image analysis with FreeSurfer

Axial T1-weighted images were analyzed using FreeSurfer software version 5.3 developed by Martinos Center for Biomedical Imaging (http://surfer.nmr.mgh.harvard.edu/). Analyzed data was used to measure brain volume in each region. Working flow of the software was shown in Figure 1 starting with T1W file in DICOM format which was converted to FreeSurfer file format following with motion correction along with affine transformation. The images were then normalized into the Talairach space along with image intensity inhomogeneity correction. Skull stripping was done automatically by FreeSurfer and manually removed

for the remaining. Finally, segmentation was done based on image intensity histogram and FreeSurfer atlas.[21-24] Volume and area of each brain region was obtained for the next statistical analysis.

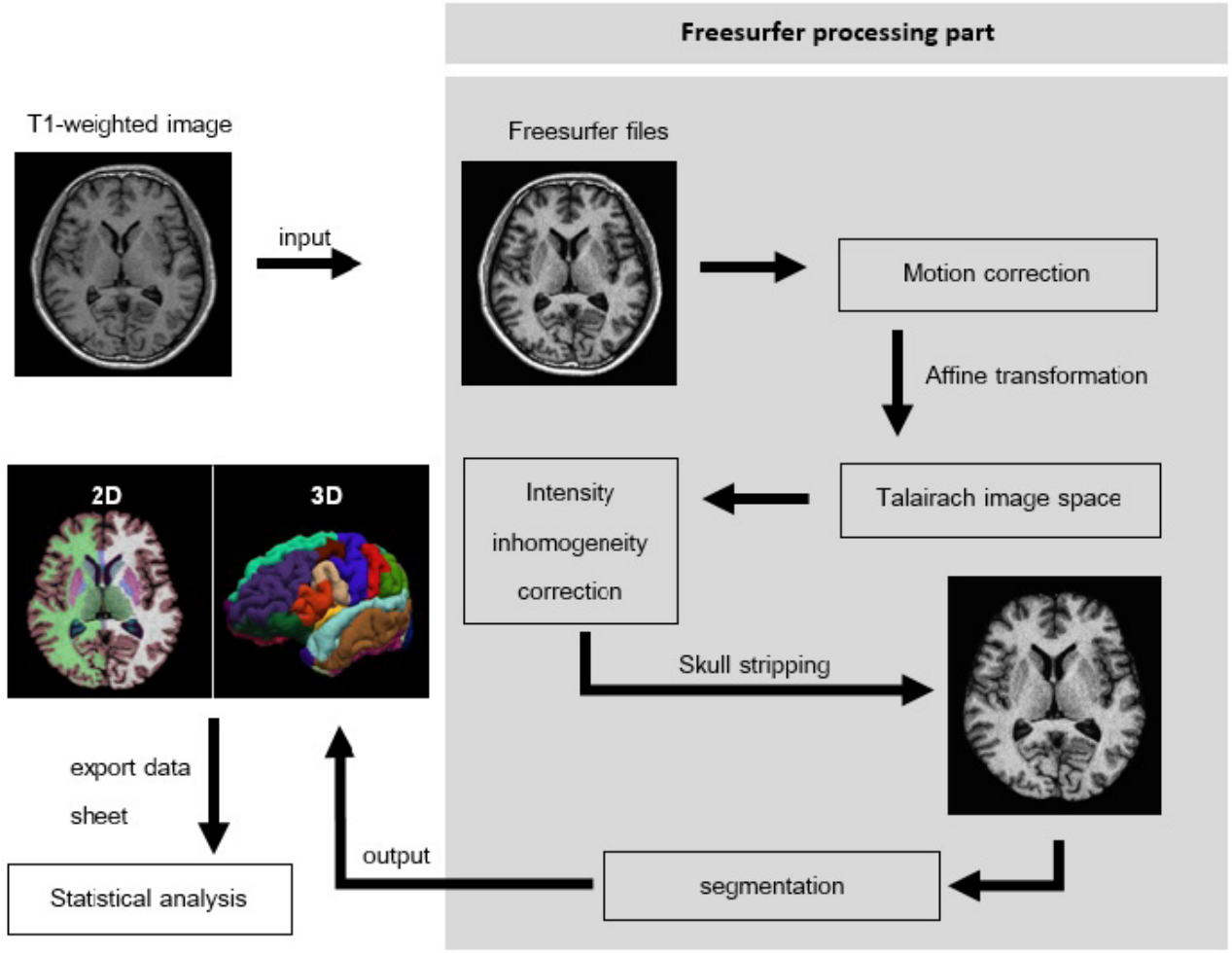

Figure 1. *Freesurfer workflow in diagram. Fist the raw data (T1-weighted image) were automated motion correction and transform to Talairach image space. Then, Freesurfer will correct a non-uniform intensity and performed skull stripping followed by segmentation for separating brain to each region. Finally, the volume data was exported in a table format for statistical analysis.*

Data analysis

In Absolute volume method, the volume in each brain region was obtained from the calculation of the FreeSurfer software. For the brain volume normalized with intracranial volume method, the absolute volume of each brain region was divided by an individual's intracranial volume.

Different parts of brain volumes were compared between groups using independent student t-test while the correlation between the 2 analysis methods and the intracranial volume were analyzed with Pearson correlation upon a confidence level of 95%, $p<0.05$.

Results

Comparison of results from the 2 analysis methods

Using the absolute volume analysis, 8 areas of brain volume from the HCs were significantly higher than MA group ($p<0.05$): at right (Rt) and left (Lt) frontal gray matters, Rt and Lt temporal gray matter, Rt parietal gray matter, Rt occipital gray matter, Lt insular gray matter and Lt insular white matter. All the 8 areas are shown in Figure 2a, mean±SD are shown in Table 3.

However, analyzing from ICV method showed 2 areas of different cortical volume between the two study group; Rt and Lt temporal gray matter (that exhibits larger volume for HC than MA) with a statistical significance ($p<0.05$). ICV approach also showed significant higher Rt insular white matter volume in MA compared to HC with $p<0.05$. All of these 3 areas are shown in Figure 2b and mean±SD are shown in Table 4.

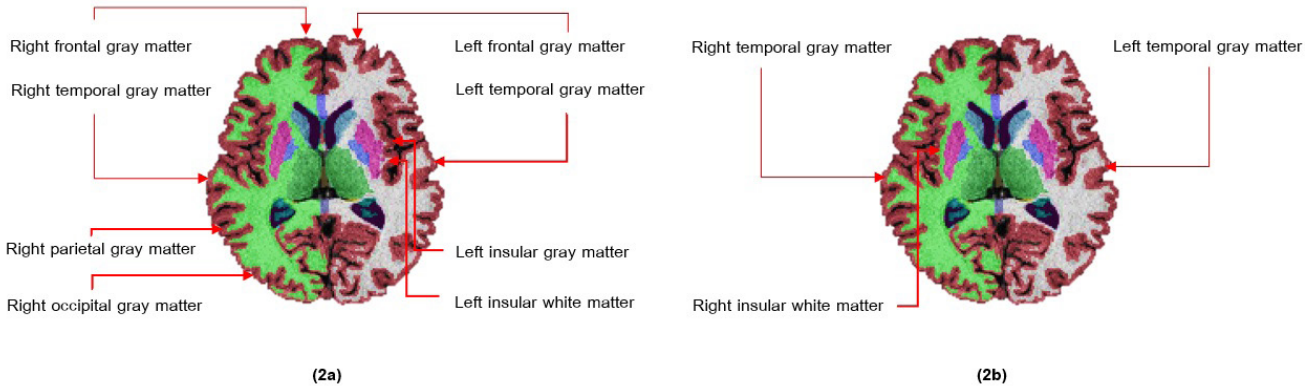

Figure 2. *Significant different regions on brain between HCs and MAs with absolute brain volume analysis (2a) and brain volume normalized with intracranial volume analysis (2b)*

Table 3 Absolute volume difference between groups of HCs and MAs on each brain region.

Regional brain volume (mm^3)	HCs (n=14)		MAs (n=10)		*p*-value
	Mean	SD	Mean	SD	
Left frontal gray matter	89587.000	7572.766	83398.800	5876.683	0.042*
Right frontal gray matter	87776.210	9071.645	80653.200	6703.253	0.047*
Left temporal gray matter	53688.290	4225.039	47538.400	2601.378	0.001*
Right temporal gray matter	51979.430	3913.410	45731.700	3797.214	0.001*
Right parietal gray matter	64302.210	6574.633	58209.400	2674.766	0.006*
Right occipital gray matter	24628.290	2567.025	22289.700	1715.632	0.020*
Left insular gray matter	7100.360	537.894	6556.300	459.313	0.017*
Left insular white matter	8831.471	694.384	8172.050	601.599	0.024*
Right insular white matter	8988.014	698.344	9057.710	607.925	0.802

** p-value <0.05 was statistically significant difference.*

Table 4 Show normalized volume difference between groups of HCs and MAs on each brain region.

Regional brain volume (mm^3)	HCs (n=14)		MAs (n=10)		*p*-value
	Mean	SD	Mean	SD	
Left temporal gray matter	0.0356	0.0016	0.0336	0.0020	0.013*
Right temporal gray matter	0.0345	0.0016	0.0323	0.0024	0.013*
Right insular white matter	0.0060	0.0004	0.0064	0.0005	0.026*

** p<0.05 was statistically significant difference.*

Correlation between two analysis methods between healthy controls (HC) and methamphetamine abusers (MA)

Figure 3 demonstrates Pearson correlation between the ICV and the two methods for volume determination in HC group. It could be observed that the analysis method from the absolute volume gave positive correlation with a statistical significance (p<0.05) in almost every area except the Lt and Rt basal ganglia, Lt parietal gray matter, Lt and Rt occipital gray matter, Lt and Rt occipital white matter and Lt insular white matter. The analysis method using the brain volume normalized with ICV gave negative correlation with a statistical significance (p<0.05) in only Lt and Rt basal ganglia areas.

The correlation of the 2 analysis methods with the ICV in the MAs group was shown in Figure 4. The analysis method from absolute volume provided positive correlation with a statistical significance (p<0.05) in Lt frontal gray matter and Rt parietal gray matter areas and with the analysis of brain volume normalized with ICV, no correlation with a statistical significance in all areas was found.

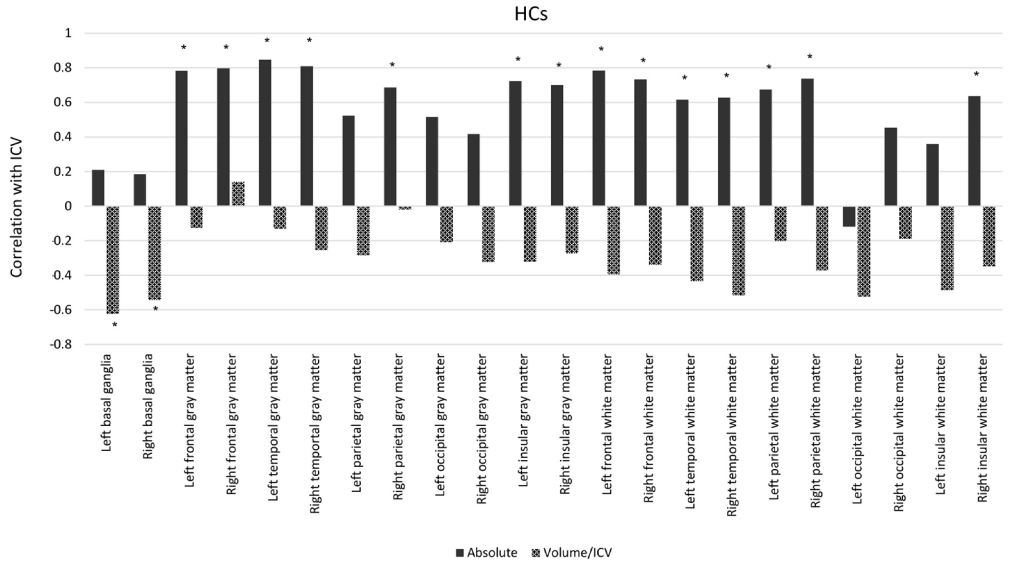

Figure 3. *Correlation pattern for each regional of brain volume in HCs using different methods. *p<0.05 was considered to indicate statistically significant correlation*

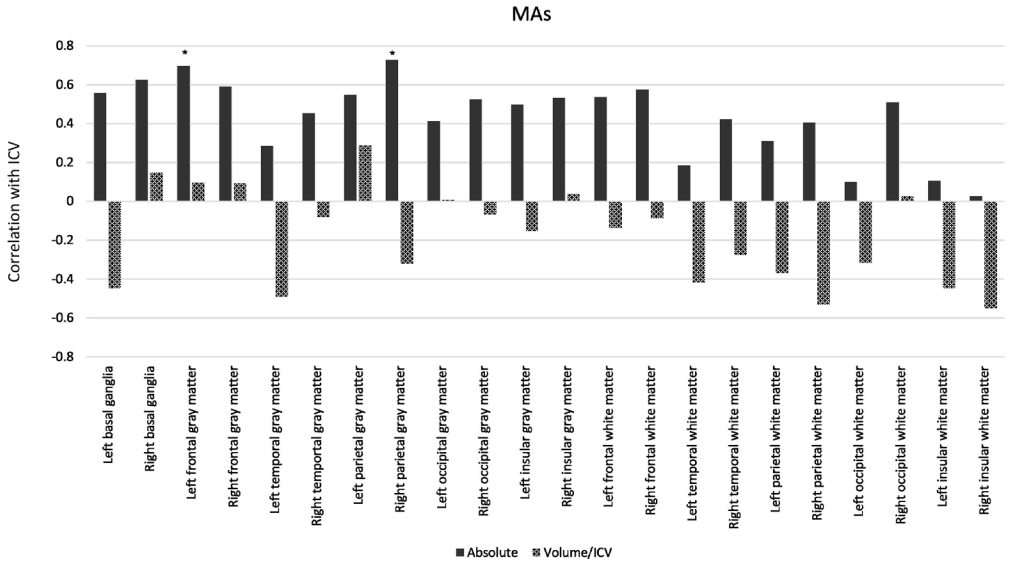

Figure 4. *Correlation pattern for each regional of brain volume in MAs using different methods. *p<0.05 was considered to indicate statistically significant correlation*

Discussion

Our study showed that the gray matter volume of both sides of the temporal lobes were lower and the white matter volume of right insular was higher in MAs compared with HCs regardless of analysis methods (Table 3 and Table 4). Brain volume in MAs group that lower than that of HCs group is most likely appeared in gray matter which was consistent with previous studies.[18, 19]

The significant larger of brain volume in Rt insular white matter in MAs was explained by Thompson[19] that it was probably from proliferation of glial (astrocyte) in MAs.

When compared with ICV, our study demonstrated positive correlation of absolute area volume in most areas in HCs. This implied larger ICV giving larger area volumes and agreed with the previous studies.[25-27] Correlation found in the 2 analysis methods with the ICV in our study agrees with the report of Voevodskaya[28] that conducted a study with 406 volunteers. The different correlation result in some

areas in our study from the previous study may be due to lower numbers of subjects in our study.

It was found in MAs group that the absolute volume gave positive correlation to the ICV in only two areas, at Lt frontal gray matter and RT parietal gray matter (Figure 4). Normalized volume did not show significant correlation with the ICV. This pattern of correlation in MAs group was different from that of the HCs group. This may be caused by the effect of methamphetamine to most of the brain volume. [15, 29]

Correlation between the two analysis methods and ICV performed in HCs and MAs groups is not the evidence to conclude for which method was superior to others. However, different patterns of the correlation found in both groups may be a trigger for brain change due to methamphetamine use in MAs. Limitation of this study is a small sample size and most of volunteers were male. Larger sample size is needed to confirm our observation. Moreover, results from other techniques such as magnetic resonance spectroscopy (MRS) and neuropsychological tests (NP test) should be applied to support for the results.

Conclusion

The study shows that using the analysis with absolute volume and the analysis with brain volume normalized with ICV provided similar results. However, the difference is numbers of significant different areas between study groups. To prove for the reliable analysis method, other techniques such as magnetic resonance spectroscopy (MRS) and neuropsychological (NP) test may be needed. In addition, ICV is an internal reference for normalization, and may not be appropriate for diseases with brain atrophy.

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References

[1] Zhou Y, Kierans A, Kenul D, Ge Y, Rath J, Reaume J, et al. Mild traumatic brain injury: longitudinal regional brain volume changes. Radiology. 2013; 267(3): 880-90.

[2] MacKenzie JD, Siddiqi F, Babb JS, Bagley LJ, Mannon LJ, Sinson GP, et al. Brain atrophy in mild or moderate traumatic brain injury: a longitudinal quantitative analysis. AJNR Am J Neuroradiol. 2002; 23(9): 1509-15.

[3] Prasad KM, Shirts BH, Yolken RH, Keshavan MS, Nimgaonkar VL. Brain morphological changes associated with exposure to HSV1 in first-episode schizophrenia. Mol Psychiatry. 2007; 12(1): 105-13, 1.

[4] Ernst T, Chang L, Jovicich J, Ames N, Arnold S. Abnormal brain activation on functional MRI in cognitively asymptomatic HIV patients. Neurology. 2002; 59(9): 1343-9.

[5] Robbins TW, Ersche KD, Everitt BJ. Drug addiction and the memory systems of the brain. Ann N Y Acad Sci. 2008; 1141: 1-21.

[6] Filippi M, Rovaris M, Comi G. Neurodegeneration in Multiple Sclerosis. Milan: Springer; 2007.

[7] Nakama H, Chang L, Fein G, Shimotsu R, Jiang CS, Ernst T. Methamphetamine users show greater than normal age-related cortical gray matter loss. Addiction. 2011; 106(8): 1474-83.

[8] Jan RK, Lin JC, Miles SW, Kydd RR, Russell BR. Striatal volume increases in active methamphetamine-dependent individuals and correlation with cognitive performance. Brain Sci. 2012; 2(4): 553-72.

[9] Keller SS, Gerdes JS, Mohammadi S, Kellinghaus C, Kugel H, Deppe K, et al. Volume estimation of the thalamus using freesurfer and stereology: consistency between methods. Neuroinformatics. 2012; 10(4): 341-50.

[10] Keller SS, Roberts N. Measurement of brain volume using MRI: software, techniques, choices and prerequisites. J Anthropol Sci. 2009; 87: 127-51.

[11] Im K, Lee JM, Lyttelton O, Kim SH, Evans AC, Kim SI. Brain size and cortical structure in the adult human brain. Cereb Cortex. 2008; 18(9): 2181-91.

[12] Dewey J, Hana G, Russell T, Price J, McCaffrey D, Harezlak J, et al. Reliability and validity of MRI-based automated volumetry software relative to auto-assisted manual measurement of subcortical structures in HIV-infected patients from a multisite study. Neuroimage. 2010; 51(4): 1334-44.

[13] Nordenskjold R, Malmberg F, Larsson EM, Simmons A, Brooks SJ, Lind L, et al. Intracranial volume estimated with commonly used methods could introduce bias in studies including brain volume measurements. Neuroimage. 2013; 83: 355-60.

[14] Ge Y, Grossman RI, Babb JS, Rabin ML, Mannon LJ, Kolson DL. Age-related total gray matter and white matter changes in normal adult brain. Part I: volumetric MR imaging analysis. AJNR Am J Neuroradiol. 2002; 23(8): 1327-33.

[15] Jernigan TL, Gamst AC, Archibald SL, Fennema-Notestine C, Mindt MR, Marcotte TD, et al. Effects of methamphetamine dependence and HIV infection on cerebral morphology. Am J Psychiatry. 2005; 162(8): 1461-72.

[16] Chang L, Cloak C, Patterson K, Grob C, Miller EN, Ernst T. Enlarged striatum in abstinent methamphetamine abusers: a possible compensatory response. Biol Psychiatry. 2005; 57(9): 967-74.

[17] Chang L, Smith LM, LoPresti C, Yonekura ML, Kuo J, Walot I, et al. Smaller subcortical volumes and cognitive deficits in children with prenatal methamphetamine exposure. Psychiatry Res. 2004; 132(2): 95-106.

[18] Bartzokis G, Beckson M, Lu PH, Edwards N, Rapoport R, Wiseman E, et al. Age-related brain volume reductions in amphetamine and cocaine addicts and normal controls: implications for addiction research. Psychiatry Res. 2000; 98(2): 93-102.

[19] Thompson PM, Hayashi KM, Simon SL, Geaga JA, Hong MS, Sui Y, et al. Structural abnormalities in the brains of human subjects who use methamphetamine. J Neurosci. 2004; 24(26): 6028-36.

[20] Orikabe L, Yamasue H, Inoue H, Takayanagi Y, Mozue Y, Sudo Y, et al. Reduced amygdala and hippocampal volumes in patients with methamphetamine psychosis. Schizophr Res. 2011; 132(2-3): 183-9.

[21] Jovicich J, Czanner S, Greve D, Haley E, van der Kouwe A, Gollub R, et al. Reliability in multi-site structural MRI studies: effects of gradient non-linearity correction on phantom and human data. Neuroimage. 2006; 30(2): 436-43.

[22] Takao H, Abe O, Ohtomo K. Computational analysis of cerebral cortex. Neuroradiology. 2010; 52(8): 691-8.

[23] Sled JG, Zijdenbos AP, Evans AC. A nonparametric method for automatic correction of intensity nonuniformity in MRI data. IEEE Trans Med Imaging. 1998; 17(1): 87-97.

[24] Buckner RL, Head D, Parker J, Fotenos AF, Marcus D, Morris JC, et al. A unified approach for morphometric and functional data analysis in young, old, and demented adults using automated atlas-based head size normalization: reliability and validation against manual measurement of total intracranial volume. Neuroimage. 2004; 23(2): 724-38.

[25] Barnes J, Ridgway GR, Bartlett J, Henley SM, Lehmann M, Hobbs N, et al. Head size, age and gender adjustment in MRI studies: a necessary nuisance? Neuroimage. 2010; 53(4): 1244-55.

[26] Pell GS, Briellmann RS, Chan CH, Pardoe H, Abbott DF, Jackson GD. Selection of the control group for VBM analysis: influence of covariates, matching and sample size. Neuroimage. 2008; 41(4): 1324-35.

[27] Pfefferbaum A, Sullivan EV, Swan GE, Carmelli D. Brain structure in men remains highly heritable in the seventh and eighth decades of life. Neurobiol Aging. 2000; 21(1): 63-74.

[28] Voevodskaya O, Simmons A, Nordenskjold R, Kullberg J, Ahlstrom H, Lind L, et al. The effects of intracranial volume adjustment approaches on multiple regional MRI volumes in healthy aging and Alzheimer's disease. Front Aging Neurosci. 2014; 6: 264.

[29] Salo R, Fassbender C. Structural, functional and spectroscopic MRI studies of methamphetamine addiction. Curr Top Behav Neurosci. 2012; 11: 321-64.

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Rapid alternative methods for erythrocyte sedimentation rate measurement

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ABSTRACT

Background: Erythrocyte sedimentation rate (ESR) measurement is the most widely used in laboratory test to assess an inflammation or acute phase response.

Objectives: To introduce two rapid alternative methods for ESR measurement.

Materials and methods: ESR was performed in 449 blood samples using Westergren, Wintrobe with centrifugation (Win-C) and capillary tube (Cap-C) with centrifugation methods.

Results: Win-C and Cap-C methods were significantly correlated with Westergren method by using Spearman rank correlation. Both Win-C and Cap-C methods were demonstrated the good % of sensitivity, % of specificity, % of positive predictive value, % of negative predictive value and cut-off point by using the receiver operating characteristic (ROC) curves analysis.

Conclusion: Both methods demonstrated acceptable agreement with the conventional ESR measurements and appear to be a faster, easier and safety.

Introduction

Erythrocyte sedimentation rate (ESR) measurement is one of the most widely used laboratory test to assess an inflammation or acute phase response. The ESR phenomenon is the combination of aggrega¬tion, precipitation, and packing of erythrocytes. These are included with fibrinogen, immuno¬globulins, amount and shape of erythrocytes facilitation.[1] Although it is not considered a specific diagnostic test but useful for the monitoring and follow-up for certain groups of patients, such as rheumatoid arthritis, temporal arthritis, polymyalgia rheumatica, and Hodgkin' s disease. Besides it is used in prognostic value in acute coronary syndrome and stroke. The Westergren method has been used and proposed as a conventional ESR measurement by several agencies.[2] In general, anticoagulant and diluted blood samples were used in the Westergren tube, which determines the sedimentation of erythrocytes after 1 hour at room temperature in a strictly vertical by the mounted tube of defined length and bore size. The distance of the erythrocytes fallen was measured in millimeters (mm) as ESR result. This manual method has many processes in the pre-analytical and analytical step which had many factors that can affect ESR results and high biohazard risk. Thus, many research studies have tried to look the better suitable and speed methods for the modern clinical laboratories.

Nevertheless, a number of modifications of the Westergren method are now available including smaller sample volume, less manual manipulations, shorter testing times, automation, and interfacing with the laboratory information system. We aim to demonstrate and propose our two rapid alternative ESR measurement methods.

Materials and methods

Blood samples

A total of 449 blood samples from the participants in Cardiovascular and Diabetes Prevention in Elderly Project were used to test ESR examination during October 2012-December 2014. We did not have any specific inclusion and exclusion criteria. These blood samples were from 204 men (77 with aged <50 yrs, 127 with aged ≥50 yrs) and 245 women (121 with aged <50 yrs, 124 with aged ≥50 yrs). All blood samples were collected by sterile venipuncture of

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K3-EDTA vacuum tubes (Vacutainer, Becton Dickinson, UK) and carried out all ESR measurements within 4 hr. The study protocol was approved by the Ethics Committee of the Naresuan University.

Erythrocyte sedimentation rate measurements

(i) Westergren Method:

In the beginning, we performed the conventional ESR measurement by using Westergren method in our laboratory, 3 by using Westergren tube, a 200-millimeters (mm) long vertically graduated glass tubes and 2.55 mm in diameter with open at each end (Curtin Matheson Scientific, Houston, TX). Briefly, 1 mL of K3-EDTA blood samples were diluted to 4:1 with 0.9% normal saline solution (250 µL) and manual gently inversion mixed and transferred into the Westergren tube to reach 200 mm mark and vertically into supporting stand at room temperature. ESR result, red blood cell sedimentation was read and recorded as the mm mark after 1 hr (mm/hour).

(ii). Wintrobe with centrifugation (Win-C) method:

This method used the Wintrobe tube and centrifugation force for ESR measurement. Briefly, K3-EDTA undiluted blood sample was filled into the Wintrobe tube (with 0 to 100 mm mark) and centrifuged at 500 rpm for 3 min at room temperature. After that the distance of the fallen RBC and plasma front was immediately measured and recorded (mm mark on the Wintrobe tube).

(iii). Capillary tube with centrifugation (Cap-C) Method:

Briefly, K3-EDTA undiluted blood samples were gently mixed and drawn into the micro-capillary tubes (glass plain blue tip) and sealed with the sealing clay at the end of the tube. Then, capillary tubes were centrifuged at 500 rpm for 3 min at room temperature. After that the distance of the fallen RBC and plasma front was immediately measured and recorded with the ruler as millimeter.

Both Win-C and Cap-C methods were termed the sedimentation index according to the study of Alexy et al.[4] that using centrifugation force as the special hydrodynamic force for increased the sedimentation rate. Westergren methods, Win-C and Cap-C methods were performed by the same time and same researcher for all blood samples.

Statistical analysis

All results were demonstrated as median and interquartile. Spearman rank correlation was used to test the correlation of Win-C and Cap-C with Westergren methods. The receiver operating characteristic (ROC) curve were analyzed the comparison results of these 3 methods. A ROC curve is a plot between sensitivity (Y-axis) versus false positive (X-axis), obtained for different cut-off points. Areas under the curve (AUC) of the ROC curves and 95% confidence intervals (CI) were evaluated as the accuracy of diagnostic measurement. A discriminate analysis was performed to identify these parameters that provided the best differentiation between Win-C and Cap-C methods. Greater AUC of the ROC curve indicated better markers of the study. In general, an AUC of a ROC of <0.5 suggests no discrimination, whereas a maximal AUC of a ROC of 1 suggests outstanding discrimination.[5] The $p<0.05$ (two tailed) were considered as significant.

Results

The results of ESR measurement with Westergren method were categorized to 202 (44.7%) normal-ESR and 247 (55.3%) Elevated-ESR as demonstrated in Table 1 with reference ranges of the method. Results of ESR measurement by both Win-C and Cap-C methods were demonstrated in Table 2. The results of ESR measurement by Win-C and Cap-C methods were significantly correlated with Westergren method (r=0.929, $p<0.001$ and r=0.859, $p<0.001$), respectively. Both Win-C and Cap-C methods were demonstrated discrimination as AUC>0.5 and good % sensitivity, % specificity, % positive predictive value (PPV) and % negative predictive value (NPV). The AUCs, optimal cut-off point, % sensitivity, % specificity, %PPV and %NPV of Win-C and Cap-C methods were demonstrated in Table 3.

Table 1 Demonstration the results of 449 ESR measurements by using Westergren method.

ESR results	Samples (%)
Normal-ESR	202 (44.7%)
Elevated-ESR	247 (55.3%)
* Normal reference range of men age	
<50 yrs.	0-15 mm/hr
≥50 yrs.	0-20 mm/hr
*Normal reference range of women age	
<50 yrs.	0-20 mm/hr
≥50 yrs.	0-30 mm/hr

Nor-ESR: normal ESR results, Ab-ESR: higher ESR results

Table 2 Demonstration of median, interquartile, minimum and maximum results of 449 ESR measurements by each method.

Methods	ESR results		
	Median (interquartile)	Minimum	Maximum
Westergren (mm/hr)	24.0 (12.0–50.5)	1.0	155.0
Win-C (mm)	36.2 (28.0–49.0)	2.0	77.0
Cap-C (mm)	46.7(40.0–54.3)	4.0	73.0

Table 3 Demonstration of the cut-off points, AUC, %sensitivity, %specificity, %PPV and % NPV of the ESR measurements with Win-C and Cap-C methods.

Methods	Group (sex, age)	n	cut-off (mm)	AUC	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Win-C	Men <50 yrs.	77	0-28	0.886	87.8	86.1	87.9	84.1
	Men ≥50 yrs.	127	0-37	0.918	90.7	82.1	94.4	70.9
	Women <50 yrs.	121	0-35	0.879	85.9	72.0	80.0	85.9
	Women ≥50 yrs.	124	0-40	0.872	88.8	77.8	79.1	100.0
Cap-C	Men <50 yrs.	77	0-40	0.848	80.5	71.2	79.1	77.8
	Men ≥50 yrs.	127	0-47	0.863	80.1	78.6	88.0	65.4
	Women <50 yrs.	121	0-45	0.829	79.1	78.0	79.3	84.9
	Women ≥50 yrs.	124	0-50	0.825	80.5	79.4	64.9	95.8

AUC: area under the curve, PPV: positive predictive value, NPV: negative predictive value.

Discussion

ESR is widely used as a clinical guide to aid in the diagnosis, follow up and management in rheumatoid arthritis, temporal arthritis, osteomyelitis and clinical significant in stroke, coronary artery disease, prostate cancer, tuberculosis and Hodgkin disease.[6-8] Generally, ESR measurement may affect by age, race, and blood storage which importance in the clinical conditions. Sedimentation is increased in anemia (megaloblastic higher than iron-deficiency anemia) while polycythemia inhibits sedimentation. Red cells morphology (spherocytosis, acantocytosis, and sickle cells) are also affected sedimentation rate,[8,9] and combination with hemoglobin, lipid concentrations, plasma proteins ratio and plasma pH.[10]

Many new methods for ESR measurement have been developed and proposed for clinical laboratories.[4, 6, 11] These new methods are shorter time testing and guarantee safety both in automated and manual systems. Both Win-C and Cap-C methods were performed with Wintrobe tube and micro-capillary tubes which required less blood sample (suitable for pediatric use), advantages of speed, safety and uniform specimen handling. Centrifugation force in both Win-C and Cap-C methods was used as the special hydrodynamic force accelerated the erythrocytes sedimentation. Win-C and Cap-C methods were suitable for the high workload clinical laboratories and emergency laboratories. Consideration in the limitations of this present study, no strict inclusion and exclusion criteria were implemented. All results of ESR measurements were carried out in one time and we do not estimate the reproducibility of the results.

Conclusion

Our rapid alternative ESR measurement, Win-C and Cap-C methods appear to be the faster, reliable and safety. Win-C may suitable for routine and emergency laboratory while Cap-C may suitable for Pediatric Division with a smaller sample volume.

References

[1] Vennapusa B, De-La-Cruz L, Shah H, Michalski V, Zhang QY. Erythrocyte sedimentation rate (ESR) measured by the Streck ESR-Auto Plus is higher than with the Sediplast Westergren method: a validation study. Am J Clin Pathol 2011; 135: 386-90.

[2] Jou J, Lewis S, Briggs C, Lee SH, De La Salle B, McFadden S, et al. ICSH review of the measurement of the erythrocyte sedimentation rate. Int J Lab Hematol 2011; 33: 125-32.

[3] Westergren A. The technique of the red cell sedimentation reaction. Am Rev Tuberc 1926; 14: 94-101.

[4] Alexy T, Pais E, Meiselman HJ. A rapid method to estimated Westergren sedimentation rates. Rev Sci Instrum 2009; 80: 096102.

[5] Swets JA. Measuring the accuracy of diagnostic systems. Science 1988; 240: 1285-93.

[6] Arikan S, Akalin N. Comparison of the erythrocyte sedimentation rate measured by the micro test 1 sedimentation analyzer and the conventional Westergren method; Ann Saudi Med 2007; 27: 362-5.

[7] Saadeh C. The erythrocyte sedimentation rate: old and new clinical applications; South Med J. 1998; 91: 220-25.

[8] Vennapusa B, DeLaCruz L, Shah H, Michalski V, Zhang QY. Erythrocyte sedimentation rate (ESR) measured by the streck ESR-auto plus is higher than with the sediplast Westergren method. Am J Clin Pathol 2011; 135: 386-90.

[9] ICSH Expert Panel. Guidelines on the selection of laboratory tests for monitoring the acute phase response. J Clin Pathol 1998; 41: 1203-12.

[10] Pawlotsky Y, Goasguen J, Guggenbuhl P, Veillard E, Jard C, Pouchard M, et al. Sigma ESR: an erythrocyte sedimentation rate adjusted for the hematocrit and hemoglobin concentration. Am J Clin Pathol 2004; 122(5):802-10.

[11] Hashemi R, Majidi A, Motamed H, Amini A, F Najari, Tabatabaey A. Erythrocyte sedimentation rate measurement using as a rapid alternative to the Westergren method. Emergency 2015; 3(2):50-3.

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Feasibility of high resolution melting curve analysis for rapid serotyping of *Salmonella* from hospitalised patients

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ABSTRACT

Background: Serum agglutination test is the gold standard phenotyping method widely used for *Salmonella enterica* characterisation.This conventional method is limited by its complicated and time-consuming procedures. High resolution melting curve (HRM) analysis is introduced as a rapid and labour-saving method.

Objectives: To compare the results of conventional serum agglutination and quantitative PCR-HRM analysis to assess the feasibility of this alternative approach for *Salmonella* serotyping.

Materials and methods: *Salmonella* strains from 38 human-originating samples were serotyped using the conventional serum agglutination method and HRM analysis.

Results: The conventional serum agglutination assay detected 14 serotypes, while the HRM analysis identified 10 HRM profiles. There was a correlation between most of the serotyping results obtained by the two methods. Nine of the HRM profiles were unique to a single serotype, of each. One exception was HRM_3. Many of the indistinct curves that were grouped in this HRM pattern belonged to five *Salmonella* serotypes, including Weltevreden, Corvallis, Derby, Kedougou and Kentucky.

Conclusion: It is difficult to determine all *Salmonella* serotypes by HRM analysis. However, this method can be used as an alternative to the conventional serum agglutination assay for rapid and labour-saving serotyping.

Introduction

The foodborne pathogen *Salmonella enterica* causes human gastroenteritis and is considered a world-wide public health problem.[1-3] Contaminated, raw or poorly prepared foods, particularly those originating fresh from farms, are involved in most cases.[4] Characterization of the organism is an essential part of epidemiological surveillance and outbreak investigation. Serotyping is the most common and simple characterization method in use.[5,6] More than 2,500 serotypes of *S. enterica* have been identified,[7] and the disease appears to be spreading. For example, *Salmonella* Typhimurium and Rissen are the main serotypes in pigs,[1] while *S.* Enteritidis and *S.* Dublin are the main serotypes in poultry and dairy products, respectively.[4,8,9] Seroprevalence data could be used as baseline information to manage and control the disease.

Conventionally, the gold standard serotyping method is based on the Kauffmann-White classification scheme, which involves combining the cell wall O and H antigenic determinations obtained from the agglutination test with matching antiserum.[5,10] However, this method is time

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consuming and requires technical skills. The ISO 2002[11] recommends that samples be cultured for at least a week before agglutination is performed. Large numbers of specific sera are also required. To overcome these limitations, high resolution melting curve (HRM) analysis is introduced.[2,12] HRM is a post-quantitative PCR method used to identify variations in gene polymorphism. Serotype recognition based on the differences in PCR melting curves is generated by real-time PCR,[13] providing a fast workflow and high throughputs of data.[2,12] For *Salmonella* serotyping, polymorphisms of the *fljB*, *gyrB* and *ycfQ* genes have been targeted.[13]

The objective of this study was to compare *Salmonella* serotyping obtained from quantitative PCR-HRM analysis and conventional serum agglutination. The results will demonstrate the feasibility of an alternative serotype screening method.

Materials and methods

Salmonella strains and serum agglutination tests

A total of 38 *Salmonella* strains were isolated from stool samples of hospitalised patients from the Phayao Ram Hospital, Phayao Province, Thailand from March 2015 to June 2016. The study was conducted under ethical approval (No. 57 02 04 0020) granted by the Ethics Committee of the University of Phayao. Stool samples were initially identified following the ISO protocol.[13] Then, the presumptive *Salmonella* colonies were confirmed with biochemical and serum agglutination tests at the WHO National *Salmonella* and *Shigella* Center, the National Institute of Health, Ministry of Public Health, Nonthaburi Province, Thailand.

HRM analysis serotyping

For each sample, DNA extraction was completed following the method previously described by Cheng and Jiang.[14] In brief, a 1 mL aliquot of an overnight culture was centrifuged at 8,000 g for 2 min. The pellet was washed twice with 400 µL of STE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA and 100 mM NaCl) and re-suspended in 200 µL of TE buffer (10 mM Tris HCl, pH 8.0, and 1 mM EDTA). Then, 100 µL of Tris HCl (pH 8.0) saturated phenol solution was added to the suspension, which was subsequently mixed and cen-trifuged at 13,000 g for 5 minutes at 4°C. A 160 µL aliquot of the upper aqueous layer was mixed with 40 µL of TE buffer and 100 µL of chloroform and centrifuged for 5 minutes at 13,000 g at 4°C. The upper aqueous solution was extracted with chloroform, and a 150 µL aliquot was kept at -20°C until used.

Multiplex HRM analysis was performed using a combination of primers to amplify the following genes: *fljB* (5' GTGAAAGATACAGCAGTAACAACG 3' and reverse 5' GCGTCTATTATTGTTCATGAAAC 3'), *gyrB* (5' AAACGCCGATCCACCCGA 3' and reverse 5' GAAGGCACGCCGCTACT 3') and *ycfQ* (5' GCCTACTCTCTATGCGGAATTCAC 3' and reverse 5' GCGGAGGAGCGCGCTATAG 3'). Sizes of the targeted genes were 170, 171 and 241 bps, respectively. Real-time PCR was conducted using a BIO-RAD CFX96TM real-time system (Bio-Rad, Hercules, CA, USA). The HRM master mixture (10 µL) contained 1 µL of DNA, 0.1 pmol of *gyrB* primer pairs, 0.075 pmol of *fljB* primer pairs, 0.075 pmol of *ycfQ* primer pairs and 2 µL of HOT FIRE Pol EvaGreen no ROX mix (Solis Biodye, Tartu, Estonia). The thermocycling conditions were programmed according to the protocol of Zeinzinge et al.[13] The HRM profiles were generated based on the temperature shift of the melting curves, using *S*. Bareilly as a baseline reference.

Data analysis

Simpson's diversity index was used to estimate the discriminatory power of the two serotyping methods. In the index range, a minimum value of zero represents no power to discriminate any type in the sample population.[15] Maximum value of one denotes the ability to distinguish all samples in a population. The predictive values of the HRM analysis and the conventional serum agglutination test were evaluated using the Wallace coefficient. This coefficient indicates the probability that a pair of strains assigned to the same type by one typing method is also typed as identical by another method. In the range of 0-1, the maximum value of 1 indicates 100% predictability of one method to another.[16]

Results

Of the 38 *Salmonella* strains tested, fourteen serotypes were detected by serum agglutination, with the most frequency of *Salmonella* 4, 5, 12 : i : - (12 strains; 32%), followed by *S*. Stanley (9 strains; 23%) and *S*. Weltevreden (5 strains; 13%), respectively (Figure 1). While, the HRM analysis created nine unique HRM patterns corresponding to *S*. 4,5,12:i-, *S*. Stanley, *S*. Newport, *S*. Montevideo, *S*. Enteritidis, *S*. Give, *S*. Yoruba, *S*. Bareilly, *S*. 9,12:-:1,5. However, HRM_3 demonstrated several un-specified curves, which were regarded in 5 strains of *S*. Weltevreden and a strain of each belonging *S*. Derby, *S*. Corvallis, *S*. Kentucky and *S*. Kedougou (Figure 2).

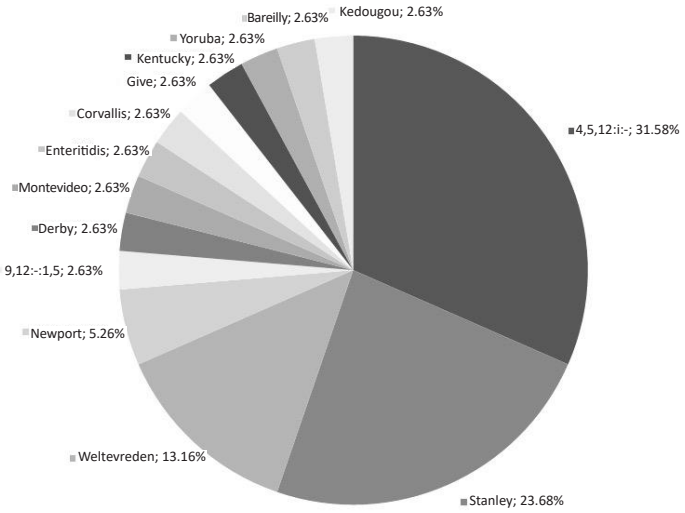

Figure 1. *Sero-distribution of Salmonella strains isolated from hospitalised patients in Phayao Ram Hospital during March 2015 to June 2016 (serum agglutination test)*

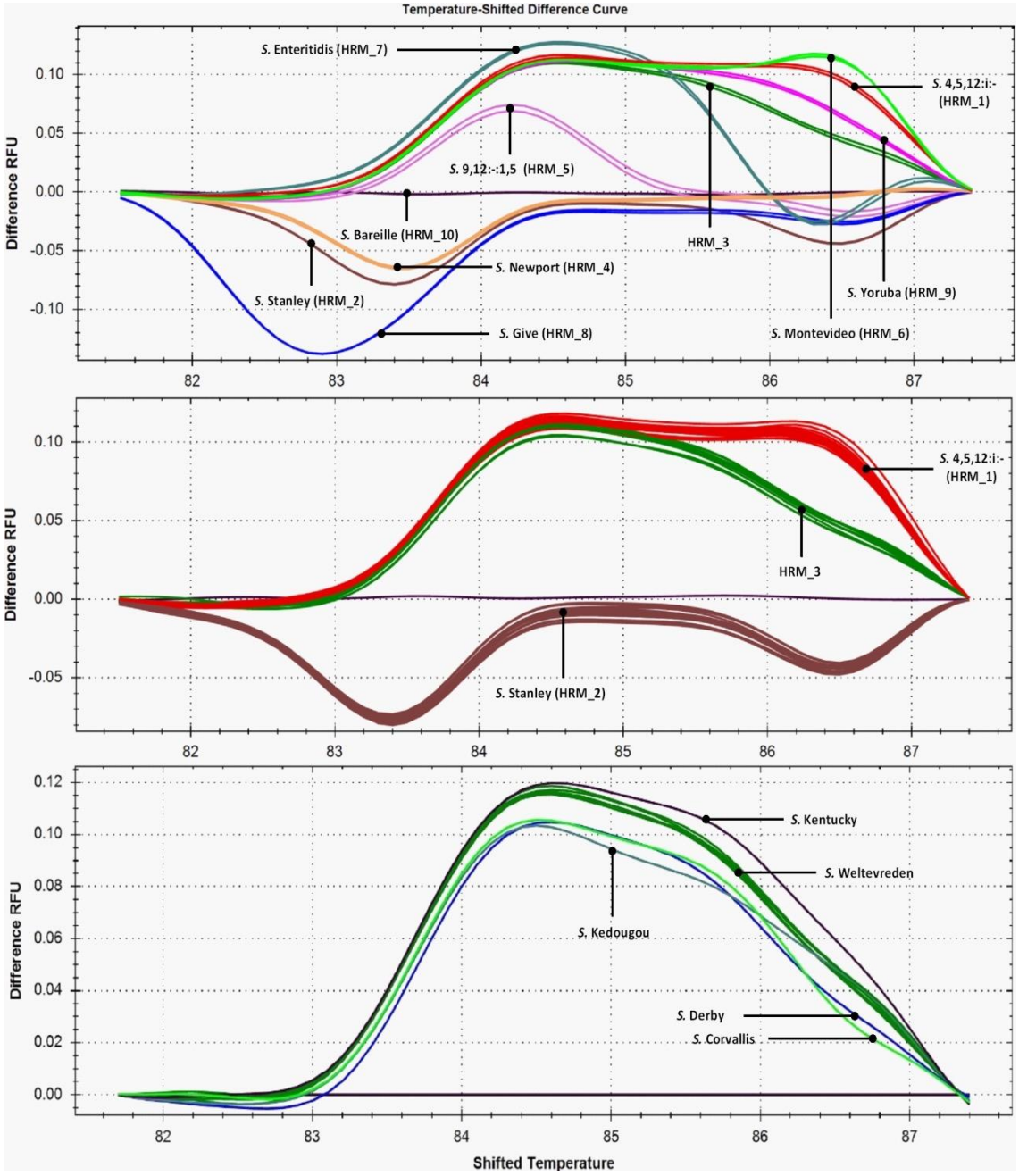

Figure 2. *HRM profiles of Salmonella spp. serotypes following multiplex PCR amplification of fljB, gyrB and ycfQ. Top panel: 10 HRM profiles of all Salmonella tested (assigned HRM_1 to HRM_10). Middle panel: 3 HRM profiles (HRM_1 to HRM_3) in extended RFU scale range. Bottom panel: HRM curves of HRM_3 in ultra-extended RFU scale range.*

Simpson's diversity index indicates a power of distinguishability of the strains in population. The values of two serotyping methods were demonstrated as 0.839 (95% CI; 0.820-0.858) and 0.802 (95% CI; 0.781-0.823) for conventional serum agglutination and HRM anaylsis, respectively. For the point of Wallace coefficient measurement, value of HRM analysis to the conventional serotyping was 0.813 (95% CI; 0.730-0.896) (Table 1).

Table 1. Simpson's diversity index and Wallace coefficients with the 95% confidence intervals of two serotyping methods.

Serotyping method	Simpson's index (95% CI)	Wallace coefficients (95% CI)	
		Conventional	HRM-analysis
Conventional	0.839 (0.820-0.858)	-	1.000* (1.000-1.000)
HRM analysis	0.802 (0.781-0.823)	0.813** (0.730-0.896)	-

* Wallace coefficients of conventional serotyping method to HRM analysis
** Wallace coefficients of HRM analysis to conventional serotyping method

Discussion

Salmonella is an important foodborne zoonotic pathogen, and it recognized as one of public health concerns, worldwide.[4,7,12] In Thailand, from the surveillance of Bureau of Epidemiology in 2017, an approxi-mate of food poisoning cases in 77 provinces was 108,153 cases or 165.30 per 100,000 of Thai population. In addition, salmonellosis is considered as the top three organisms involving to the problem for a decade, indicating that the disease has been a significant and rising problem in the country.[17]

Thirty-eight *Salmonella* strains recovered from stool of hospitalised patients in Phayao Ram hospital during March 2015 to June 2016, fourteen serotypes were identified by gold standard serum agglutination method. *Salmonella* 4, 5, 12 : i : - is the most frequently detected, followed by *S.* Stanley and *S.* Weltevreden, respectively. Considered in the serotype and their potential origins belonged, an antigenic formula of *Salmonella* 4, 5, 12 : i : - is likely allied with *S.* Typhimurium and *S.* 4,5,12:i:1,2. Absent of second phase in flagella antigens could be explained in its monophasic variant.[18] Moreover, clonal genetic relatedness among *S.* 4, 5, 12 : i : - and *S.* Typhimurium have been identified in pig-associated samples.[19] Besides, sharing of DNA fingerprinting pattern among human-originated and pig-originated *Salmonella* Stanley is also noticed.[20] For *S.* Weltevreden, it was reported as the most frequently found from chicken meats and human patients in Thailand.[4,21] As a result, pork and poultry consumption are potential reflected as the main source of human salmonellosis in the province.

HRM analysis serotyping has been applied in field practice, recently. A turnaround time of 8-12 hours is approximated. That fewer than a performing in conventional standard method, which is carried out for 3-5 days.[22] One hundred specificity result was proved by Ren et al. (2017), which was performed in *S.* Gallinarum and *S.* Pollorum.[23] However, in the study, results showed the moderate to high degree of its correlation toward the standard conventional sero-agglutination. Nine of HRM profiles were unique to the single serotype, of each. One exception is the HRM_3, which were belonged to five *Salmonella* serotypes. Higher variation of *Salmonella* serotypes tested in the current study, as well as analogous of nucleotide sequence of the three PCR-targeted genes, *fljB*, *gyrB* and *ycfQ* are possibly played the role on the circumstance.

Although, HRM analysis could not discriminate the five of 14 *Salmonella* serotypes in the study. However, overlapping of the 95% confidence intervals of Simpson's diversity index suggests the discriminatory power of the two methods is not much different. Measured in Wallace coefficient, the value of HRM analysis to the conventional serotyping was 0.813. *Salmonella* strains which are found to show a similar HRM profile will have 81.3% chance of being grouped in a similar serotype when assessed by conventional serum agglutination method. Serotyping predictability by HRM analysis may be accepted for applied in field practice, in some serotypes.

Conclusion

HRM analysis method was successfully introduced to *Salmonella* characterization. Dissociation curves profiles based on the polymorphism of three unique genes, *fljB*, *gyrB* and *ycfQ* could differentiate almost *Salmonella* serotypes in this study. Nevertheless, it can be currently revealed as an alternative approach for direct *Salmonella* serotyping, with the speedy and labor-saving procedure.

Acknowledgements

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Antunes P, Mourão J, Pestana N., Peixe L. Leakage of emerging clinically relevant multidrug-resistant *Salmonella* clones from pig farms. J Antimicrob Chemother 2011; 66(9): 2028-32.

[2] Leader BT, Frye JG, Hu J, Fedorka-Cray PJ, Boyle DS. High-throughput molecular determination of *Salmonella enterica* serovars by use of multiplex PCR and capillary electrophoresis analysis. J Clin Microbiol 2009; 47(5): 1290-9.

[3] Lopes GV, Michael GB, Cardoso M, Schwarz S. Antimicrobial resistance and class 1 integron-associated gene cassettes in *Salmonella enterica* serovar Typhimurium isolated from pigs at slaughter and abattoir environment. Vet Microbiol 2016; 194: 84-92.

[4] Bangtrakulnonth A, Pornreongwong S, Pulsrikarn C, Sawanpanyalert P, Hendriksen RS, Lo Fo Wong DM., et al. *Salmonella* serovars from humans and other sources in Thailand, 1993-2002. Emerg Infect Dis 2004; 10(1): 131–6.

[5] Popoff MY, Bockemuhl J, McWhorter-Murlin A. Supplement 1991 (no. 35) to the Kauffmann-White scheme. Res Microbiol 1992; 143(8): 807-11.

[6] Sirichote P, Bangtrakulnonth A, Tianmanee K, Unahalekhaka A, Oulai A, Chittaphithakchai P, et al. Serotypes and antimicrobial resistance of *Salmonella enterica* spp. in central Thailand, 2001-2006. Southeast Asian J Trop Med Public Health 2010; 41(6): 1405-1415.

[7] Herikstad H, Motarjemi Y, Tauxe RV. *Salmonella* surveillance: a global survey of public health serotyping. Epidemiol Infect 2002; 129(1): 1–8.

[8] Chuanchuen R, Ajariyakhajorn K, Koowatananukul C, Wannaprasat W, Khemtong S, Samngamnim S. Antimicrobial resistance and virulence genes in *Salmonella enterica* isolates from dairy cows. Food-borne Pathog Dis 2010; 7(1): 63-9.

[9] Foley SL, Lynne AM, Nayak R. *Salmonella* challenges: prevalence in swine and poultry and potential pathogenicity of such isolates. J Anim Sci 2008; 86 (14 Suppl): E149-62. doi: 10.2527/jas.2007-0464

[10] Grimont PAD, Weill FX. Antigenic formulas of the *Salmonella* serovar. 9th ed. Paris: Institut Pasteur; 2007.

[11] International Standard Organization. Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp. 4th ed. Geneva: ISO; 2002.

[12] Alvarez J, Sota M, Vivanco AB, Perales I, Cisterna R, Rementeria A, et al. Development of a multiplex PCR method for detection and epidemiological typing of *Salmonella* in human clinical samples. J Clin Microbiol 2004; 42(4): 1734-8.

[13] Zeinzinger J, Pietzka AT, Stöger A, Kornschober C, Kunert R, Allerberger F. One-step triplex high-resolution melting analysis for rapid identification and simultaneous subtyping of frequently isolated *Salmonella* serovars. Appl Environ Microbiol 2012; 78(9): 3352-3360.

[14] Cheng HR, Jiang N. Extremely rapid extraction of DNA from bacteria and yeasts. Biotechnol Lett 2006; 28(1): 55-9

[15] Hunter PR, Gaston MA. Numerical index of the discriminatory ability of typing system: an application of Simpson's index of diversity. J Clin Microbial 1988; 26(11): 2465-6.

[16] Fowlkes EB, Mallows CL. A method for comparing two hierarchical clusterings. J Am Stat Assoc 1983; 78(383): 553-69.

[17] Bereau of Epidemiology, Thailand. 2017. National Disease Surveillance (Report 506): Food poisoning 2017 [internet]. [Place unknown]: Bereau of Epidemiology; 2017 [cite 2018 April 8]. Available from: http://www.boe.moph.go.th/boedb/surdata/506wk/y60/d03_5360.pdf.

[18] Huoy L, Pornruangwong S, Pulsrikarn C, Chaturongakul S. Molecular characterization of Thai *Salmonella enterica* serotype Typhimurium and serotype 4,5,12:i:- reveals distinct genetic deletion pat-terns. Foodborne Pathog Dis 2014; 11(8): 589-92.

[19] Tadee P, Boonkhot P, Pornruangwong S, Patchanee P. Comparative phenotypic and genotypic characterization of *Salmonella* spp. in pig farms and slaughterhouses in two provinces in northern Thailand. Plos One 2015; 10(2): e0116581. doi: 10.1371/journal.pone.0116581.

[20] Hendriksen RS, Hello SL, Bortolaia V, Pulsrikarn C, Nielsen EM, Pornruangmong S, et al. Characterization of isolates of *Salmonella enterica* Serovar Stanley, a serovar endemic to asia and associated with travel. J Clin Microbiol 2012; 50(3): 709-20.

[21] Padungtod P, Kaneene JB. *Salmonella* in food animals and humans in northern Thailand. Int J Micro-biol 2006; 108(3): 346-54.

[22] O'Regan E, McCabe E, Burgess C, McGuinness S, Barry T, Duffy G, et al. Development of a real-time multiplex PCR assay for the detection of multiple *Salmonella* serotypes in chicken samples. BMC Mi-crobiol. 2002; 8: 156. doi: 10.1186/1471-2180-8-156.

[23] Ren X, Fu Y, Xu C, Feng Z, Li M, Zhang L, et al. High resolution melting (HRM) analysis as a new tool for rapid identification of *Salmonella enterica* serovar Gallinarum biovars Pullorum and Gallinarum. Poult Sci 2017; 96(5): 1088-93.

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Accuracy performance of an oral fluid-based HIV rapid diagnostic test to scale up the opportunity for treatment and prevention in Thailand

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ABSTRACT

Background: Rapid HIV tests increase an opportunity to access HIV testing, especially for high risk groups. One of the interesting approaches is oral HIV self-testing. However, performance of oral HIV test has not yet been evaluated in Thailand.

Objectives: To evaluate the performance of an oral fluid HIV rapid test for detecting recent HIV infection

Materials and methods: Men who have sex with men (MSM), transgender (TG) and female sex workers (FSW) were recruited in Bangkok, Chonburi, and Phuket. All participants were screened HIV status by oral fluid (OraQuick), whole blood (Alere Determine HIV-1/2 Ab), and plasma (Elecsys HIV combiPT). Discordant results were confirmed by nucleic acid amplification test. Performance of oral fluid and whole blood HIV rapid tests were evaluated by MedCalc's Diagnostic test. MacNemar's exact test was used to compare the numbers of detected HIV-infected participants.

Results: Five hundred and twenty nine participants were enrolled to perform HIV testing, including MSM/TG (n=289, 54.63%) and FSW (n=240, 45.37%). There were 68, 69 and 71 reactive cases from oral fluid, whole blood and plasma, respectively. Concordant reactive results among three tests were found in 64 participants, whereas 11 participants showed discordant results. Four false positive and seven false negative cases with oral fluid test were exhibited. Among false negative participants, two cases were recent infection, by which one case has received antiretroviral drugs during last 60 days. Oral fluid test had 90.14% (95% CI 80.74-95.94) sensitivity, 99.13% (95% CI 97.78-99.76) specificity and 97.92% (95% CI 96.31-98.96) accuracy. This test could detect fewer infections than those of whole blood (p=0.0019) and plasma (p=0.0057).

Conclusion: This study demonstrated that oral fluid test could detect fewer HIV infections than blood-based HIV tests since recent HIV-infected MSM/FSW were undiagnosed. Thus, this test might be inappropriate for high risk and general populations who receiving antiretroviral therapy.

Introduction

HIV is one of the most frequently addressed pathogens that has been targeted in the millennium. At the end of 2016, there were approximately 36.7 million persons living with HIV (PLWH) and 3.5 million persons in South-East Asia.[1] Knowing of HIV status is the key to early access to HIV treatment and prevention services. In 2014, UNAIDS announced 90-90-90 target and goal to end AIDS by 2030.[2] The strategies of this target and goal were at least 90% of HIV-infected people knew their status, and at least 90% of those who knew their status obtained antiretroviral therapy (ART), and at least 90% of those who received ART were

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viral suppressed. The initial step to reach the United Nations' 90-90-90 targets to end the HIV epidemic was 90% of people living with HIV to learn their HIV status. Therefore, HIV testing is essential for achieving "the first 90". Approximately 30% of people with HIV are unaware of their infection in world-wide.[1] The only way to determine a person's HIV status is the HIV testing. In many countries, critical gaps exist in HIV services, including prevention, testing and treatment. There is an opportunity to prevent 1.5 million infections per year by 2020 and reach the "fast-track" goals if we can improve prevention and testing services, as well as ensure high-quality, well-adhered-to treatment and care for all.[3] In Thailand, Reach-Recruit-Test-Treat-Retain (RRTTR) program has urged to scale up HIV prevention and treatment services among undiagnosed PLWH, especially for high risk groups, such as men who have sex with men (MSM), transgender (TG), sex workers, prisoners, people who inject drugs (PWID) and migrants. However, passive services and stigma are barriers to approach HIV testing. Mobile service at Drop in Center (DIC) and brothels using whole blood rapid HIV tests has been implemented at health care centers for professional use since 2014 in order to expand the access for HIV testing to non-clinical sites and provide an opportunity for faster linkage to treatment and care.[4-5] Moreover, rapid HIV tests have given the same-day results within 30-60 minutes. In 2004, rapid test using oral fluid (OraQuick Advance Rapid HIV-1/2 Antibody Test; OraSure Technologies, INC., Bethlehem, Pennsylvania, USA) was approved by US Food and Drug Administration (FDA) and it was approved for home use in 2012.[6] The widespread enlargement of home-based HIV testing in USA and new supervised self-testing initiatives in sub-Saharan Africa have occurred since 2006. Oral fluid HIV testing has constituted as one of the most favorite point-of-care test (POCT).[7,8] It is likely to satisfy patients due to the rapid result, non-invasive and pain-free specimen collection.[9-11] Nevertheless, HIV tests using oral fluid have not yet been approved for diagnostic purpose in Thailand.[12-13] The aim of this study was to compare the performance of oral fluid HIV test (OraQuick) with whole blood (Alere Determine HIV-1/2 Ab) and plasma (Elecsys HIV combiPT) in order to detect recent HIV infection.

Materials and methods

Study design and participants

During January-June 2015, this study was conducted in RRTTR program, AIDS ZERO Plan of Thailand at DIC and three mobile HIV testing services, including Bangkok, Chon Buri, and Phuket. High risk populations (MSM, TG and FSW) aged more than 15 years old were recruited, by which they had unknown or negative HIV status and no antiretroviral uptake (pre-exposure Prophylaxis (PrEP) and post-exposure prophylaxis (PEP)) within last two months to avoid false negative results by using oral fluid. Writing or verbal consent was given by all participants. They were received counseling before getting the HIV test in oral fluid, whole blood and EDTA plasma. This study was approved by Department of Disease Control, Ministry of Public Health, Thailand.

HIV testing

One rapid HIV test in oral fluid (OraQuick Advance Rapid HIV-1/-2 Antibody Test, OraSure Technologies, Bethlehem, PA, USA, 5 µL) and three whole blood HIV POCTs were used. Alere Determine HIV-1/2 (Inverness Medical Japan Co., Ltd., Japan, 50 µL) was used for screening. Any HIV-reactive whole blood specimens were confirmed by using DoubleCheckGold Ultra HIV1&2 (Organics LTD., Israel, 25 µL) and SD Bioline HIV-1/2 (Standard Diagnostics, Inc. Korea, 10 µl). All EDTA plasma specimens were also tested by 4thgen EIA (Elecsys HIV Combi PT, Electrochemiluminescence Immunoassay, Roche Diagnostics GMBH, Germany, 40 µL). Any HIV-reactive plasma specimens were tested by using Alere Determine HIV-1/2, DoubleCheckGold HIV1&2 and SD Bioline. Discordant result cases were confirmed by HIV Ag EIA (Elecsys HIV Ag, Electrochemiluminescence Immunoassay, Roche Diagnostics GMBH, Germany, 40 µL) and nucleic acid amplification test (NAAT, Cobas ampliPrep/Cobas TaqMan HIV-1 test, version 2.0, Roche Molecular Systems, Inc., USA, 1,000 µL). For the participants who had discordant HIV results among three specimens, new EDTA plasma were collected within 14 days in order to identify recent infection by using HIV Ag EIA, Determine HIV-1/2 Ag/Ab Combo (Determine Combo, Alere Inc., Japan, 50 µL) and NAAT.

Quality control measurements of HIV tests were done. Medical technologists who performed OraQuick were well-trained on biological principles of the test, kit storage, specimen collection, testing, interpretation and quality control by OraSure personnel staffs. Other HIV tests were assayed together with internal quality control (IQC) panels provided by Department of Medical Sciences, Ministry of Public Health. The OraQuick and Determine Combo have not yet been approved in Thailand. Therefore, method verification is needed before use. Both test kits passed our verification with in-house panels (three known HIV status volunteers) including HIV-negative antigen/antibody, HIV-positive antigen and HIV-positive antibody.

Statistical analysis

The diagnostic performance of oral fluid and whole blood HIV rapid tests were comprised of accuracy, sensitivity, specificity, false positive (FP), false negative (FN), positive predictive value (PPV), and negative predictive value (NPV) by using MedCalc's Diagnostic test evaluation calculator (free statistical calculators).[14] McNemar's exact test was used to compare the numbers of detected HIV-infected participants.[15]

Results

A total of 529 participants were recruited including 289 MSM/TGs and 240 FSWs. Results of OraQuick, Determine HIV-1/2 and Elecsys HIV Combi PT tests were shown in Figure 1A. OraQuick was found to be reactive in 68 cases. Sixty-nine HIV-reactive cases by Determine HIV-1/2 had concordantly reactive results with doubleCheckGold, SD Bioline HIV-1/2 tests and Elecsys HIV Combi PT. Two participants, who were HIV- reactive with Elecsys HIV Combi PT but non-reactive with doubleCheckGold, SD Bioline HIV-1/2

tests and Determine HIV -1/2 tests, were positive by Elecsys HIV Ag and NAAT.

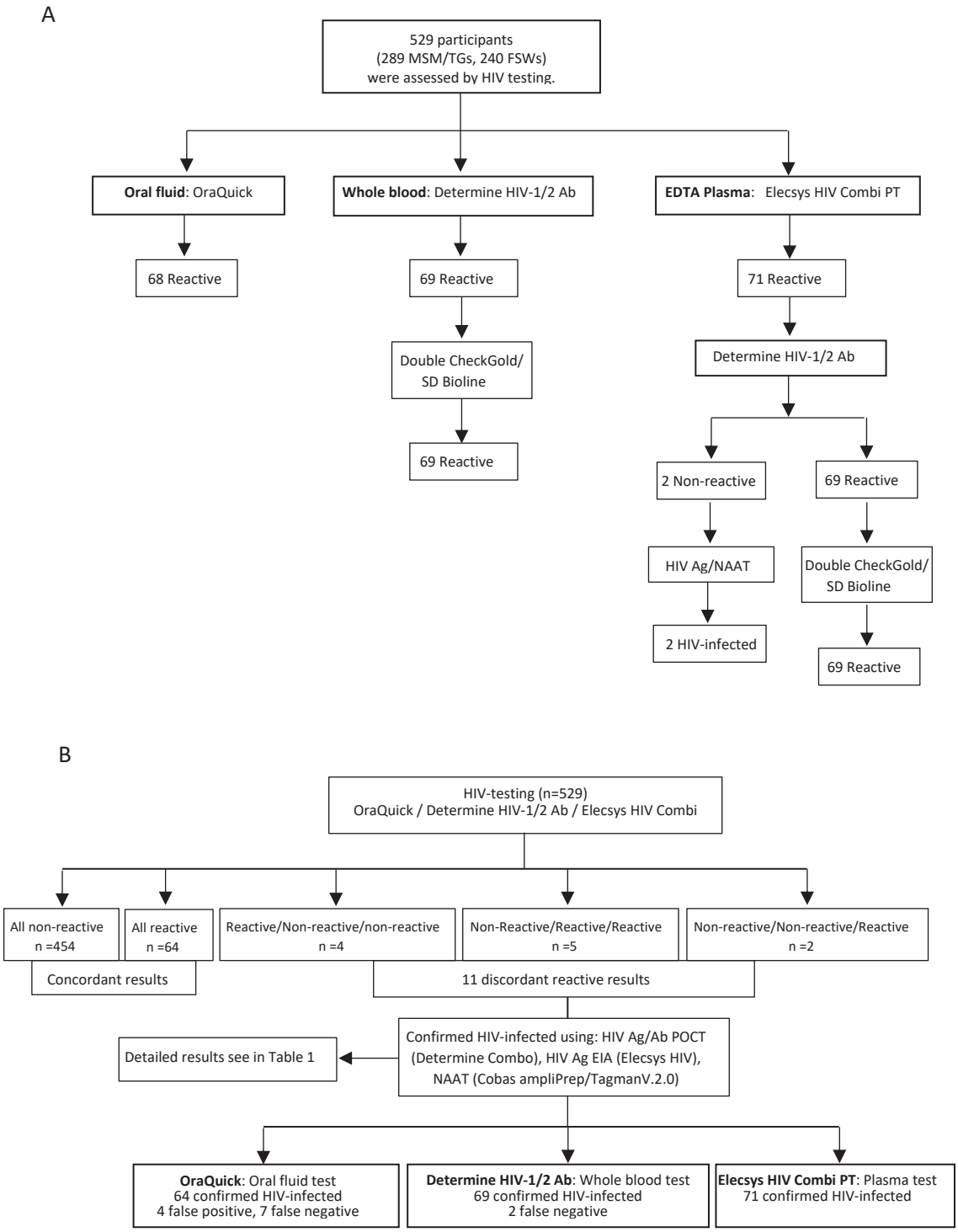

Figure 1 *The algorithm and HIV testing results in this study A) The HIV results of oral fluid, whole blood and plasma-based HIV testing. B) Eleven discordant results were identified for early HIV infection.*
OraQuick, OraQuick Rapid HIV-1/2 antibody test; 4th gen EIA, 4th generation Electrochemiluminescence Immunoassay - Elecsys HIV Combi PT; HIV Ag EIA, Electrochemiluminescence Immunoassay - Elecsys HIV Ag; HIV Ab POCT, HIV antibody point of care test, NAAT, Nucleic-acid amplification test - Cobas AmpliPrep/Tagman V.2.0; For screening test, Determine HIV-1/2 Ab; For supplementary tests, Double CheckGold and SD Bioline.

Concordant reactive results among three specimen types were found in 64 (12.10%) cases, whereas 11 (2.08%) cases had discordant results as shown in Figure 1B and Table 1. Four out of 11 cases were only reactive by OraQuick (false positive), while seven cases were non-reactive (false negative). Only five participants were concordantly reactive by Determine HIV-1/2, Elecsys HIV Combi PT and NAAT. Interestingly, two participants that were non-reactive by both OraQuick and Determine HIV -1/2, were positive by Elecsys HIV Combi PT, Elecsys HIV Ag and NAAT. Overall, 71 (13.42%) HIV-infected participants were detected. There were significantly different between OraQuick compared to 4th gen EIA, Elecsys HIV Combi PT (p=0.0057) and Alere Determine HIV -1/2 (p=0.0019) (Table 1).

Table 1. Discordant results of HIV testing among participants in this study.

No	Risk groups	Rapid HIV testing[a]						Elecsys HIV Combi PT (4th gen EIA)	HIV Ag Elecsys HIV (EIA)	HIV RNA (copies/mL)
		OraQuick[b]	Determine HIV-1/2 Ab	Double check gold	SD Bioline	Determine combo				
						Ag	Ab			
1	MSM	-	+	+	+	+	+	+	+	380,000
2	MSM/TG	-	+	+	+	+	+	+	+	474,000
3	MSM	-	+	+	+	-	+	+	+	291,000
4	MSM	-	+	+	+	-	+	+	-	49,200
5	MSM/TGc	-	+	+	+	-	+	+	-	29,800
6	MSMd	-	-	-	-	+	-	+	+	10,000,000
7	FSWe	-	-	-	-	-	-	+	+	117,800
8	MSM	+	-	-	-	-	-	-	-	ND
9	MSM/TG	+	-	-	-	-	-	-	-	ND
10	MSM/TG	+	-	-	-	-	-	-	-	ND
11	MSM	+	-	-	-	-	-	-	-	ND

Ag, antigen; Ab, antibody; EIA, enzyme Immunoassay; ND, Not done; MSM, men have sex with men; TG, transgender; FSW, female sex worker

a Oral fluid-based test: OraQuick; Whole blood-based test: Determine HIV-1/2 Ab, Double check gold, SD Bioline.

b Significant differences were found when compared to Elecsys HIV Combi PT (p=0.0057) and Determine HIV-1/2 Ab (p=0.0019).

c This participant had the history of antiretroviral drug uptake (Prep and PEP) for several times during last 60 days.

d This participant was acute Infection that OraQuick and Determine HIV-1/2Ab gave the non-reactive results but Determine Combo antigen was reactive. HIV RNA was >10,000,000 copies/mL and EIA HIV Ag/Ab was reactive. After two weeks, all HIV Ab tests were reactive, while oral fluid test was still non-reactive.

e OraQuick and Determine HIV-1/2 Ab had the non-reactive results. HIV RNA was 117,800 copies/mL and HIV Ag/Ab EIA test was reactive. After two weeks, all HIV Ab tests were reactive, while oral fluid test was still non-reactive.

Performance evaluation of OraQuick and Alere Determine HIV-1/2 compared with HIV status was shown in Table 2. OraQuick sensitivity (90.14%, 95% CI 80.74-95.94) was lower than Alere Determine HIV-1/2 (96.97%, 95% CI 89.48-99.63), while Alere Determine HIV-1/2 specificity (100%, 95% CI 99.21-100.00) was slightly greater than OraQuick (99.13%, 95% CI 97.78-99.76). The PPV of OraQuick (94.12%, 95% CI 85.74-97.71) was lower than Alere Determine HIV-1/2 (100.00%) although the NPV of OraQuick (98.48%, 95% CI 96.98-99.24) and Alere Determine HIV -1/2 (99.57%, 95% CI 98.34-99.89) were comparable. Accuracy of OraQuick and Alere Determine HIV-1/2 were 97.92% (95% CI 96.31-98.96) and 99.62% 95% CI 98.64-99.95), respectively. Additionally, there were 0.87% FP and 9.86% FN with OraQuick, as well as 0% FP and 3.03% FN with Alere Determine HIV-1/2.

Table 2. Performance evaluations of oral fluid rapid HIV test and whole blood HIV test compared with HIV status[a]. (n=529)

Statistical parameter	OraQuick Advance Rapid HIV-1/2[b]	Alere Determine HIV-1/2[c]
	Value (95% CI)	Value (95%CI)
Sensitivity	90.14% (80.74-95.94)	96.97% (89.48-99.63)
Specificity	99.13% (97.78-99.76)	100% (99.21-100.00)
Positive Likelihood Ratio	103.21 (38.79-274.66)	-
Negative Likelihood Ratio	0.10 (0.05-0.20)	0.03 (0.01-0.12)
Disease prevalence	13.42% (10.63-16.63)	12.48% (9.78-15.60)
Positive Predictive Value	94.12% (85.74-97.71)	100.00%
Negative Predictive Value	98.48% (96.98-99.24)	99.57% (98.34-99.89)
Accuracy	97.92% (96.31-98.96)	99.62 (98.64-99.95)
False Positive	0.87%	0%
False Negative	9.86%	3.03%

[a] HIV status: diagnosed by physical examination and risk behavior in the past. More than two-thirds of HIV Ab tests were reactive. HIV RNA was detected by NAAT and HIV Ag tests were reactive.

[b] Oral fluid-based test

[c] Whole blood-based test

Discussion

This study demonstrated that performance (sensitivity, specificity, PPV, NPV, accuracy) of oral fluid HIV test was lower than those of whole blood HIV test. In contrast, FP and FN of HIV test with oral fluid were greater than whole blood. Oral fluid test was unable to diagnose in seven HIV-infected MSM/TG/FSWs, by which early infection was found in two cases. One of them was acute infection confirmed by HIV Ag test and NAAT (HIV RNA 10,000,000 copies/ml). However, this participant was able to be detected by rapid HIV Ag/Ab test (Determine Combo). The other participant seemed to be in window period since it could not be detected by all rapid HIV tests (Table 1). The NPV of oral fluid HIV test was 98.48%, suggesting that the non-reactive result with oral fluid was more correct in high risk populations (MSM/TG/FSW) when no recent HIV infection has occurred. Moreover, the infected participants who had several antiretroviral drug uses (Prep and PEP) for last 60 days (No. 5, Table 1) showed the false non-reactive result with oral fluid test. Similarly, the previous studies have found false-negative results in participants who had ART.[16-21] Nevertheless, non-reactive results of oral fluid test in participants taking antiretroviral drugs were found to be HIV-positive afterward.[16-18] One study has explained that antiretroviral drugs might decrease glycoprotein (gp) 41 production, which used as the target antigen to detect HIV Ab in OraQuick test.[16] In fact, the quantity of HIV Ab in oral fluids was lower than whole blood and plasma, especially who had effective antiretroviral drugs.[19] A study in PWID from Thailand has indicated that oral fluid test in participants who received pre-exposure prophylaxis took longer to develop the reactive result. Blood-based HIV tests might thus be more appropriate.[20]

The PPV of 94.12% in these high risk populations also suggested that the oral fluid HIV test was beneficial for screening because it could provide the rapid result with non-invasive handing. However, the reactive result was needed to be confirmed by other HIV tests.[22] In this study, rapid HIV testing in whole blood using Alere Determine HIV-1/2 showed the comparable performance with Elecsys HIV Combi PT (EIA). No false reactive and two false non-reactive were observed in participants who had HIV antigen positive. Antigen reactive band was detected by whole blood HIV Ag/Ab test in one case (No. 6, Table 1). Whole blood HIV Ag/Ab test would thus be suitable for rapid screening in high risk and hard accessible groups.

According to our study, the performance of oral fluid HIV test was less accurate than blood-based tests that have also been shown in several studies.[23-26] In high HIV prevalence populations, the rapid HIV testing are appropriate for enhancing epidemic HIV control in mobile setting. If possible, whole blood rapid HIV Ag/Ab test should be the first screening test since it is able to detect both HIV antigen and antibody. In addition, it is ease of use, quick turnaround time, no requirement of cold chain and specialized equipment. However, confirmation at HIV care centers is still required for rapid HIV tests. Those who have sexual risk behavior or negative HIV test should be regularly followed up until 3 months after post-exposure. Mobile setting and decentralization of laboratory services may be useful to scale up the opportunity to detect HIV-infected population and approach them to treat with antiretroviral drugs in order to reduce transmission. Nonetheless, rapid HIV tests should be confirmatory tested by supplementary tests, e.g., HIV antigen-antibody combination assays, HIV antigen test and NAAT.[27]

Conclusion

Our findings suggested that whole blood rapid HIV tests should be used for high risk populations. If non- invasive practice is required, oral fluid tests would be one of the tools of choice but window period and ART uptake must be concerned. Furthermore, rapid HIV tests should be confirmed by other HIV tests.

Disclaimer

The content of this report is those of the authors and do not necessarily reflect the policy and views of Department of Disease Control, Ministry of Public Health, Thailand.

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References

[1] World Health Organization (WHO). Global Health Observatory (GHO) data: HIV/AIDS. Geneva: World Health Organization; 2017.

[2] The Joint United Nations Programme on HIV/AIDS (UNAIDS). 90-90-90: an ambitious treatment target to help end the AIDS epidemic. Geneva: The Joint United Nations Programme on HIV/AIDS; 2014.

[3] World Health Organization (WHO). HIV/AIDS topical information: HIV testing services. Geneva: World Health Organization; 2017.

[4] Cherutich P, Bunnell R, Mermin J, HIV testing: current practice and future directions. Curr HIV/AIDS Rep 2013; 10(2): 134-41.

[5] Thailand National AIDS Committee. National strategic information and monitoring and evaluation plan for HIV/AIDS Thailand 2012 to 2016. Bangkok: Thailand National AIDS Committee; 2013.

[6] US Food and Drug Administration. OraQuick® In-home HIV test summary of safety and effectiveness. Silver Spring, MD: US Food and Drug Administration; 2012.

[7] CLIA database. CLIA record BP010047. Rockville, MD: US Food and Drug Administration; 2008.

[8] Wesolowski LG, MacKellar DA, Facente SN, Dowling T, Ethridge SF, Zhu JH, et al. Post-marketing surveillance of OraQuick whole blood and oral fluid rapid HIV testing. AIDS 2006; 20(12): 1661-6.

[9] Pant Pai N, Joshi R, Dogra S, Taksande B, Kalantri SP, Pai M, et al. Evaluation of diagnostic accuracy, feasibility and client preference for rapid oral-based diagnosis of HIV infection in rural India. PLoS One 2007; 2(4): e367.

[10] Pai NP, Barick R, Tulsky JP, Shivkumar PV, Cohan D, Kalantri S, et al. Impact of round-the-clock , rapid oral fluid HIV testing of women in labor in rural India. PLoS Med 2008; 5(5): e92.

[11] Hutchinson AB, Branson BM, Kim A, Farmham PG. A meta-analysis of the effectiveness of alternative HIV counseling and testing methods to increase knowledge of HIV status. AIDS 2006; 20(12): 1597-604.

[12] Thailand Food and Drug Administration. Medical device control in Thailand. Nonthaburi: Thailand Food and Drug Administration; 1990.

[13] Department of Medical Sciences, Thailand Ministry of Public Health. Diagnostic HIV tests available in Thailand. Nonthaburi: Department of Medical Sciences; 2014.

[14] MedCalc. Diagnostic test evaluation calculator [Internet]. Ostend: MedCalc Software bvba; 2015 [cited 2015 Jul 31]. Available from: https://www.medcalc.org/calc/diagnostic_test.php.

[15] Lowry R. Vassarstats: statistical computation - McNemar's Test [Internet]. [Place unknown]: Vassarstats; 2015 [cited 2015 Jul 31]. Available from: http://vassarstats.net/propcorr.html.

[16] O'Connell RJ, Merritt TM, Malia JA, VanCott TC, Dolan MJ, Zahwa H, et al. Performance of the OraQuick rapid antibody test of diagnosis of human immunodeficiency virus type 1 infection in patients with various levels of exposure to highly active antiretroviral therapy. J Clin Microbiol 2003; 41(5): 2153-5.

[17] Choko AT, MacPherson P, Webb EL, Willey BA, Feasy H, Sambakunsi R, et al. Uptake, accuracy, safety, and linkage into care over two years of promoting annual self-testing for HIV in Blantyre, Malawi: a community-based prospective study. PLoS Med 2015; 12(9): e1001873.

[18] Mavedzenge SN, Sibanda E, Mavengere Y, Hatzold K, Mugurungi O, Ncube G, et al. Supervised HIV self-testing to inform implementation and scale up of self-testing in Zimbabwe. J Int AIDS Soc 2015; 18 (Suppl 4): 96.

[19] Stekler JD, O'Neal JD, Lane A, Swanson F, Maenza J, Stevens CE, et al. Relative accuracy of serum, whole blood and oral fluid HIV tests among Seattle men who have sex with men. J Clin Virol 2013; 58(Suppl 1): e19-22.

[20] Suntharasamai P, Martin M, Choopanya K, Vanichseni S, Sangkum U, Tararut P, et al. Assessment of oral fluid HIV test performance in an HIV pre-exposure prophylaxis trial in Bangkok, Thailand. PLoS One 2015; 10(12): e0145859.

[21] McNeil Jr DG. Rapid H.I.V. home test wins federal approval. The New York Times 2012 Jul 3: A1.

[22] Facente SN, Dowling T, Vittinghoff E, Sykes DL, Colfax GN. False positive rate of rapid oral fluid HIV tests increases as kits near expiration date. PLoS One 2009; 4(12): e8217.

[23] Delaney KP, Branson BM, Uniyal A, Phillips S, Candal D, Owen SM, et al. Evaluation of the performance characteristics of 6 rapid HIV antibody tests. Clin Infect Dis 2011; 52(2): 257-63.

[24] Pant Pai N, Balram B, Shivkuma S, Martinez-Cajas JL, Claessens C, Lambert G, et al. Head-to-head comparison of accuracy of a rapid point-of-care HIV test with oral versus whole-blood specimens; a systematic review and meta-analysis. Lancet Infect Dis 2012; 12(5): 373-80.

[25] Pavie J, Ruchline A, Loze B, Niedbalski L, Delaugerre C, Laforgerie E, et al. Sensitivity of five rapid HIV tests on oral fluid or finger-stick whole blood: a real-time comparison in a healthcare setting. PLoS One 2010; 5(7): e11581.

[26] Centers for Disease Control and Prevention (CDC). False-positive oral fluid rapid HIV tests–New York City, 2005-2008. MMWR Morb Mortal Wkly Rep 2008; 57: 660-5.

[27] Stekler JD, Swenson PD, Coombs RW, Dragavon J, Thomus KK, Brenan CA, et al. HIV testing in a high-incidence population: is antibody testing alone good enough?. Clin Infect Dis 2009; 49(3): 444-53.

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Molecular characterization and liquid chromatography-mass spectrometric multiple reaction monitoring-based detection in case of suspected phalloides syndrome poisoning

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ABSTRACT

Background: The most common lethal mushrooms are invariably attributed to amanitas, which contain several types of lethal peptide toxins. During 2015 to 2018, the suspect in phalloides syndrome case reported to the Thai National Institute of Health included 33 patients with 3 deaths. This syndrome is characterized by a long latent period and having two phases of gastrointestinal irritation followed by progressive liver dysfunction.

Objectives: The aims of this study were to identify mushroom samples from four clinically reported cases based on nuclear internal transcribed spacer (ITS) sequence data and diagnose lethal peptide toxins using liquid chromatography (LC)-tandem mass spectrometry (MS/MS) with multiple reaction monitoring (MRM).

Materials and methods: Nucleotide similarity was identified using BLAST search of the NCBI database. Phylogenetic analysis of nuclear internal transcribed spacer (ITS) region was conducted by maximum likelihood method. Mushroom peptide toxins were analyzed using LC-MS/MS with MRM.

Results: Based on BLAST search yielded 98% to 100% of mushroom samples from four clinically reported cases to *Amanita brunneitoxicaria*. Phylogenetic analysis showed all mushroom samples placed closely related to *A. brunneitoxicaria* with a strong bootstrap value (BS=100%). The presence of three lethal peptide toxins in clinical mushroom samples was confirmed by MS/MS spectra acquired from a reference standard, including α-amanitin (*m/z* 919.0, RT=2.157 min), β-amanitin (*m/z* 920.1, RT=2.167 min) and phalloidin (*m/z* 789.3, RT=2.189 min). The product ions of *m/z* 259.3, 259.0 and 330.3 were the most abundant and stable ions for the α-amanitin, β-amanitin and phalloidin analyses, respectively.

Conclusion: This study revealed that the toxic mushrooms ingested by patients were confirmed to be a species of amanitas closely related to *Amanita brunneitoxicaria*. Furthermore, rapid detection using a high-throughput LC-MS/MS with MRM represents an effective method in identifying lethal peptide toxins from poisoning caused by mushrooms.

Introduction

A checklist of Thai Basidiomycetes mushrooms was reported, including 1,978 species.[1] Several mushroom species has proven to be edible and become important in economics. However, the toxicity of some mushrooms species is still inadequate. According to mushroom poisoning case report of Toxicology center (Thai National Institute of Health) were identified approximately 76% for gastrointestinal irritants, 14% for amanitin-containing mushrooms and 10% for alkaloid muscarine-containing mushrooms, respectively.[2] The most common lethal wild mushrooms inhabited are

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amanitas. These mushrooms are sometimes misidentified as their young fruiting bodies; furthermore, there are misconceptions about ethnomycological knowledge such as mushrooms that are ingested by animals are safe for humans and boiling of mushrooms can detoxify toxins.[3] In cases of poisoning, a rapid identification of mushroom samples consumed by the patients is required for appropriate medical treatments. However, the available mushroom samples are not always well-preserved and the standard morphological identification of particular samples needs experienced mycologists. Hence, molecular-based identification as well as LC-MS/MS with MRM method should be employed as tools to identify the poisonous mushroom samples in the absence of morphological characteristics. For molecular marker, the internal transcribed spacer (ITS) region of nuclear ribosomal DNA has successful identification in various groups of mushroom. This locus was proposed as the universal barcode region for fungal identification[4,5] and used in forensic investigations to confirm several cases of mushroom poisoning.[2,3,6,7] Moreover, we developed a rapid and convenient high-throughput method to identify the lethal peptide toxins in fatal case of mushroom poisoning. To analyze the lethal peptide toxins, LC-MS/MS confirmatory method with multiple reaction monitoring (MRM) was performed to measure the product ions of interest.[8] This method could become a practical method for toxicological purpose.

Materials and methods

Clinical mushroom samples and case reports

Mushroom samples obtained from clinically reported cases during 2015 to 2018 with case reports of poisoning were used in this study. In each case hospitalization was required. A total of 36 patients were suspected phalloides syndrome after mushroom ingestion. All patients represented a latent period about 4 to 24 hours that exhibits two phases of gastrointestinal irritations and liver dysfunction. Three deaths occurred in patients due to acute hepatic and renal failure.

DNA extraction and PCR amplification

Fruiting bodies of 2-15 mg were ground in liquid nitrogen. DNA was extracted using the PureLinkTM Plant total DNA purification kit (Invitrogen, USA) according to the manufacturer's instructions. The entire nuclear ITS regions was simultaneously amplified using the universal primers ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3').[9,10] PCR condition was as described previously.[3]

Fungal identification and phylogenetic analyses

The nuclear ITS sequences obtained from the clinical mushroom samples were compared for nucleotide similarity against the GenBank database using BLASTN 2.8.0 server.[11,12] DNA sequences alignment was done using Geneious R8 (http://www.geneious.com/). Ambiguously aligned portions were removed manually. The ITS sequences belonging to core taxa of section of *Phalloidea*e was downloaded from GenBank (Figure 1). These sequences were selected based on current phylogeny of the genus *Amanita.*[13] Phylogenetic analysis was conducted using maximum likelihood (ML) method. ML analysis was performed in RAxML 7.2.6 using the GTRGAMMA model with 25 rate parameter categories.[14] Branch support was estimated by using 1000 bootstrap pseudoreplicates. Only clades that received bootstrap support ≥70% under ML was considered as strongly supported. Phylogenetic tree was depicted using the program FigTree 1.4.3 (http://tree.bio.ed.ac.uk).

Sample preparation and purification

Five grams of clinical mushroom samples were blended and extracted with 20 mL of methanol. The extract was incubated at 65 °C for 10 min, followed by centrifugation at 14,000 rpm for 5 min. Supernatant (100 µL) was then re-extracted with methanol-chloroform-deionized water (4:2:3, v/v/v). The tube was gently inverted 3 or 4 times followed by 3 min of centrifugation at 14,000 rpm to separate the layers. Polar (lower) phase was transferred to a clean microcentrifuge tube. Supernatant was homogenized with 300 µL of methanol and centrifuged for 3 min at 14,000 rpm. Clear supernatant was decanted to dryness under a stream of nitrogen. Residue was dissolved in acetonitrile and filtered with VertiPure™ PVDF(HL) syringe filters (13 mm, 0.2 µm). The filtrate was then submitted for subsequent analysis.

Peptide toxins detection using LC-MS/MS

Peptide toxins including α-amanitin, β-amanitin, phallacidin and phalloidin obtained from Sigma-Aldrich (St. Louis, USA) were used as standard reference material. Separation of peptide toxins and determination of molecular weight as well as the precursor ion fragmentation were using LC-MS/MS method on Agilent 6495 Triple Quadrupole LC/MS and 1290 infinity LC modules with Agilent MassHunter software (California, USA). In the chromatographic system, a Z0RBAX SB-C18 narrow-bore (21 mm x 150 mm, 3.5 µm) and ZIC-HILIC (20 mm x 2.1 mm, 3.5 µm) columns were used for separation of amatoxins (α-amanitin & β-amanitin) and phallotoxins (phallacidin & phalloidin), respectively. Amatoxins mobile phases were 10 mM ammonium acetate in 0.1% (v/v) formic acid (mobile phase A) and 0.1% (v/v) formic acid in acetonitrile (mobile phase B) for the separation of α-amanitin and β-amanitin. Phallotoxins mobile phases were 2 mM ammonium formate in deionized-H_2O (mobile phase A) and acetonitrile (mobile phase B). A program was started with 10% mobile phase A and 90% mobile phase B. Total run time for each sample was 15 min. Flow rate was 0.2 mL/min, and the injection volume was 5 µL. The column temperature was 40°C.

Mass spectrometer was performed in MS/MS mode using a multiple reaction monitor (MRM) to detect a specific precursor ion (MS1) to product ion (MS2) transitions for each analysis. The MS conditions were as follows: gas temperature, 200°C; gas flow, 14 L/min; nebulizer, 40 psi; and capillary voltage, 4,000 V.

Results

Fungal Identification using NCBI-BLAST

The remnant mushroom samples were amplified by PCR based amplification of the nuclear ITS regions. A PCR amplification and purification product was approximately 700 bp long. Result of BLAST search showed the highest pairwise identify for all samples tested with scores ranging from 98 % to 100% identity, which exhibited identical species identification for *Amanita brunneitoxicaria* (KY747436) and the type material of *A. brunneitoxicaria* (NR_151655).

Phylogenetic analysis of mushroom samples consumed by the patients

A matrix of 731 unambiguously aligned nucleotide characters was constructed. ML analysis of the nuclear ITS dataset yielded a tree with the final optimization likelihood of lnL = -3167.926. In this study, we focused on the clinical mushroom samples that suspected phalloides syndrome poisoning and resulted in death of the patients. All clinical mushroom samples are clustered with *Amanita brunneitoxicaria* (BS=100%) and *A. fuligineoides* as a sister taxa (Figure 1). Based on the remnant mushroom samples, its general features include pileus darkest at centre towards greyish at margin, convex to plane; cylindrical stipe, white to pale grey; context stuffed and bulb subglobose with saccate volva (Figure 2).

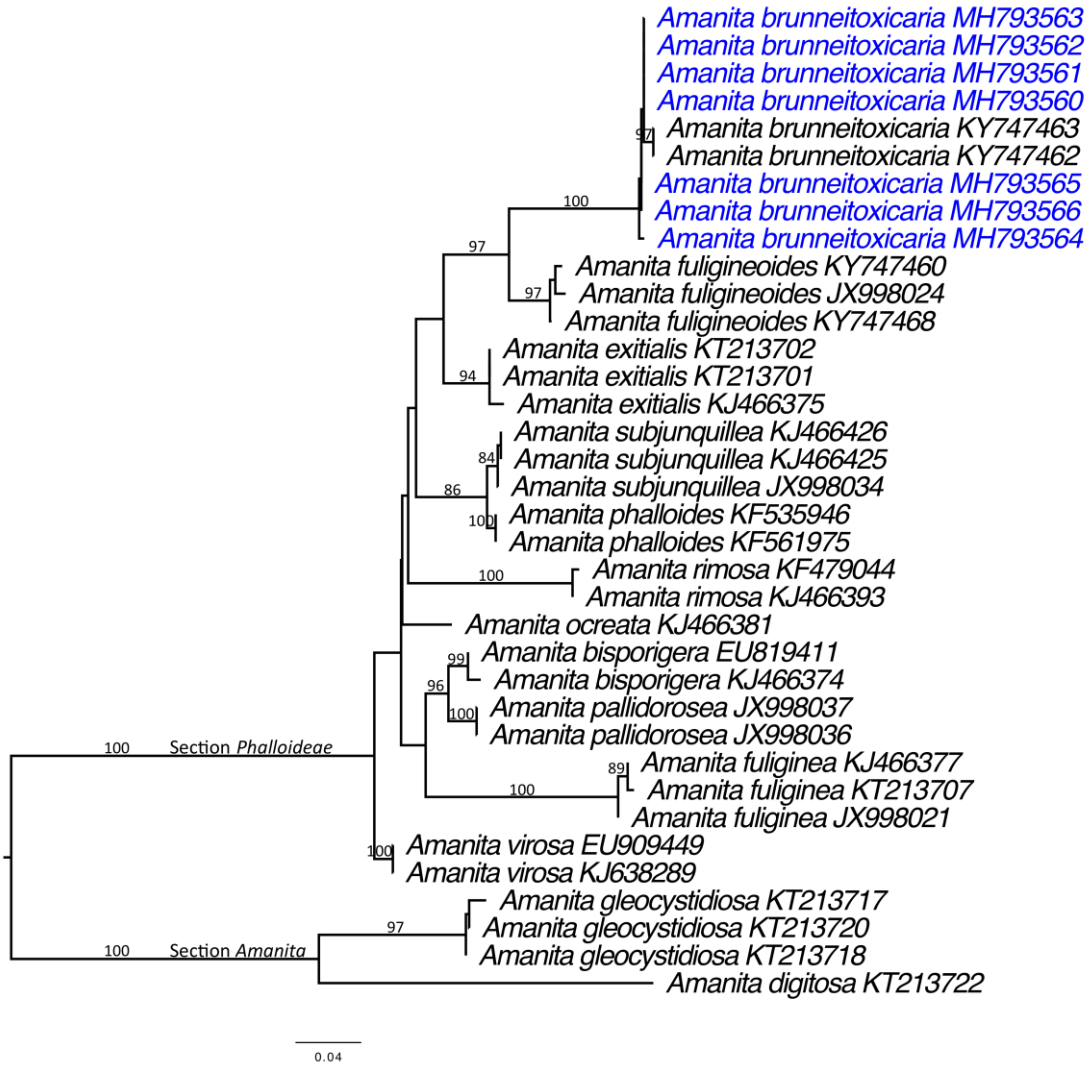

Figure 1 *Best-scoring maximum likelihood tree based on the nuclear ITS rDNA sequences of selected mushroom species of section Phalloideae. Clinical mushroom samples ingested by patients are highlighted. Bootstrap support values performed with RAxML 7.2.6 are given in number above branches. Reference sequences were downloaded from GenBank.*

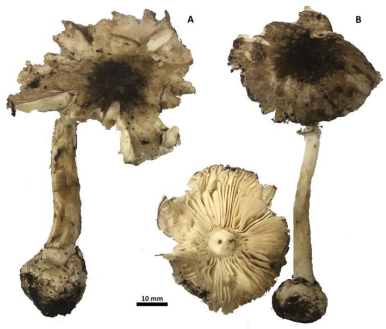

Figure 2. *The remnant clinical mushroom samples (A) Amanita sp. D344 and (B) Amanita sp. D346.*

Peptide toxins

The reference standard and purified compounds obtained from the clinical mushroom samples were analyzed with MS/MS spectra and the corresponding molecular weights were performed based on the fragmentation of the precursor ion (Figure 3 & 4). The lower limit of quantification was 11 µg/L for α-amanitin, 11 µg/L for β-amanitin, 100 µg/L for phallacidin and 10 µg/L for phalloidin. The ion acquisition timeframe revealed in the range of 100-1,000 (*m/z*). Multiple reaction monitoring (MRM) was performed at unit resolution using a mass transition ion pair (Table 1). The product ions *m/z* 259.3, 259.0, 811.5 and 330.3 were the most abundant and stable ions for the α-amanitin, β-amanitin, phallacidin and phalloidin analyses. Three lethal peptide toxins of α-amanitin, β-amanitin and phalloidin were detected in all mushroom samples (Figure 4).

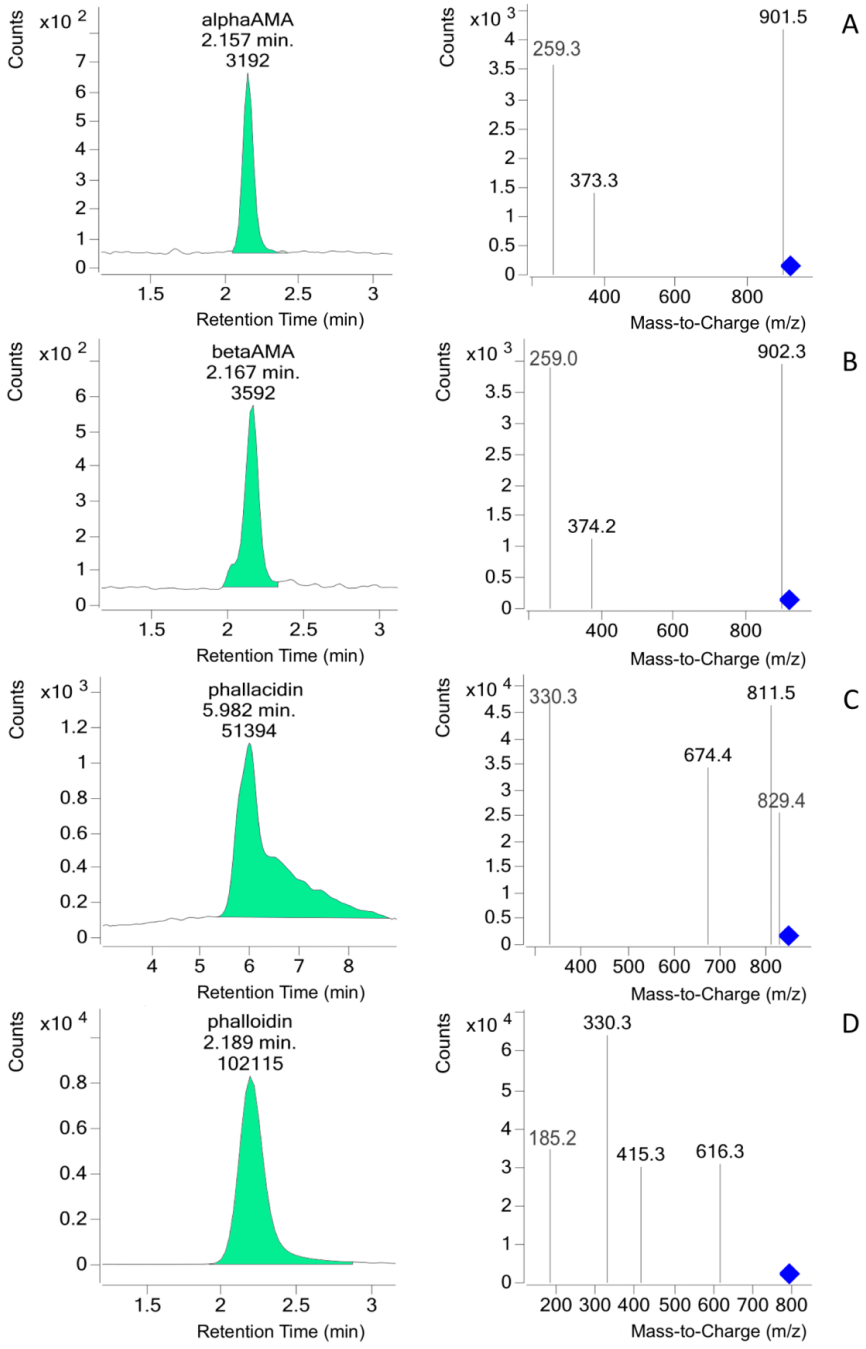

Figure 3. *Liquid chromatography-tandem mass spectrometric MRM chromatograms of standard lethal peptide toxins (A) alpha amanitin, (B) beta amanitin, (C) phallacidin and (D) phalloidin. Diamond-shape represents the molecular ion.*

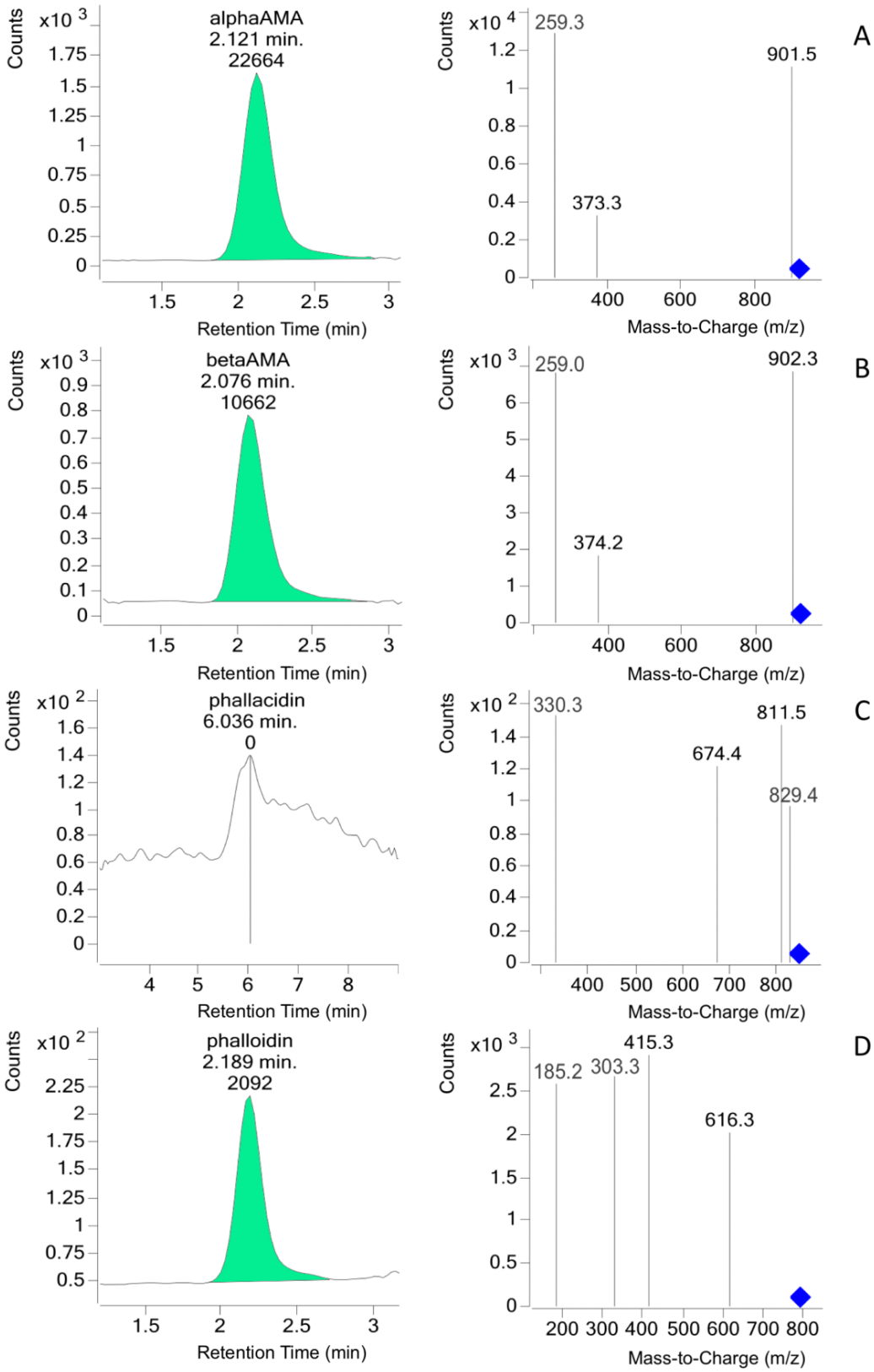

Figure 4 *Liquid chromatography-tandem mass spectrometric MRM chromatograms of lethal peptide toxins obtained from clinical mushroom samples (A) alpha amanitin, (B) beta amanitin, (C) phallacidin and (D) phalloidin. Diamond-shape represents the molecular ion.*

Table 1. Lists of characteristic ions of standard lethal peptide toxins during MRM acquisition.

Compounds	Molecular ion (*m/z*)	Product ions (*m/z*)	Peak area (%)
α-amanitin	919.0	919.0→259.3	3192 (49)
		919.0→373.3	525 (8)
		919.0→901.5	2813 (43)
β-amanitin	920.1	920.1→259.0	3592 (52)
		920.1→374.2	369 (5)
		920.1→902.3	2968 (43)
phallacidin	847.3	847.3→330.3	46803 (31)
		847.3→674.4	31610 (21)
		847.3→811.5	51394 (34)
		847.3→829.4	21241 (14)
phalloidin	789.3	789.3→185.2	50013 (21)
		789.3→330.3	102115 (42)
		789.3→415.3	43275 (18)
		789.3→616.3	45387 (19)

Discussion

Food poisoning by amatoxins-containing mushroom is found abundantly in the genus *Amanita* (Basidiomycota). This genus was traditionally divided into seven sections including *Amanita, Amidella*, *Caesareae*, *Lepidella, Phalloideae*, *Vaginatae* and *Validae*.[15] The most toxic species belongs to the sections *Amanita* and *Phalloideae* such as *Amanita bisporigera*, *A. brunneitoxicaria*, *A. exitialis, A. gleocystidiosa*, *A. phalloides* and *A. virosa*.[3,13,16–19] Both sections synthesize controlled cyclic peptide toxins which divided into amatoxins, phallotoxins and virotoxins.[20,21] Amatoxins are bicyclic octapeptides with an indole-(R)-sulphoxide bridge, while phallotoxins and virotoxins contains bicyclic heptapeptides with an indole/thio-ether bridge and monocyclic peptides with 2-methylsulphonyltryptophan, respectively.[20,21] These toxins can function in different ways. Amatoxins are specific inhibitors of RNA polymerase and prevent subsequent protein synthesis, whereas phallotoxins and virotoxins form complexes with actin and blocks microfilaments depolymerization of the cytoskeleton from liver and muscle cells.[13–15]

In Thailand, several poisoning cases of amanitas have been recorded including *Amanita digitosa*, *A. exitialis, A. fuliginea*, *A. gleocystidiosa*, *A. pyriformis* and *A. virosa*.[3,19,23] Our previous studies showed that the species containing amatoxins and phallotoxins were *Amanita exitialis*, *A. fuliginea* and *A. gleocystidiosa*.[3,23] In addition, we also found a highest alpha-amanitin concentration in *A. gleocystidiosa* of the section *Amanita*. Outbreaks of above toxic *Amanita* species were found in the northern and northeastern part of Thailand.[3] In this study, we focused on four major mushroom poisoning cases during 2015 to 2018. These cases were suspected phalloides syndrome poisoning. The first poisoning case (August 2015) reported from Yasothon Province included 7 patients and 1 death. The second confirmed case (July 2017) from Mahasarakham Province included 12 patients and 1 death. The last two cases (May to June 2018) reported from Ubon Ratchathani and Chanthaburi Provinces included 14 patients and 1 death. The remnant mushroom samples harvested by the patients were delivered to the Toxicology Center (National Institute of Health, Department of Medical Sciences). Based on their morphology, the remnant mushroom samples were primarily identified as the genus *Amanita*. However, the mushroom samples are not well preserved for species level diagnosis. Thus, we used the molecular techniques to help clarify their species. The nuclear ITS marker is one of the universal DNA barcode marker has been applied in various group of mushrooms for toxicological and clinical purposes.[2,3,7,24–27] Comparison with the ITS sequences deposited in GenBank showed that all mushroom samples were closely related to *Amanita brunneitoxicaria*. These sequences matched BLAST reference sequence with scores ranging from 98 % to 100% identity. Our phylogenetic analysis confirmed that the toxic mushrooms ingested are genetically similar to *A. brunneitoxicaria*. This species was a newly discovered species in Thailand.[13] Its general features include small to medium pileus, dark greyish brown, darkest at centre, non-striate margin; gill free, white; stipe cylindrical, white to greyish white; bulb subglobose with saccate volva and the presence of alpha-amanitin.[13] In northeastern region, amanitas are favourite food source and very common in local market during the wet season including *Amanita princeps*, *A. javanica, A. hemibapha* and *A. vaginata*. According to the poisoning case report revealed that most cases of poisoning occur in people who are not familiar with the local mushroom. Most of the cases revealed that the patients ate wild mushrooms that were thought to be the edible *A. vaginata*. Toxic *Amanita brunneitoxicaria* and *A. vaginata* belong to different sections. Morphologically,

A. brunneitoxicaria is similar to *A. vaginata* in having small to medium cap with dark greyish brown, stipe cylindrical with white to greyish white; however, *A. brunneitoxicaria* can be distinguished from *A. vaginata* by its non-striate of pileus margin.

So far, several liquid chromatography methods have been used for the detection of mushroom peptide toxins.[2,3,8,28,29] Here, we analysed lethal peptide toxins via multiple reactions monitoring (MRM) using tandem mass spectrometer with electrospray (ESI) source operating in positive mode. The MRM method was performed on a triple quadrupole mass spectrometer. The precursor ions of interest was preselected with the mass filter and induced to fragment of product ions by collision-induced dissociation (CID). Our result confirmed that three lethal peptide toxins of α-amanitin, β-amanitin, phallacidin were found in all mushroom samples ingesting by patients. The most abundant and stable product ions obtained from MRM-MS analysis were *m/z* 259.3, 259.0 and 330.3, respectively. According to Duffy, amanitins are potently toxic to humans with an approximate lethal dose of α-form 0.1 mg/kg of body weight which can be found in a single mushroom sample.[30] Based on our findings *Amanita brunneitoxicaria* contained α-amanitin with an average level of 0.04 to 0.5 µg/g wet weight. Having ingested the toxic *A. brunneitoxicaria*, all patients showed the first latent period within 4 to 14 hours of gastrointestinal phase, which is characterized by nausea, vomiting, abdominal pain and cramps as well as severe secretory diarrhea. The second latent period (14-24 hrs.) starts with the first signs of liver and renal damage. The clinical symptoms described here were similar to those of phalloides syndrome.[3,20,30]

In conclusion, we suggest that molecular based diagnostics as well as LC-MS/MS method are suitable for the rapid detection of toxic *Amanita* samples, independent of their complete morphology. These methods can separate the lethal peptide toxin-producing mushroom species; thus, they are urgently needed for the appropriate medical treatments of mushroom poisoning cases. Furthermore, the toxic *Amanita brunneitoxicaria* provided a new informative data for clinical studies.

Conflict of interest

The authors declare that there is no conflict of interest.

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References

[1] Chandrasrikul A, Suwanarit P, Sangwanit U, Lumyong S, Payapanon A, Sanoamuang N, et al. Checklist of Mushrooms (Basidiomycetes) in Thailand. Bangkok, Thailand: Office of Natural Resources and Environmental Policy and Planning; 2011.

[2] Leudang S, Sikaphan S, Parnmen S, Nantachaiphong N, Polputpisatkul D, Ramchiun S, et al. DNA-based identification of gastrointestinal irritant mushrooms in the genus *Chlorophyllum*: A food poisoning case in Thailand. J Heal Res 2017; 31(1): 41–9.

[3] Parnmen S, Sikaphan S, Leudang S, Boonpratuang T, Rangsiruji A, Naksuwankul K. Molecular identification of poisonous mushrooms using nuclear ITS region and peptide toxins: a retrospective study on fatal cases in Thailand. J Toxicol Sci 2016; 41(1): 65–76.

[4] Ratnasingham S, Hebert PDN. bold: The Barcode of Life Data System (http://www.barcodinglife.org). Mol Ecol Notes 2007; 7(3): 355–64.

[5] Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, et al. From the Cover: Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc Natl Acad Sci 2012; 109(16): 6241–6.

[6] Judge BS, Ammirati JF, Lincoff GH, Trestrail JH, Matheny PB. Ingestion of a newly described North American mushroom species from Michigan resulting in chronic renal failure: *Cortinarius orellanosus*. Clin Toxicol (Phila) 2010; 48(6): 545–9.

[7] Gausterer C, Penker M, Krisai-Greilhuber I, Stein C, Stimpfl T. Rapid genetic detection of ingested *Amanita phalloides.* Forensic Sci Int Genet 2014; 9(1): 66–71.

[8] Zhang S, Zhao Y, Li H, Zhou S, Chen D, Zhang Y, et al. A simple and High-Throughput analysis of amatoxins and phallotoxins in human plasma, serum and urine using UPLC-MS/MS combined with PRiME HLB µElution Platform. Toxins (Basel) 2016; 8(5): 128. doi: 10.3390/toxins 8050128

[9] Gardes M, Bruns TD. ITS primers with enhanced specificity for basidiomycetes--application to the identification of mycorrhizae and rusts. Mol Ecol 1993; 2(2): 113–8.

[10] White TJ, Bruns TD, Lee SB, Taylor JW. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J. & White T., editor. PCR Protocols: a guide to methods and applications. New York: Academic Press; 1990.

[11] Zhang Z, Schwartz S, Wagner L, Miller W. A greedy algorithm for aligning DNA sequences. J Comput Biol 2000; 7(1–2): 203–14.

[12] Morgulis A, Coulouris G, Raytselis Y, Madden TL, Agarwala R, Schaffer AA. Database indexing for production MegaBLAST searches. Bioinformatics 2008; 24(16): 1757–64.

[13] Thongbai B, Miller SL, Stadler M, Wittstein K, Hyde KD, Lumyong S, et al. Study of three interesting *Amanita* species from Thailand: Morphology, multiple-gene phylogeny and toxin analysis. PLoS One 2017; 12(8): e0182131.

[14] Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 2014; 30(9): 1312–3.

[15] Zhang L, Yang J, Yang Z. Molecular phylogeny of eastern Asian species of *Amanita* (Agaricales, Basidomycota): taxonomic and biogeographic implications. Fungal Divers 2004; 17: 219–38.

[16] Li P, Deng W, Li T. The molecular diversity of toxin gene families in lethal *Amanita* mushrooms. Toxicon 2014; 83: 59–68.

[17] Cai Q, Tulloss RE, Tang LP, Tolgor B, Zhang P, Chen ZH, et al. Multi-locus phylogeny of lethal amanitas : Implications for species diversity and historical biogeography. BMC Evol Biol 2014; 14: 1–16.

[18] Yang Z-L, Li T-H. Notes on three white *Amanitae* of section *Phalloideae* (Amanitaceae) from China. Mycotaxon 2001; 78: 439–48.

[19] Chaiear K, Limpaiboon R, Meechai C, Poovorawan Y. Fatal mushroom poisoning caused by *Amanita virosa* in Thailand. Southeast Asian J Trop Med Public Health 1999; 30(1): 157–60.

[20] Bresinsky A, Besl H. A colour atlas of poisonous fungi: A handbook for pharmacists, doctors, and biologists. London: Wolfe Publishing Ltd; 1990.

[21] Garcia J, Costa VM, Carvalho A, Baptista P, de Pinho PG, de Lourdes Bastos M, et al. A*manita phalloides* poisoning: Mechanisms of toxicity and treatment. Food Chem Toxicol 2015; 86: 41–55.

[22] Hallen HE, Luo H, Scott-Craig JS, Walton JD. Gene family encoding the major toxins of lethal *Amanita* mushrooms. Proc Natl Acad Sci U S A 2007; 104(48): 19097–101.

[23] Li GJ, Hyde KD, Zhao RL, Hongsanan S, Abdel-Aziz FA, Abdel-Wahab MA, et al. Fungal diversity notes 253--366: taxonomic and phylogenetic contributions to fungal taxa. Fungal Divers 2016;1–237.

[24] Nugent KG, Saville BJ. Forensic analysis of hallucinogenic fungi: A DNA-based approach. Forensic Sci Int 2004; 140(2–3): 147–57.

[25] Kowalczyk M, Sekuła A, Mleczko P, Olszowy Z, Kujawa A, Zubek S, et al. Practical aspects of genetic identification of hallucinogenic and other poisonous mushrooms for clinical and forensic purposes. Croat Med J 2015; 56(1): 32–40.

[26] Zuber A, Kowalczyk M, Sekuła A, Mleczko P, Kupiec T. Methods used in species identification of hallucinogenic and other poisonous mushrooms in forensic investigations. Problem Forensic Sci 2011; 86: 151–61.

[27] Maeta K, Ochi T, Tokimoto K, Shimomura N, Maekawa N, Kawaguchi N, et al. Rapid species identification of cooked poisonous mushrooms by using real-time PCR. Appl Environ Microbiol 2008; 74(10): 3306–9.

[28] Li C, Wei F, Muhammad S, Yang G, Wang S, Liu X. A cost-effective LC-MS/MS method for identification and quantification of alpha-amanitin in rat plasma: Application to toxicokinetic study. J Chromatogr B 2017; 1064: 36–9.

[29] Helfer AG, Meyer MR, Michely J a., Maurer HH. Direct analysis of the mushroom poisons α- and β-amanitin in human urine using a novel on-line turbulent flow chromatography mode coupled to liquid chromatography-high resolution-mass spectrometry/mass spectrometry. J Chromatogr A 2014; 1325: 92–8.

[30] Duffy TJ. Toxic Fungi of Western North America [Internet]. MykoWeb; 2008. [cited 2018 August 14]. Available from: www.mykoweb.com

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Prevalence of single and double thalassemia carriers in pregnant women and spouses: Case study of Sawanpracharak Hospital

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ABSTRACT

Background: Thalassemia is common in Thailand. Knowing the prevalence of thalassemia carriers in married couples would lead to proper management of this disease.

Objectives: This study was aimed to survey the prevalence of thalassemia carriers in pregnant women and spouses attending the antenatal care (ANC) unit at Sawanpracharak Hospital, Nakhorn Sawan Province, Thailand. Criterions to differentiate double thalassemia carriers and single thalassemia carriers were also aimed to propose.

Materials and methods: A retrospective study was conducted. Data of red cell parameters, hemoglobin typing, and globin gene genotyping were collected during 2012 to 2017. Study protocol was reviewed and approved by the Research Ethic Committee, Sawanpracharak Hospital.

Results: Both thalassemia diseases and carriers were found in the studied cohort. Prevalence of thalassemia carriers was 56.0% in which 7.6% found to be double thalassemia carriers of HbE/α thalassemia 1 (SEA and non-SEA type). HbE ($+A_2$), RBC count, MCV, MCH, and RDW were significantly different between the single HbE carriers and the double HbE/SEA-α thalassemia 1 carriers. Testing the previously established HbE, MCV, and MCH cutoff points demonstrated high efficiency in detecting this major double carrier.

Conclusion: Thalassemia and hemoglobinopathies were common in the married couples attending the ANC clinic at Sawanpracharak hospital. Both single and double thalassemia carriers were existed. The double HbE/SEA-α thalassemia 1 carrier was predominated and could be detected efficiently by the previously established cutoff points of HbE, MCV, and MCH.

Introduction

Thalassemia and hemoglobinopathies are inherited chronic anemia commonly found in Thailand. Genes of these disorders are inherited in an autosomal recessive fashion. Those having these genes in heterozygous or doubly heterozygous forms are called single and double thalassemia carriers, respectively. These carriers are in fact clinically asymptomatic. However, those having the genes for thalassemia and hemoglobinopathies in homozygote or compound heterozygote form have the clinical symptoms of thalassemia disease.[1] The most severe cases of thalassemia disease; *e.g*. Hb Bart's hydrops fetalis, always die before birth and just after birth and may induce toxemia of pregnancy in mother. In contrast, those severe thalassemia patients who are alive always require regular blood transfusion, and suffer from several clinical complications.

Prevalence of thalassemia carriers is high in Thailand, reaching the nationwide frequency of about 40%. The prevalence

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of the α-thalassemia carriers (both α-thalassemia 1 and α-thalassemia 2) is about 20-30%, while that of the β-thalassemia, HbE and Hb Constant Spring are 3-9%, 54%, and 8%, respectively.[2] Thus, it is highly possible that both single and double carriers exist in the thalassemia carriers encountered in Thailand.

Presence of double α/β-thalassemia carriers and double HbE/α-thalassemia carriers in samples initially diagnosed as the β-thalassemia carriers and HbE carriers has be noted in several studies. In Malaysia, prevalence of double α/β-thalassemia carriers were found to be 12.7%, in which 7.8% were the double carriers of the SEA-α thalassemia 1/β-thalassemia.[3] In China, 4.4% prevalence of double the SEA-α thalassemia 1/β-thalassemia was demonstrated.[4] In Thailand, survey in Lampang and Chiang Mai found that 6.4% of the β-thalassemia carriers and 15.2% of the HbE carriers had the co-existing α-thalassemia. Prevalence of the double SEA-α thalassemia 1/β-thalassemia carriers and double HbE/SEA-α thalassemia 1 carriers were found to be 5.2% and 11.2%, respectively, in this study.[5] Thus, it is highly possible for the double thalassemia carriers to exist in other parts of Thailand. Most importantly, survey of this kind has never been substantially conducted in the central part of Thailand.

Materials and methods

Study design

A retrospective study was performed in this research. A total of 382 laboratory records of married couples attending the antenatal care (ANC) unit, Sawanpracharak Hospital, during the years 2012-2017 were collected. These records included red blood cell parameters (red blood cell count (RBC), hemoglobin concentration (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and red cell distribution width (RDW)), hemoglobin typing, SEA-α thalassemia 1 genotype. The protocol was initially reviewed and approved by the Research Ethic Committee, Swanpracharak Hospital (COE No. 02/2561).

Red blood cell parameters determination

Red blood cell parameters including RBC count (10^6/μL), Hb (g/dL), Hct (%), MCV (fL), MCH (pg) were determined by an automated blood cell analyzer (Sysmex XN-1000™ Hematology Analyzer, Kobe, Japan).

Hemoglobin (Hb) typing

Hb typing to determine the relative quantities of hemoglobin in blood was performed by both the cation-exchange high performance liquid chromatography (HPLC) (Beta-Thalassemia Short Program, Variant™Hb Testing System; Bio-Rad Laboratories, Hercules, California, USA) and capillary zone electrophoresis (CZE) (Capillarys 2 System; Sebia, EvryCedex, France). Normal Hb types of A_2A was seen in normal individuals, α-thalassemia carriers, and β-thalassemia carriers. Normal individuals and α-thalassemia carriers had HbA_2<3.5% (2.6±0.36% for normal, 2.3±0.47% for α-thalassemia 1 carriers), while the β-thalassemia carriers had HbA_2 from 3.5 to 10% (5.5±1.26%).[6] The HbE carriers had Hb types of AE (+A_2) by the cation exchange HPLC with the HbE (+A_2) levels of 27.8±7.50%.[6] In the CZE, Hb types of A, E, and A_2 were seen with HbE levels of 24.0±3.0% and HbA_2 levels of 4.0±0.4%.[7] However, HbE levels plus HbA_2 levels were reported in cases of CZE method.

SEA-α thalassemia 1 genotyping

The SEA deletion of α-thalassemia 1 (SEA-α thalassemia 1) was identified by modified Gap-PCR and relative quantitative PCR with dissociation curve analysis described elsewhere.[8,9] In the modified Gap-PCR, the amplified products sized 185 bp were specific for the SEA deletion, while those of 314 bp were specific for wild type allele. Thus, carriers of the SEA-α thalassemia 1 had two PCR products sized 185 bp and 314 bp. In contrast, normal individuals had only one PCR products sized 314 bp. In the relative quantitative PCR, the dissociation temperature (Td) of SEA-α thalassemia 1 was 86 °C, being the same for both heterozygote and homozygote. Heterozygote of the SEA-α thalassemia 1 was characterized, however, by calculating the threshold cycle (Ct) difference or delta Ct (ΔCt) values between the SEA-α thalassemia 1 gene and the albumin gene. The ΔCt values of normal, SEA-α thalassemia 1 heterozygote or carrier, and SEA-α thalassemia 1 homozygote were 11.99±2.88, 6.87±1.80 and 2.26±0.62, respectively.

Criterion of making the final diagnosis

RBC parameters, types and quantities of hemoglobin, and Gap-PCR results were taken together to establish the final diagnosis of thalassemia in all recorded subjects as shown in Table 1.

Table 1. Criterion for the final diagnosis of thalassemia and hemoglobinopathies used in the study.

Diagnosis	Hb (g/dL)/Hct (%)	MCV (fL)/MCH (pg)	Hb types	Gap-PCR for SEA-α thalassemia 1
Normal	≥12/≥36	≥80/≥27	A_2A, A_2≤3.5%	Negative
Single SEA-α thalassemia 1 carrier	Variable	<80/<27	A_2A, A_2≤3.5%	Positive
Single β-thalassemia carrier	Variable	<80/<27	A_2A, A_2:3.6-8%	Negative
Single HbE carrier	Variable	Variable	EA	Negative
Double HbE/SEA-α thalassemia 1 carriers	Variable	Variable	EA	Positive
HbE/β-thalassemia	Variable	Variable	EF	Negative
Double HbE/β-thalassemia with SEA-α thalassemia 1 carriers	Variable	Variable	EF	Positive
Homozygous HbE	Variable	Variable	EE	Negative

Table 1. Criterion for the final diagnosis of thalassemia and hemoglobinopathies used in the study. (continuous)

Diagnosis	Hb (g/dL)/Hct (%)	MCV (fL)/MCH (pg)	Hb types	Gap-PCR for SEA-α thalassemia 1
Double homozygous HbE and SEA-α thalassemia 1 carriers	Variable	Variable	EE	Positive
Double HbE/non SEA-α thalassemia 1 carriers	Variable	Variable	AE, E ≤20%	Negative
HbH disease	Variable	Variable	A_2AH	Positive
Non-SEA α thalassemia 1 carrier	Variable	<80/<27	A_2A, A_2≤3.5%	Negative
Hb Constant Spring homozygote	Variable	Variable	A_2ACS	Negative
AE Bart's disease	Variable	Variable	AE Bart's	Positive
HbE/HbKU	Variable	Variable	E with KU	Negative
Other abnormal hemoglobin	Variable	Variable	A2A with Abn Hb	Negative

Note. HbKU: Hb Korle-BU (β73:Asp->Asn)

Statistical analysis

Statistical analysis including descriptive statistics (mean, standard deviation (SD), standard error of the mean (SE), and inferential statistics (Student's t-test) were carried out using the statistical software. The p value of less than 0.05 was considered significantly different.

Results

Demographic data

Records of 382 married couples including 190 males aged 26.06±6.83 years old and 192 females aged 22.94±5.69 years old were studied. The subjects were 367 Thai (96.1%) and 15 Burmese (3.9%). For Thais, 345 (90.3%) were from the central Thailand, 13 (3.4%) were from the northern Thailand, 8 (2.1%) were from the northeastern Thailand, and 1 (0.3%) was from the southern part of Thailand. Majority of the cases were from Nakhon Sawan Province.

Prevalence of thalassemia carriers

Based on the results of Hb typing and the modified Gap-PCR, 66.2% of the subjects had thalassemia phenotypes (carriers and diseases), while 56.0% were carriers of α-thalassemia, β-thalassemia and HbE. Additionally, 7.6% of the subjects were found to be double carriers of HbE/α-thalassemia 1 (SEA and non SEA types). The rest double forms of thalassemia included double carriers of the HbE/β-thalassemia with SEA-α thalassemia 1, and of homozygous HbE and SEA-α thalassemia 1 (Table 2).

Table 2. Thalassemia phenotypes in 382 subjects.

Diagnosis	Thai No. (%)	Burmese No. (%)	Possible genotypes
Normal	119 (31.2)	11 (2.9)	β^A/β^A, αα/αα
Single SEA-α thalassemia 1 carrier	53 (13.9)		$^{--SEA}$/αα
Single β-thalassemia carrier	60 (15.7)	1 (0.3)	β^T/β^A
Single HbE carrier	67 (17.5)	1 (0.3)	β^E/β^A
Double HbE/SEA-α thalassemia 1 carriers	24 (6.3)		β^E/β^A, $^{--SEA}$/αα
HbE/β-thalassemia	8 (2.1)		β^E/β^T
Double HbE/β-thalassemia with SEA-α thalassemia 1 carriers	1 (0.3)		β^E/β^T, $^{--SEA}$/αα
Homozygous HbE	17 (4.4)	1 (0.3)	β^E/β^E
Double homozygous HbE and SEA-α thalassemia 1 carriers	2 (0.5)		β^E/β^E, $^{--SEA}$/αα
Double HbE/non SEA-α thalassemia 1 carriers	4 (1.0)	1 (0.3)	β^E/β^A, --/αα
HbH disease	5 (1.3)		$^{--SEA}$/-α
Non SEA-α thalassemia 1 carrier	3 (0.8)		--/αα
Hb Constant Spring homozygote	1 (0.3)		$\alpha^{CS}\alpha/\alpha^{CS}\alpha$
AEBart's disease	1 (0.3)		β^E/β^A, $^{--SEA}$/ -α
HbE/HbKU	1 (0.3)		β^E/β^{KU}
Other abnormal hemoglobin	1 (0.3)		β^{Abn}/β^A

Note. β^A: Normal β-globin chain, β^E: HbE globin chain, β^T: β-thalassemia globin chain, β^{KU}: Hb Korle-Bu globin chain (β73;Asp->Asn), β^{Abn}: Unidentified abnormal β-globin chain, α^{CS}: Hb Constant Spring globin chain

Hemoglobin pattern in single and double forms of thalassemia.

Hemoglobin pattern of A_2A was observed in both single β-thalassemia carrier and double β-thalasemia/SEA-α thalassemia 1 carriers. AE patterns were observed for both single HbE carrier and double HbE/SEA-α thalassemia 1 carriers. In addition, the EF pattern was observed in single HbE/β-thalassemia and double HbE/β-thalassemia with SEA-α thalassemia 1 carriers. Finally, the EE pattern was the seen in single homozygous HbE and double homozygous HbE/SEA-α thalassemia 1 carriers.

Although types of hemoglobin in blood of both single and double form of thalassemia were the same, the levels of these hemoglobins were found to be obviously different. HbE ($+A_2$) levels were lower in double HbE/SEA-αthalassemia 1 carriers than those of single HbE carrier. Levels of HbE and HbF were higher in the single HbE/β-thalassemia than those of double HbE/β-thalassemia with SEA-α thalassemia 1 carrier. In addition, the HbE levels were higher in double homozygous HbE/SEA-α thalassemia 1 carriers than those of the single homozygous HbE. Detail of this information is shown in Table 3 and Figure 1.

Table 3. Hb typing patterns in single and double forms of thalassemia. "a" and "b" indicate statistically significant difference (p<0.05), "c" indicates no difference (p>0.05).

Diagnosis	Hb types	HbA_2 level (%)	$HbE(+A_2)$ level (%)
Normal (128)	A_2A	2.9±2.2 [a, c]	
Single SEA-α thalassemia 1 carrier (53)	A_2A	2.8±2.1[a, c]	
Single β-thalassemia carrier (61)	A_2A	5.7±0.7[a]	
Single HbE carrier (68)	EA		26.1±3.5[b]
Double HbE/SEA-α thalassemia 1 carriers (24)	EA		20.3±2.9[b]
Single homozygous HbE (18)	EE		87.3±3.7[c]
Double homozygous HbE/SEA-α thalassemia 1 carriers (2)	EE		92.2±9.9[c]

Note: a and b: statistically significant difference (p<0.05), c: no difference (p>0.05)

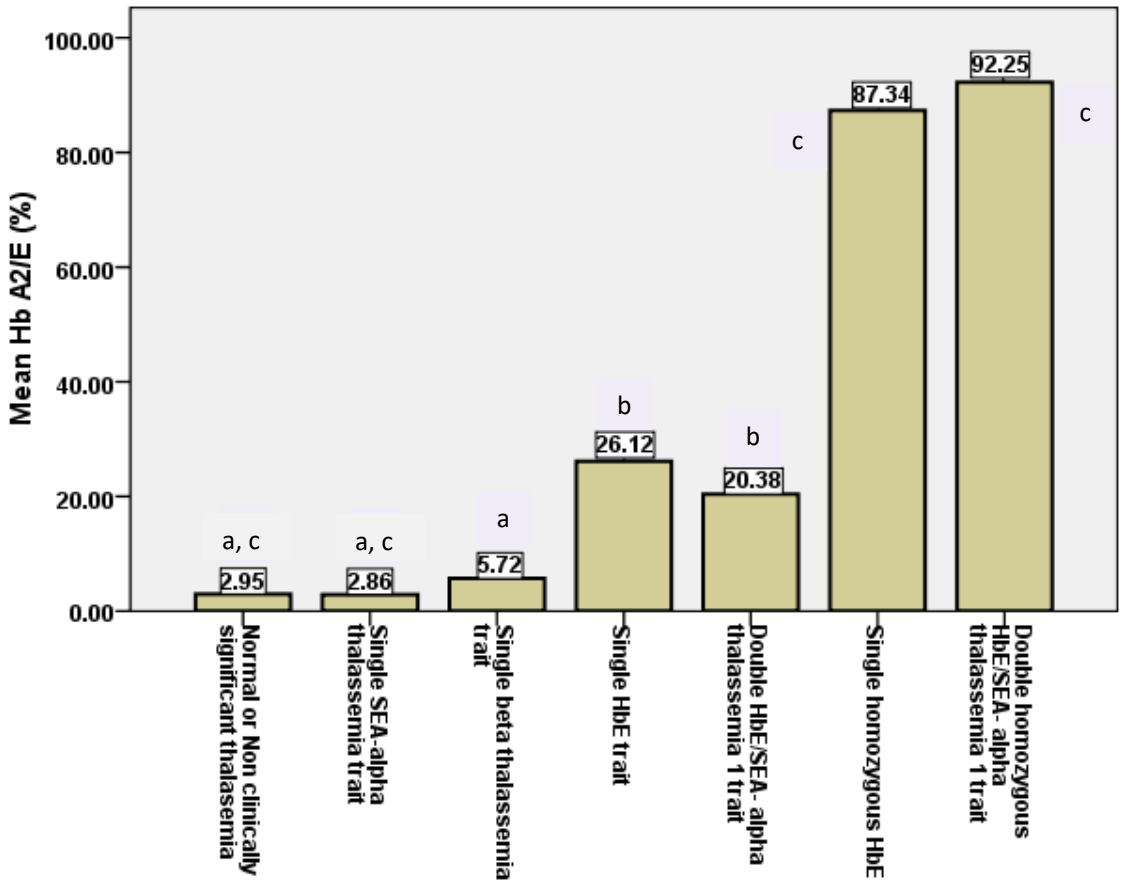

Figure 1 *Bar chart demonstrating the levels of Hbs A_2/E in single HbE carrier and double HbE/SEA-α thalassemia 1 carriers.*
Note: *a, b: significant difference within the tested groups (p<0.05), c: no difference in the pair tested (p>0.05). Mean values are placed at the top of each bar. Trait is a synonym for carrier.*

Red cell parameters of single and double form of thalassemia and hemoglobinopathies.

In HbE carrier group, RBC count, MCV, and MCH were significantly different between the single HbE carrier and double HbE/SEA-α thalassemia 1 carriers. RBC count was higher, while MCV and MCH were significantly lower in double HbE/SEA-α thalassemia 1 carriers than the single HbE carrier (Student's t test $p<0.05$) (Table 4, Figure 2).

In homozygous HbE group, 2 cases of double homozygous HbE/SEA-α thalassemia 1 carrier and homozygous HbE were encountered. RBC count, Hb, Hct, and MCV tended to be higher and RDW seemed to be lower in double homozygous HbE/SEA-α thalassemia 1 carrier than the single homozygous HbE. In contrast MCH were almost similar (Table 4, Figure 2).

Presence of the SEA-α thalassemia 1 carrier in the HbE/β-thalassemia seemed to reduce the hematological severity compared to the HbE/β-thalassemia without the SEA-α thalassemia 1 carrier. This was shown by higher levels of RBC count, Hb, Hct, and lower RDW in the HbE/β-thalassemia with co-existence of the SEA-α thalassemia 1 than in the HbE/β-thalassemia without the SEA-α thalassemia 1 trait.

Table 4. Red blood cell parameters (mean±SD) in the single and double form of thalassemia analyzed in this study.

Diagnosis	RBC count (10^6/uL)	Hb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	RDW (%)
Normal (105)	5.06±0.79	12.3±1.8	37.9±5.3	75.4±4.9	24.6±1.9	15.3±2.5
Single SEA-α thalassemia 1 carrier (49)	5.66±0.99	12.2±1.8	38.1±6.7	67.4±3.9	21.2±3.5	16.7±2.8
Single β-thalassemia carrier (52)	5.48±0.88	12.0±1.9	37.2±5.7	68.5±7.2	22.1±2.7	16.1±1.3
Single HbE carrier (56)	4.85±0.8	12.2±2.0	37.1±5.9	77.1±5.8	25.4±2.2	15.1±1.5
Double HbE/SEA-α thalassemia 1 carriers (24)	5.52±1.1	11.6±2.3	36.0±7.3	68.0±6.8	22.0±2.5	16.4±2.5
Single homozygous HbE (11)	5.50±1.00	11.8±1.7	34.8±7.3	64.8±4.8	20.7±1.5	16.8±1.5
Double homozygous HbE/SEA-α thalassemia 1 carriers (2)	5.78±0.58	12.0±1.5	37.8±5.5	65.3±3.1	20.7±0.6	16.1±0.1

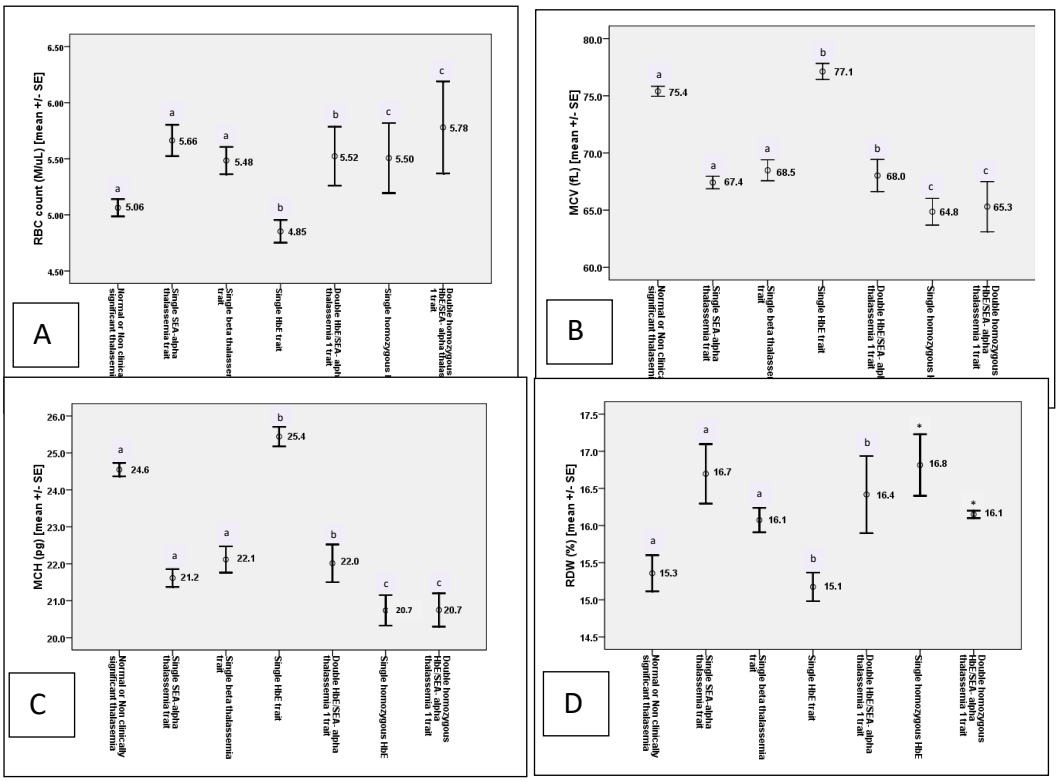

Figure 2 *Comparisons of RBC count (A), MCV (B), MCH (C), and RDW (D) between normal and single thalassemia carriers and double thalassemia carriers.*
Note: *a, b, *: significant difference within the tested groups (p<0.05), c: indicates no difference in the pair tested (p>0.05)*

Testing the previously established cutoff values to detect double HbE/SEA-α thalassemia 1 carriers.

As it was clear that the double HbE/SEA-α thalassemia 1 carriers predominated in this cohort, we then tested the previously established cutoff points in these 24 cases of this phenotype. These cutoff points relied on Hb levels. For Hb<10 g/dL, the cutoff points for HbE, MCV, MCH were 21.1%, 64.9 fL, 21.0 pg, respectively. For Hb 10-11.9g/dL, the cutoff points were 25.6%, 72.8%, 23.9 pg for HbE, MCV, MCH, respectively. Finally, for Hb≥12.0 g/dL, the cutoff points for HbE, MCV, MCH were 27.1%, 76.7 fL, 25.3 pg, respectively.[10] These cutoff points were found to have 94.7% sensitivity, 83.3% specificity, 64.3% positive predictive value (PPV), and 98.0% negative predictive value (NPV) in detecting the double HbE/SEA-α thalassemia 1 carriers in this cohort.

Discussion

This study has surveyed among the married couples for the prevalence of thalassemia in both carrier and disease forms. Majority of the subjects was from Nakhon Sawan and other provinces in the central Thailand. The high prevalence with the mixture of thalassemia found in this study should indicate that this disorder was also common in the central Thailand. Prevention and control scheme to detect the carriers of thalassemia must be implemented in order to prevent and control the emerge of new thalassemia patients.

Survey for thalassemia in antenatal care unit was performed previously in Mahararaj Nakorn Chiang Mai Hospital in which most of the subjects were from Chiang Mai Province.[11] This study found 25.6% prevalence of thalassemia (both carrier and disease) in their series. The finding in this study contrasted that in Chiang Mai survey by the higher prevalence of thalassemia and hemoglobinopathies. This emphasized the problem of this disorder in this geographic region, especially Nakhon Sawan Province since most of the subjects were of Nakhon Sawan origin.

Presence of double form of thalassemia and hemoglobinopathies were obvious in this study, being higher than that observed in Chiang Mai by Wanapirak, et al.[11] This information highlighted the problem of the double form of thalassemia in Nakhon Sawan Province and, possibly, other provinces in the central Thailand. Therefore, the laboratory personnel involved in thalassemia diagnosis must be greatly aware. Failure to detect double α/β-thalassemia carriers or HbE/SEA-α thalassemia 1 carriers can lead to the birth of the Hb Bart's hydrops fetalis babies which is not initially concerned.

The double α/β-thalassemia carriers and double HbE/α-thalassemia carriers have some laboratory results different from those in the single carriers. MCV, for instance, tends to be higher in double α/β-thalassemia carriers than the single β-thalassemia carriers.[12-14] However, these differences were not obvious. In contrast, both MCV, MCH, and HbE were shown previously to be significantly lower in double HbE/α-thalassemia 1 carriers than the single HbE carriers.[5,10,15,16] The result of the present study was identical to that observed in those studies. These similar findings indicated that MCV, MCH and HbE were the best parameters to be used to detect double HbE/SEA-α thalassemia 1carriers. Thus, we tested the cutoff points of MCV, MCH, and HbE as set up by Leckngam et al in this series of HbE carriers. The high sensitivity and moderate specificity demonstrated in this study indicated that MCV, MCH, HbE were suitable to screen out the double HbE/SEA-α thalassemia 1 carriers from the single HbE carriers.

In conclusion, this study clearly demonstrated the occurrence of variety of thalassemia as well as double thalassemia carriers in Sawanpracharak Hospital. The double HbE/SEA-α thalassemia 1 carriers predominated and could be simply screened by using MCV, MCH, and HbE, which are easily accessible for medical technologists. The information emerged from this study should alert clinicians, nurses, medical technologists, and other allied health personnel involved in thalassemia diagnosis in this part of Thailand to be aware of the existence of double thalassemia carriers, *i.e*. to prevent misdiagnosis of this fatal disorder.

Conflict of interest

None

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References

[1] Weatherall DJ, Clegg JB. The Thalassaemia Syndromes (Fourth edition). Oxford: Blackwell Scientific; 2001.

[2] Fucharoen S, Winichagoon P, Thonglairuam V. β-thalassemia associated with α-thalassemia in Thailand. Hemoglobin 1988; 12(5-6): 581-92.

[3] Wee YC, Tan KL, Kuldip K, Tai KS, George E, Tan PC, et al. α-thalassaemia in association with β-thalassaemia patients in Malaysia: a study on the co-inheritance of both disorders. Community Genet 2008; 11(3): 129-34.

[4] Li D, Liao C, Li J, Xie X, Huang Y, Zhong H. Detection of α-thalassemia in β-thalassemia carriers and prevention of Hb Bart's hydrops fetalis through prenatal screening. Haematologica 2006; 91(5): 649-51.

[5] Tatu T, Kiewkarnkha T, Khuntarak S, Khamrin S, Suwannasin S, Kasinrerk W. Screening for co-existence of α-thalassemia in β-thalassemia and in HbE heterozygotes via an enzyme-linked immunosorbent assay for Hb Bart's and embryonic ζ-globin chain. Int J Hematol 2012; 95(4): 386-93.

[6] Fucharoen S, Winichagoon P, Wisedpanichkij R, Sae-Ngow B, Sriphanich R, Oncoung W, et al. Prenatal and postnatal diagnoses of thalassemias and hemoglobinopathies by HPLC. Clin Chem 1998; 44(4): 740-8.

[7] Sangkitporn S, Sangkitporn SK, Tanjatham S, Suwannakan B, Rithapirom S, Yodtup C, et al. Multicenter validation of fully automated capillary electrophoresis method for diagnosis of thalassemias and hemoglobinopathies in Thailand. Southeast Asian J Trop Med Public Health 2011; 42(5): 1224-32.

[8] Sangkitporn SK, Wangkahat K, Sangnoi A, Songkharm B, Charoenporn P, Sangkitporn S. Rapid diagnosis of α^{O}-thalassemia using the relative quantitative PCR and the dissociation curve analysis. Clin Lab Haematol 2003; 25(6): 359-65.

[9] Siriratmanawong N, Pinmuang-Ngam C, Fucharoen G, Fucharoen S. Prenatal diagnosis of Hb Bart's hydrops fetalis caused by a genetic compound heterozygosity for two different α-thalassemia determinants. Fetal Diagn Ther 2007; 22(4): 264-8.

[10] Leckngam P, Limweeraprajak E, Kiewkarnkha T, Tatu T. The Hb E (HBB: c.79G>A), Mean corpuscular volume, mean corpuscular hemoglobin cutoff points in double heterozygous Hb E/- -(SEA) α-thalassemia-1 carriers are dependent on hemoglobin levels. Hemoglobin 2017; 41(1): 38-43.

[11] Wanapirak C, Muninthorn W, Sanguansermsri T, Dhananjayanonda P, Tongsong T. Prevalence of thalassemia in pregnant women at Maharaj Nakorn Chiang Mai Hospital. J Med Assoc Thai 2004; 87(12): 1415-8.

[12] Melis MA, Pirastu M, Galanello R, Furbetta M, Tuveri T, Cao A. Phenotypic effect of heterozygous α and β^{O}-thalassemia interaction. Blood 1983; 62(1): 226-9.

[13] Siriratmanawong N, Fucharoen G, Sanchaisuriya K, Ratanasiri T, Fucharoen S. Simultaneous PCR detection of β - thalassemia and α - thalassemia 1 (SEA type) in prenatal diagnosis of complex thalassemia syndrome. Clin Biochem 2001; 34(5): 377-80.

[14] Rosatelli C, Falchi AM, Scalas MT, Tuveri T, Furbetta M, Cao A. Hematological phenotype of the double heterozygous state for α and β thalassemia. Hemoglobin 1984; 8(1): 25-35.

[15] Charoenkwan P, Wanapirak C, Thanarattanakorn P, Sekararithi R, Sae-Tung R, Sittipreechacharn S, et al. Hemoglobin E levels in double heterozygotes of hemoglobin E and SEA-type α-thalassemia. Southeast Asian J Trop Med Public Health 2005; 36(2): 467-70.

[16] Sanchaisuriya K, Chirakul S, Srivorakun H, Fucharoen G, Fucharoen S, Changtrakul Y, et al. Effective screening for double heterozygosity of Hb E/α^{O}-thalassemia. Ann Hematol 2008; 87(11): 911-4.

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High-resolution melting-curve analysis for serotyping of *Salmonella* spp. group B isolated from minced pork in the Northern part of Thailand

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ABSTRACT

Background: Nontyphoidal *Salmonella* spp. is the major bacterial cause of food poisoning. Conventional serotyping is complicated and time consuming.

Objectives: To establish a rapid molecular - based screening for *Salmonella* serotypes

Materials and methods: Several aspects of *Salmonella* isolates were characterized by both rapid multiplex real-time PCR and high-resolution melting-curve (HRM) analysis. Group B *Salmonella* isolates (n=29) were isolated from 165 of minced pork samples randomly collected from local markets in six provinces of the Northern Thailand. Several genetic determinants responsible to specific phenotypes were selected, including *Salmonella* spp. (*Inv*A), *Salmonella* Serotypes (*flj*B, *gyr*B and *ycf*Q), Beta lactam resistance including serious ESBL determinants (*bla*TEM, *bla*CTX-M).

Results: HRM serotyping successfully revealed the epidemiological prevalence of three *Salmonella* serotypes from all group B *Salmonella* isolates, including 38% *S.* Stanley, 24% *S.* Typhimurium, and 17% *S.* Monophasic. Further conventional serotyping showed five unknown HRM patterns as *S.* Agona, *S.* Schwarzengrund, *S.* Saintpaul, *S.* Brandenburg and one unknown serotype. Fifty-five percent of the isolates showed multidrug-resistant phenotype. The high prevalence of *bla*TEM gene totally corresponded to the observed ampicillin-resistant phenotype. However, the presence of *bla*CTX-M group 1 was widely observed but not corresponded to its expected ESBL phenotype. Melt curve analysis of the observed *bla*CTX-M group 1 amplicons compared with the positive ESBL gene (*bla*CTX-M -55) showed the high difference in the melting temperature (T_m) of those amplicons which indicated that the observed *bla*CTX-M group 1 amplicons were less likely to be ESBL gene. Only one ESBL *Salmonella* isolate from Nan province showed the presence of *bla*CTX -M group 9 with ESBL phenotype. The highly virulent ESBL *Salmonella* serovar Typhimurium encoding *bla*CTX-M group 9 in contaminated minced pork from the Nan province suggested the high alert for the rapid screening of ESBL producing *Salmonella* spp. in meat and animals to prevent a potential future outbreak.

Conclusion: By performing the molecular analysis, this study successfully revealed the importantly epidemiological aspects of the *Salmonella* isolates group B collected from the Northern Thailand. This approach should simplify the screening for *Salmonella* serotypes in minced pork.

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Introduction

Nontyphoidal *Salmonella* (NTS) is the major pathogen causing gastroenteritis to mostly young children.[1] Commonly, *Salmonella*-infected victims were initially exposed to the contaminated environment and usually infected by the non-hygienic behaviour of the victims. Nontyphoidal *Salmonella* spp. has been classically classified by the different cellular appearance of somatic antigens (O antigens) combined with flagellar antigens (H antigens). To initially determine *Salmonella* serogroups, only unique O antigen was initially diagnosed to more than 50 distinct groups.[2] For the complete serotyping, the determination of phase 1 and 2 H antigens was executed to derive the serotype or unique antigenic combinations between O and H antigens according to the complete standard *Salmonella* typing system (Kauffmann-White scheme).[2]

In Thailand, the incidence of salmonellosis was documented through the epidemiological survey of both governmental agencies[3] and independent research.[4] The conventional typing of *Salmonella* serotypes was usually performed as the standard technique for identifying *Salmonella* serotypes. The epidemiological results showed that some specific *Salmonella* serogroups predominated in certain area causing regional health problems; nevertheless, different types of samples showed various distribution of some serogroups especially group B which was reported to be the most predominated and virulent serogroup.[4] The distribution of *Salmonella* serovars in Thailand is slightly different throughout various geographical regions[5] but significantly different among continents.[6] In Thailand, the most common serovars of *Salmonella* spp. isolated from humans was *Salmonella* Group B including Typhimurium, Anatum, Derby, and Stanley.[7]

Multi-Drug Resistance (MDR) was observed frequently in the major prevalent nontyphoidal *Salmonella* serovars found in human gastroenteritis and the current occurrence has been increased and related significantly to *Salmonella* spp. group B.[4] In Thailand, the significant increase of the second-line antibiotic ceftriaxone, amikacin and kanamycin resistance was noticed and could possibly be related to the over-use of antibiotics to livestock feed and medical treatment.[8] In addition, the resistance to the effective quinolone drugs such as nalidixic acid was also reported.[9] The failure of antibiotic treatment from nearly all third-generation cephalosporin antibiotics, classified as Extended Spectrum β-Lactamases (ESBLs) has created the major threat to the unresponsive cases toward the first and second line therapies which currently has been the subject of active clinical epidemiological research.[10] ESBL genes include those encoding various beta-lactamases such as the classical *bla*TEM, *bla*SHV and *bla*OXA group and the newer *bla*CTX-M, *bla*CMY and *bla*DHA group usually observed with different levels of virulence and epidemiological capability of transmission.[11] ESBL *Salmonella* spp. encoding *bla*CTX-M are of concern due to their capability for global dissemination through both clonal and horizontal transfer of the gene.[12]

The standard method for *Salmonella* identification is based on the culture method recommended by ISO system.[13] The assay employs different selective media to identify *Salmonella* spp. and then performs serotyping by serological identification based on different combinations of O and H antigenic determinants.[1] The method requires skilled personnel to perform the standardized protocols, which are quite laborious and time-consuming. In order to reduce the turnaround time for subtyping *Salmonella* spp., multiplex PCR of specific gene determinants was introduced.[14] The molecular subtyping for *Salmonella* serovar identification was based on the sequence polymorphism of *rfb* locus and flagellar alleles as gene targets.[15] Other molecular modifications such as High - resolution melting - curve analysis has been coupled to the multiplex PCR for detection of polymorphisms from 16S rDNA[16], *flj*B, *gyr*B and *ycf*Q. [17]

The objective of this study was to reduce operational cost and turnaround time of the traditional culture assay by performing the rapid molecular-based assays of sufficient efficacy for analyzing some important epidemiological data from 29 *Salmonella* spp. isolates group B in 165 minced pork collected from local grocery stores of five provinces in the Northern part of Thailand during June to October 2017. In this study, *Salmonella* serotyping was performed by the multiplex HRM serotyping together with the conventional multiplex PCR for the identification of common beta-lactamase genes corresponding to ampicillin and Extended Spectrum β-Lactamases ESBL phenotype.

Materials and methods

Sample collection and Salmonella isolation and identification

The total of 29 *Salmonella* species group B isolated and identified as followed. Briefly, a total of 165 minced pork samples was randomly collected from retail markets of five provinces in the Northern part of Thailand during June to October 2017. All samples were collected in transport media and carefully kept at 4 °C until further isolation and identification process at the University of Phayao. The samples were then transferred to buffered peptone water (BPW; Oxoid, Hampshire, UK) with overnight incubation at 37 °C, later transferred to both TT broth and RVS broth (Oxoid, Hampshire, UK) with overnight incubation at 37 C° and 42 C° respectively. Both overnight TT and RVS were separately plated on XLD agar (Oxoid, Hampshire, UK) and incubated overnight at 37 C°. Black centre dot colonies referred as suspected *Salmonella* colonies were picked to perform 2 biochemical tests; triple sugar iron (TSI) slant, and motility indole lysine agar (MIL) (Biomedia, Nonthaburi, Thailand). The positive colonies were selected to identify serogroups by using *Salmonella* O Polyvalent A-I Group: A,B,C,D,E,F,G,H,I (S&A REAGENTS LAB LTD, Thailand)

Determination of antibiotic-resistance profile

Susceptibility to antibiotics was performed using the disk diffusion method of the Clinical and Laboratory Standards Institute (CLSI)[18] with Ampicillin (AMP) l0 µg, Amoxicillin-clavulanate (AMC) 20-10 µg, Ceftazidime (CAZ) 30 µg, Cefotaxime (CTX) 30 µg, Cefepime (FEP) 30 µg, Ceftriaxone (CRO) 30 µg, Nalidixic acid (NA) 30 µg, Chloramphenicol (C) 30 µg, Streptomycin (S) 10 µg and Sulphamethox/trimethoprim (SXT) 1.25 µg/23.75 µg, Tetracycline (TE) 10 µg (Oxoid, Hampshire, UK). *Escherichia coli* ATCC 25922 was used as a negative control strain. ESBL test was performed

using the combination disk method according to CLSI criteria[18] with both ceftazidime (30 µg), cefotaxime (30 µg) alone and combined with clavulanic acid (10 µg) (Oxoid, Hampshire, UK). In-house known ESBL-producing *Escherichia coli* and ESBL-negative *Escherichia coli* strains ATCC 25922 were used as controls.

Determination of *Salmonella* serotypes

Determination of *Salmonella* serotypes by multiplex PCR coupled with High-resolution melting-curve analysis was initially performed with the DNA extraction from *Salmonella* isolates as previously described.[19] In brief, 1 mL aliquot of an overnight culture was centrifuged at 8,000 rpm for 2 minutes and the pellet was washed twice with 400 µL of STE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA and 100 mM NaCl) and resuspended in 200 µL of TE buffer (10 mM Tris HCl, pH 8.0 and 1 mM EDTA). 100 µL of Tris HCl pH 8.0-saturated phenol solution was added, mixed and centrifuged at 13,000 rpm for 5 minutes at 4°C. The 160 µL aliquot of the upper aqueous layer was mixed with 40 µL of TE buffer and 100 µL of chloroform and then centrifuged for 5 minutes at 13,000 rpm at 4°C. The upper aqueous solution was extracted with chloroform and 150 µL aliquot was kept at -20°C until used. Multiplex PCR coupled with High-resolution melting-curve analysis was performed using a combination of primers to amplify *flj*B (170 bps), *gyr*B (171 bps) and *ycf*Q (241 bps) (Table 1). Multiplex PCR coupled with High - resolution melting - curve analysis was conducted in BIORAD CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA). The multiplex PCR mixture (10 µL) contained 1 µl of DNA, 0.1 pmol of *gyr*B, 0.075 pmol of *flj*B and 0.075 pmol of *ycf*Q primer pairs and 2 µL of HOT FIREPol EvaGreen: no ROX Mix (Solis Biodye, Tartu, Estonia). Thermocycling conditions were as follows: 95°C for 15 minutes, followed by 45 cycles of 95°C for 10 sec, 60°C for 10 sec and 72°C for 20 sec. Samples were then heated at 95°C for 1 minute, cooled to 40°C for 1 minute and High-resolution melting-curve analysis was performed from 70°C to 95°C, rising at 0.2°C/s, with 25 acquisitions per degree Celsius. HRM profile was generated using the Precision Melt Analysis software V 1.2 with the sensitivity setting at 0.30, temperature shift at threshold 5, pre-melt normalization range from 80.87°C to 81.51°C, and post-melt normalization range from 89.17 °C to 89.92°C. Following normalizing and temperature shifting of the melting curves, difference plots were generated by selecting HRM cluster 9, representing *S*. Bareilly as the baseline

Molecular analysis of major beta-lactamase genes

Amplifications of different *bla* alleles were performed by conventional monoplex or multiplex PCR using the primers (IDT, Singapore) listed in Table 1. The reaction mixture (10 µL) contained 1 µL of DNA, primer sets at concentration listed in Table 1 and 2 µL of HOT FIREPol Blend Master Mix Plus 10 mM $MgCl_2$ (Solis Biodye). In multiplex PCR 1 and 2, thermocycling was as follows: 95°C for 12 minutes; 40 cycles of 95°C for 40 sec, 60°C for 40 sec and 72°C for 1 minute; and the final step at 72°C for 7 min. Amplicons were visualized following 1.5% agarose gel electrophoresis by staining RedSafe dye (INiRON, Washington, United States). Data analysis for descriptive statistics was performed by using SPSS for Windows, version 10 (SPSS Inc, Chicago, USA) at the University of Phayao.

Table 1. Primers used in this study[17, 36, 37]

Primer	Genes	Sequence (5'⟶ 3')	Size of PCR- product (bps)	Primer Concentration (pmol/ul)	References
HRM Multiplex *flj*B, *gyr*B and *ycf*Q genes (HRM-rt PCR)					
fljB_f	*flj*B	GTGAAAGATACAGCAGTAACAACG	170	0.075	(17)
fljB_r		ACAAAGTACTTGTTATTATCTGCG		0.075	(17)
gyrB_f	*gyr*B	AAACGCCGATCCACCCGA	171	0.1	(17)
gyrB_r		TCATCGCCGCACGGAAG		0.1	(17)
ycfQ_f	*ycf*Q	GCCTACTCTCTATGCGGAATTCAC	241	0.075	(17)
ycfQ_r		GATATCGCGCGAGGAGGCG		0.075	(17)
Multiplex 1 *bla*TEM variants including *bla*TEM-1 and *bla*TEM-2, *bla*SHV variants including *bla*SHV-1, *bla*OXA-1-like including *bla*OXA-1, *bla*OXA-4 and *bla*OXA-30					
blaTEM_f	*bla*TEM	CATTTCCGTGTCGCCCTTATTC	800	0.4	(36)
blaTEM_r		CGTTCATCCATAGTTGCCTGAC		0.4	(36)
blaSHV_f	*bla*SHV	AGCCGCTTGAGCAAATTAAAC	713	0.4	(36)
blaSHV_r		ATCCCGCAGATAAATCACCAC		0.4	(36)
blaOXA_f	*bla*OXA	GGCACCAGATTCAACTTTCAAG	564	0.4	(36)
blaOXA_r		GACCCCAAGTTTCCTGTAAGTG		0.4	(36)

Table 1. Primers used in this study[17, 36, 37] (continued)

Primer	Genes	Sequence (5'⟶3')	Size of PCR- product (bps)	Primer Concentration (pmol/ul)	References
Multiplex 2 *bla*CTX-M group 1 and group 9 : variants of *bla*CTX-M group 1 including *bla*CTX-M-1, *bla*CTX-M-3 and *bla*CTX-M-15 : variants of *bla*CTX-M group 9 including *bla*CTX-M-9 and *bla*CTX-M- 14					
CTX 1_f	*bla*CTX-M group 1	TTAGGAARTGTGCCGCTGYA[b]	688	0.4	(36)
CTX 1_r		CGATATCGTTGGTGGTRCCAT[b]			(36)
CTX 9_f	*bla*CTX-M group 9	TCAAGCCTGCCGATCTGGT	561	0.2	(36)
CTX 9_r		TGATTCTCGCCGCTGAAG			(36)
Uniplex 1 *Inv*A specific to *Salmonella* spp.					
InvA_f	*Inv*A	GTGAAATTATCGCCACGTTCGGGCAA	284	0.125	(37)
InvA_r		GCCCCGGTAAACAGATGAGTATTGA			

[b]Y=T or C; R=A or G; S=G or C; D=A or G or T

Results

HRM serotyping of the 29 *Salmonella* isolates group B precisely revealed three dominated *Salmonella* serotypes and five unknown *Salmonella* serotypes

All 29 isolates of *Salmonella* spp. group B were the isolates from Chiang Mai (n=4), Chiang Rai (n=5), Lampang (n=4), Nan (n=2), Phrae (n=2) and Phayao Province (n=12). To perform rapid molecular typing, the HRM serotyping was performed including the real-time multiplex PCR and then followed by the High-Resolution Melting-curve analysis (HRM). HRM patterns of all *Salmonella* isolates showed principal T_m at 87 °C but some additionally produced T_m at 83 °C. To generate their unique melt curves for *Salmonella* typing, the process of melt curve analysis was performed primarily by the auto-clustering function of the machine which effectively differentiated eight unique clusters with high confidence (>99.0%) except CM 28.1 isolate as shown in Table 2.

Table 2. HRM serotyping of *Salmonella* spp. (n=29) isolated from minced pork collected during June to October 2017 from the Northern part of Thailand

Isolate no.	Isolate name*	HRM Serotyping					
		T_m Peak	Clustering	Percent confidence	Cluster	Predicted Serotyped	Serotype
1	CR6.1	2(87.8,83.6)	Auto	> 99.0	1	Stanley	Stanley
2	CR7.1	2(87.6,83.6)	Auto	> 99.0	1	Stanley	Stanley
3	CR10.1	2(87.6,83.4)	Auto	> 99.0	1	Stanley	Stanley
4	CR11.1	2(87.6,83.4)	Auto	> 99.0	1	Stanley	Stanley
5	CR17.1	2(87.6,83.6)	Auto	> 99.0	1	Stanley	Stanley
6	PY5.1	1(87.4)	Auto	> 99.0	2	Typhimurium	Typhimurium
7	PY6.1	2(87.6,83.6)	Auto	> 99.0	1	Stanley	Stanley
8	PY11.1	1(87.2)	Auto	77.1	2	Typhimurium	Typhimurium
9	PY13.1	2(87.6,83.4)	Auto	> 99.0	1	Stanley	Stanley
10	PY21.1	2(87.6,83.4)	Auto	> 99.0	1	Stanley	Stanley
11	NA6.1	1(87.2)	Auto	98..2	3	Monophasic	Monophasic
12	NA14.1	1(87.6)	Auto	> 99.0	2	Typhimurium	Typhimurium
13	CM16.1	1(87.4)	Auto	> 99.0	2	Typhimurium	Typhimurium
14	CM21.1	1(87.6)	Auto	72.1	6	**Unknown**	**Agona**
15	CM28.1	2(87.6,83.4)	Manual	N/A	1	Stanley	Stanley
16	CM35.1	1(87.4)	Auto	> 99.0	2	Typhimurium	Typhimurium
17	LP4.1	2(87.6,83.4)	Auto	N/A	5	**Unknown**	**Schwarzengrund**
18	LP12.1	2(87.0,83.4)	Auto	98.5	7	**Unknown**	**Saintpaul**
19	LP13.1	1(87.8)	Auto	> 99.0	4	**Unknown**	**Brandenburg**
20	LP17.1	1(87.4)	Auto	98.7	3	Monophasic	Monophasic
21	FM11.1	1(87.2)	Auto	94.8	3	Monophasic	Monophasic

Table 2. HRM serotyping of *Salmonella* spp. (n=29) isolated from minced pork collected during June to October 2017 from the Northern part of Thailand (continued)

Isolate no.	Isolate name*	HRM Serotyping					
		T_m Peak	Clustering	Percent confidence	Cluster	Predicted Serotyped	Serotype
22	FM19.1	1(87.2)	Auto	> 99.0	8	**Unknown**	**Unknown**
23	FM25.1	1(87.4)	Auto	> 99.0	2	Typhimurium	Typhimurium
24	FM30.1	2(87.8,83.6)	Auto	> 99.0	1	Stanley	Stanley
25	FM31.1	1(87.4)	Auto	> 99.0	4	**Unknown**	**Brandenburg**
26	FM32.2	1(87.4)	Auto	98.3	3	Monophasic	Monophasic
27	FM35.1	2(87.8,83.6)	Auto	> 99.0	1	Stanley	Stanley
28	PR5.1	1(87.4)	Auto	> 99.0	2	Typhimurium	Typhimurium
29	PR20.1	1(87.2)	Auto	98.5	3	Monophasic	Monophasic
Positive control (Standard serotyping)		2(87.8,83.6)	Auto	> 99.0	1	Stanley	Stanley
		1(87.2)	Auto	94.8	2	Typhimurium	Typhimurium
		1(87.4)	Auto	> 99.0	3	Monophasic	Monophasic
Reference		1(87.4)	Auto	> 99.0	9	Barille	Barille

** CR: Chiang Rai, PY, FM: Phayao, NA: Nan, CM: Chiang Mai, LP: Lampang, PR: Phrae Province*

Eight unique HRM patterns easily differentiated by visual differentiation were created based on their serotypes. Despite the high degree of similarity between *S.* Typhimurium and *S.* Monophasic HRM curves, they could be differentiated in this study as shown in Figure 1, A. In this study, three known serotypes of *Salmonella* group B were performed along the samples as the positive controls. Each positive control generated the HRM patterns which exactly matched the corresponding serotypes of each sample as shown in Figure 1, B. The epidemiological data concerning the serotypes from HRM serotyping showed the prevalence of detected serotypes as followed; *S.* Stanley (n=11, 38%), *S.* Typhimurium (n=7, 24%), *S.* Monophasic (n=5, 17%), and six unknown serotypes (21%) as shown in Figure 2. Further conventional serotyping revealed six unknown serotypes comprising of *S. Agona, S.* Schwarzengrund, *S.* Saintpaul, *S.* Brandenburg (2) and one nontypable isolate.

MDR phenotype was highly observed in *Salmonella* spp. group B especially *S.* Typhimurium and *S.* Monophasic

All *Salmonella* isolates group B (n=29) showed 55% (n=16) of Multi-Drug Resistance (MDR) phenotype. The prevalence of antibiotic-resistant phenotype was as described; ampicillin (n=23, 80%), tetracycline (n=18, 62%), chloramphenicol (n=9, 31%), nalidixic acid (n=5, 17%), sulphamethox/trimethoprim (n=4, 14%) and cefotaxime (n=1, 3%) as shown in Table 3. The ampicillin resistant phenotype highly corresponded to the isolates processing the common *bla*TEM determinant not *bla*SHV or *bla*OXA. Additionally, the highly virulent gene, *bla*CTX-M group 1 and 9, was also detected and expected to be responsible primarily to ESBL phenotype. However, the observed *bla*CTX-M group 1 did not correspond to their ESBL phenotype. Melting curve analysis between observed *bla*CTX-M group 1 amplicons and *bla*CTX-M - 55 ESBL gene as positive control was performed and the result showed the observed *bla*CTX-M group 1 amplicons in this study processing lower T_m (89.4 °C) than the control ESBL gene (*bla*CTX-M - 55) (T_m = 91.0 °C) as shown in Figure 3. The possible explanation might be their difference in amplicon's sequence or length, thus the observed *bla*CTX-M group 1 amplicon probably did not cause the ESBL phenotype like the virulent *bla*CTX-M - 55 ESBL gene. However, only *S.* Monophasic from Nan province containing *bla*CTX-M group 9 showed the virulent ESBL phenotype. The result implied the emergence of ESBL *Salmonella bla*CTX-M group 9 circulated in minced pork and possibly along the line of the minced pork production.

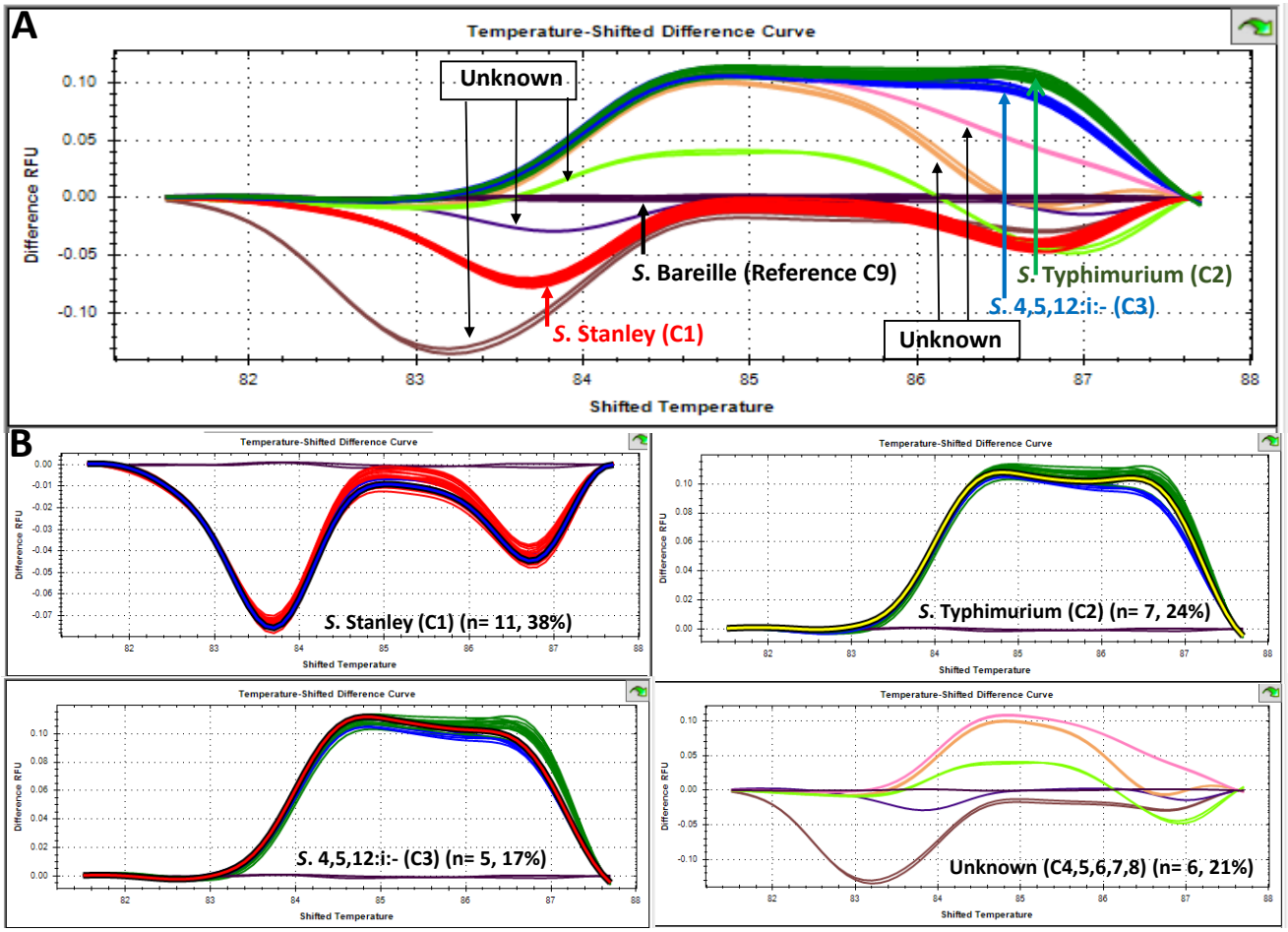

Figure 1 *HRM patterns of all 29 Salmonella isolates from minced pork collected during June to October 2017 from the Northern part of Thailand. (A) All three defined (C1-C3) and five unknown Salmonella serotypes were detected based on their unique HRM patterns. (B) The HRM patterns of each three serotype labeled with both the control (colors and bold line) and samples (colored and fine line) were presented as S.* Stanley *(red, upper left), S.* Typhimurium *(green, upper right) and S.* Monophasic *(blue, lower left). Five unique unknown HRM patterns were presented in lower right.*

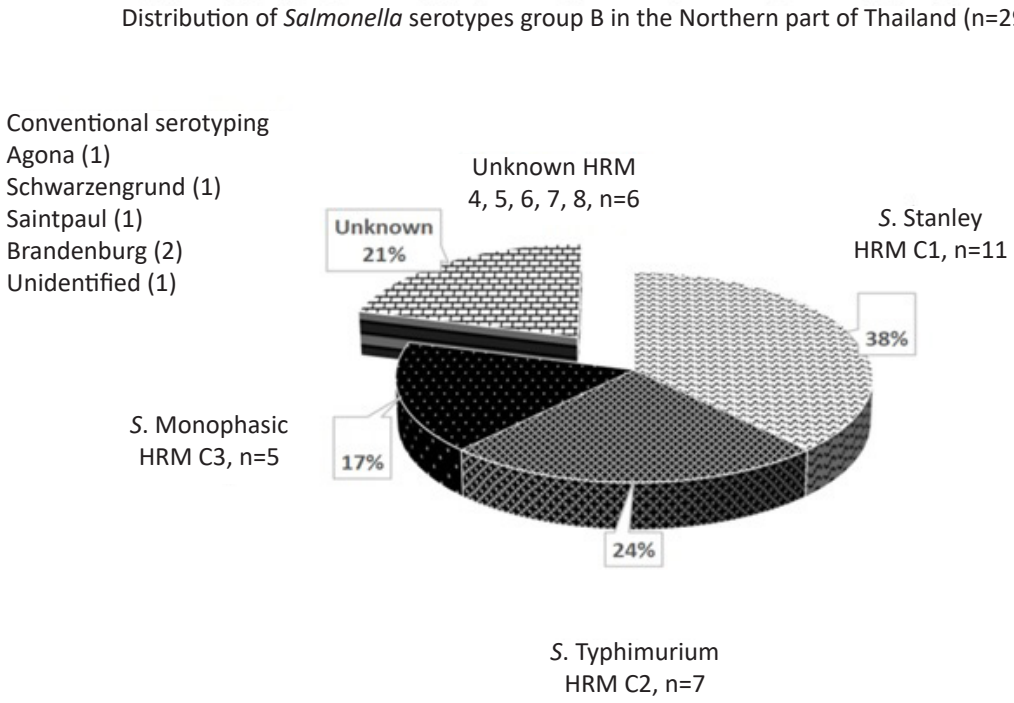

Figure 2 *HRM serotyping representing the epidemiological prevalence of three major serotypes of all 29 Salmonella isolates from minced pork collected during June to October 2017 from the Northern part of Thailand*

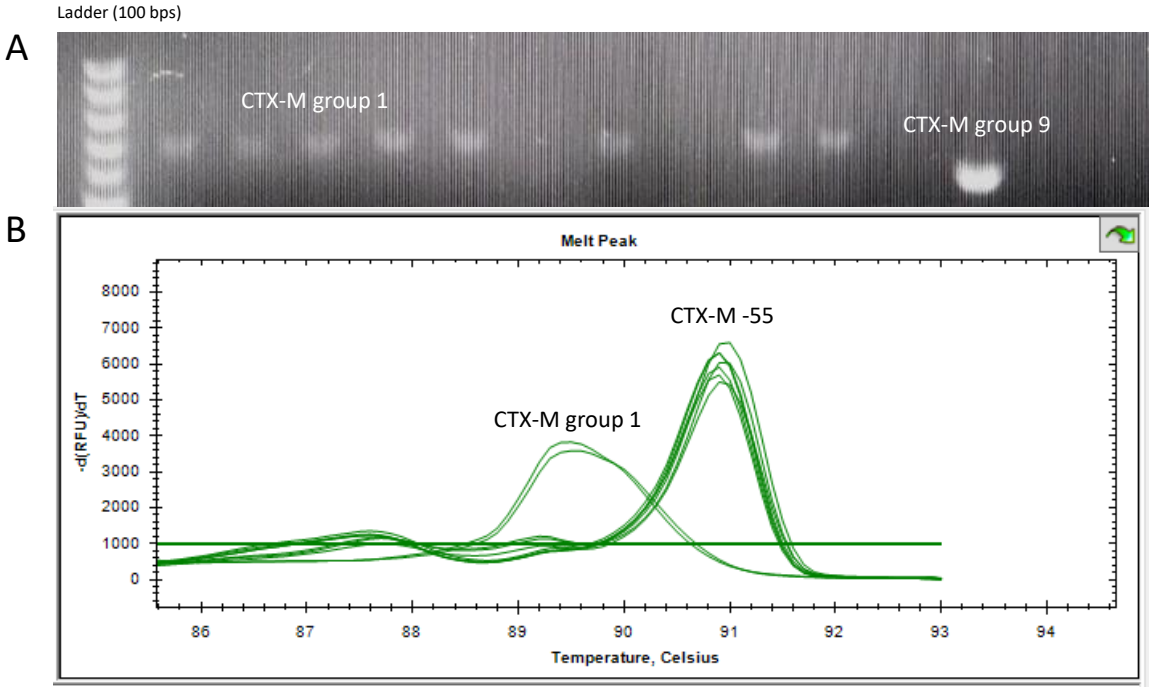

Figure 3 *(A) Multiplex PCR to determine blaCTX-M (ESBL gene) revealed both blaCTX-M group 1 and blaCTX-M group 9 (B) Melt curve analysis of the blaCTX-M group 1 fragments showed the T_m difference between the fragments and the positive control (blaCTX-M-55 or ESBL gene).*

Table 3 Epidemiological details of 29 *Salmonella* isolates from minced pork collected during June to October 2017 from the Northern part of Thailand indicating the important epidemiological data

Province	Serotypes (number of isolates)	Antibiotic-resistant phenotype profile (number of isolates)	Beta-lactam associated genotype	Predicted Beta-lactam phenotype
Chiang Rai	**Stanley[H] (5)**	AMP/TE/NA (3)*	*bla*TEM *bla*CTX-M group 1	AMP
		AMP (2)	*bla*TEM	AMP
Chiang Mai	**Typhimurium[H] (2)**	AMP/TE/SXT/C (2)*	*bla*TEM	AMP
	Stanley[H]	AMP/S/TE*	*bla*TEM *bla*CTX-M group 1	AMP
	Agony[c]	AMP	*bla*TEM	AMP
Nan	**Typhimurium[H]**	AMP/FEP/CTX/CRO/CAZ/TE/C*	*bla*TEM *bla*CTX-M group 9	AMP CTX CRO
	Monophasic[H]	AMP/S/TE*	*bla*TEM	AMP
Phrae	**Typhimurium[H]**	AMP/AMC/TE/SXT/C*	*bla*TEM	AMP
	Monophasic[H]	AMP/AMC/S/TE*	*bla*TEM	AMP
Lampang	**Monophasic[H]**	AMP/S/TE*	*bla*TEM	AMP
	Schwarzengrund[c]	AMP/NA/C*	*bla*TEM	AMP
	Saintpaul[c]	AMP/NA/C*	*bla*TEM	AMP
	Brandenburg[c]	NO	-	NO

Table 3 Epidemiological details of 29 *Salmonella* isolates from minced pork collected during June to October 2017 from the Northern part of Thailand indicating the important epidemiological data (continued)

Province	Serotypes (number of isolates)	Antibiotic-resistant phenotype profile (number of isolates)	Beta-lactam associated genotype	Predicted Beta-lactam phenotype
Phayao	**Typhimurium[H] (4)**	AMP/TE/SXT/C*	*bla*TEM	AMP
		AMP/S/TE/C*	*bla*TEM	AMP
		AMP/TE/C*	*bla*TEM	AMP
		AMP/TE	*bla*TEM	AMP
	Monophasic[H]	AMP/TE	*bla*TEM *bla*CTX-M group 1	AMP
	Stanley[H] (5)	AMP (3)	*bla*TEM *bla*CTX-M group 1	AMP
		AMP/TE (2)	*bla*TEM *bla*CTX-M group 1	AMP
	Brandenburg[c]	No	-	NO
	Unknown	TE	*bla*CTX1	NO

Note: *[H] (HRM serotyping), [c] (Conventional typing), *(MDR), Ampicillin (AMP), Amoxicillin-clavulanate (AMC), Ceftazidime (CAZ), Cefotaxime (CTX), Cefepime (FEP), Ceftriaxone (CRO), Nalidixic acid (NA), Chloramphenicol (C), Streptomycin (S), Sulphamethox/trimethoprim (SXT) and Tetracycline (TE)*

Discussion

The epidemiological research of *Salmonella* spp. is of the ultimate importance and vital procedure to provide sufficient information about the current situation of *Salmonella* prevalence and antibiotic resistance relating to each region and time frame.[20] In Thailand, *Salmonella* isolates from various sources such as clinical samples, contaminated meat, infected animals with geographical difference were collected to assess important epidemiological data such as serotypes, antibiotic-resistant genes; nevertheless, the traditional *Salmonella* serotyping coupled with antibiotic disc diffusion technique needed the long-operational time and complex conventional serotyping.[21] The other rapid modified procedure such as MALDI TOF[22], High - resolution melting - curve analysis[17] and the most resent WGS[23] were efficiently introduced to many epidemiological types of research. High-resolution melting - curve analysis capable of detecting Single Nucleotide Polymorphism (SNP) in amplified PCR products, was introduced to rapidly typing 37 different *Salmonella* serotypes.[17] In Thailand, HRM serotyping was effectively applied for analyzing 14 serotypes from 38 clinical *Salmonella* isolates revealing the most prevalent *Salmonella* spp. as *S.* Stanlay, *S.* Monophasic, and *S. Weltevreden,* respectively.[24] Significantly, *Salmonella* spp. group B or having the common O4 antigen such as *S.* Stanley, *S.* Typhimurium was found to be the predominant serotypes causing invasive salmonellosis which significantly linked to virulent phenotype, multidrug resistance (MDR).[25–27] The epidemiology of *Salmonella* isolated from mince pork in the Northern part of Thailand based on the rapid molecular platform effectively showed the most prevalent serotypes of *Salmonella* group B as *S.* Stanley concentrated mainly in Chiang Rai and Phayao Province and the second and third as *S.* Typhimurium and *S.* Monophasic concentrated mainly in Chiang Mai, Nan, Phrae and Phayao Province corresponding to the *group B Salmonella* isolates in pigs and pork[8,28] as well as in clinical samples.[24] *Salmonella* Typhimurium including variant Monophasic was intensely researched in the epidemiological survey in Thailand[29] and the other countries.[30] *S.* Monophasic was reported to be the cause of the major outbreak in the UK, Spain and Germany and could be separated as an rapidly emerging clade distributed globally possibly due to their capability of fitness to various environments.[31] Additionally, *S.* Typhimurium was reported to be the common serotype showing a high level of virulence and multidrug-resistant pattern.[32] ESBL *Salmonella* producing organisms have been reported to increase in Southeast Asia especially *Salmonella* Typhimurium.[33] The presence of *bla*CTX-M gene was reported to be the common cause of the ESBL phenotype especially *bla*CTX-M group 1 and 9.[34] The acquisition of the ESBL genes was reported through the horizontal transfer from closely related *Escherichia coli.* The ESBL producing *S.* Typhimurium harbouring *bla*CTX-M group 9 was highly observed in food-producing animals in China[35], which might explain its emergence in the Northen part of Thailand. The emergence of the virulent ESBL producing *S.* Typhimurium in Nan province was revealed in this study with the rapid molecular screening or HRM serotyping platform.

In conclusion, the study demonstrates the ability of multiplex PCR coupled with High-resolution melting - curve analysis to rapidly and conveniently serotype *Salmonella* isolates from minced pork . This knowledge and information of the associated antibiogram together with beta-lactamase genes's profile provide a powerful tool for predicting antibiotic resistance of different *Salmonella* serotypes and importantly allow facile monitoring of the emergence of ESBL *Salmonella* spp.

Conflict of Interest

The authors confirm that there are no conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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References

[1] Asmar BI, Abdel-Haq N. Nontyphoidal *Salmonella* infection in children: relation to bacteremia, age, and infecting serotype. Infect Dis (Auckl) [Internet]. 2016 Feb 1;48(2):147–51. Available from: https://doi.org/10.3109/23744235.2015.1094823

[2] A.D.Grimont, Patrick F-XW. Antigenic formulae of the *Salmonella* serovars, 9th edition. 9th ed. France; 2007.

[3] BUREAU OF EPIDEMIOLOGY. DISEASES SURVEILLANCE [Internet]. [cited 2021 Jun 7]. Available from: http://203.157.15.110/boe/home.php

[4] Pulsrikarn C, Pornreongwong S, Tribuddharat C, Meethai C, Srifuengfung S. Serogroup and Serovar Distribution of *Salmonella* in Siriraj Hospital. Siriraj Med J 2013; 65(Suppl): s34–7.

[5] Bangtrakulnonth A, Pornreongwong S, Pulsrikarn C, Sawanpanyalert P, Hendriksen RS, Lo Fo Wong DM a. *Salmonella* Serovars from Humans and Other Sources in Thailand, 1993-2002. Emerg Infect Dis. 2004; 10(1): 131–6.

[6] Kim S. *Salmonella* serovars from foodborne and waterborne diseases in Korea, 1998-2007: Total isolates decreasing versus rare serovars emerging. J Korean Med Sci 2010; 25(12): 1693–9.

[7] Hendriksen RS, Bangtrakulnonth A, Pulsrikarn C, Pornreongwong S, Hasman H, Song SW. Antimicrobial Resistance and Molecular Epidemiology of *Salmonella* Rissen from Animals, Food Products, and Patients in Thailand and Denmark. Foodborne Pathog Dis [Internet]. 2008;5(5):605–19. Available from: http://www.liebertonline.com/doi/abs/10.1089/fpd.2007.0075

[8] Padungtod P, Kaneene JB. *Salmonella* in food animals and humans in northern Thailand. Int J Food Microbiol 2006; 108(3): 346–54.

[9] Padungtod P, Kadohira M, Hill G. Livestock production and foodborne diseases from food animals in Thailand. J Vet Med Sci [Internet]. 2008;70(9): 873–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18840959

[10] Akyala AI, Alsam S. Extended Spectrum Beta Lactamase Producing Strains of *Salmonella* species - A Systematic Review. J Microbiol Res 2015; 5(2): 57–70.

[11] Liebana E, Gibbs M, Clouting C, Barker L, Clifton-Hadley F a, Pleydell E, et al. Characterization of beta-lactamases responsible for resistance to extended-spectrum cephalosporins in Escherichia coli and *Salmonella* enterica strains from food-producing animals in the United Kingdom. Microb Drug Resist 2004; 10(1): 1–9.

[12] Cantón R, González-Alba JM, Galán JC. CTX-M enzymes: Origin and diffusion. Front Microbiol 2012; 3: 1–19.

[13] ISO. 2007. ISO 6579:2007. Microbiology of food and animal feeding stuffs — Horizontal method for the detection of *Salmonella* spp. Fourth edi. Geneva, Switzerland; 2002.

[14] O'Regan E, McCabe E, Burgess C, McGuinness S, Barry T, Duffy G, et al. Development of a real-time multiplex PCR assay for the detection of multiple *Salmonella* serotypes in chicken samples. BMC Microbiol [Internet]. 2008;8(156):1–11. Available from: http://bmcmicrobiol.biomedcentral.com/articles/10.1186/1471-2180-8-156

[15] Bee KL, Kwai LT. Application of PCR-based serogrouping of selected *Salmonella* serotypes in Malaysia. J Infect Dev Ctries 2009; 3(6): 420–8.

[16] Masek BJ, Hardick J, Won H, Yang S, Hsieh YH, Rothman RE, et al. Sensitive detection and serovar differentiation of typhoidal and nontyphoidal *Salmonella* enterica species using 16S rRNA gene PCR coupled with high-resolution melt analysis. J Mol Diagnostics [Internet]. 2014;16(2):261–6. Available from: http://dx.doi.org/10.1016/j.jmoldx.2013.10.011

[17] Zeinzinger J, Pietzka AT, Stöger A, Kornschober C, Kunert R, Allerberger F, et al. One-step triplex high-resolution melting analysis for rapid identification and simultaneous subtyping of frequently isolated *Salmonella* serovars. Appl Environ Microbiol 2012; 78(9): 3352–60.

[18] CLSI. M100S: Performance Standards for Antimicrobial Susceptibility Testing. 27th ed. M100S, 26th Edition. Wayne, PA, USA: Clinical and Laboratory Standards Institute; 2016.

[19] Cheng HR, Jiang N. Extremely rapid extraction of DNA from bacteria and yeasts. Biotechnol Lett 2006; 28(1): 55–9.

[20] Eng S-K, Pusparajah P, Ab Mutalib N-S, Ser H-L, Chan K-G, Lee L-H. Salmonella: A review on pathogenesis, epidemiology and antibiotic resistance. Front Life Sci 2015; 8(3): 284–93.

[21] Sinwat N, Angkittitrakul S, Coulson KF, Pilapil FMIR, Meunsene D, Chuanchuen R. High prevalence and molecular characteristics of multidrug-resistant *Salmonella* in pigs, pork and humans in Thailand and Laos provinces. J Med Microbiol [Internet]. 2016;65(10):1182–93. Available from: http://jmm.sgmjournals.org/%0Ahttps://www.cabdirect.org/cabdirect/abstract/20173008111

[22] Shafini AB, Son R, Mahyudin NA, Rukayadi Y, Tuan Zainazor TC. Prevalence of *Salmonella* spp. in chicken and beef from retail outlets in Malaysia. Int Food Res J 2017; 24(1): 437–49.

[23] Bale J, Meunier D, Weill FX, DePinna E, Peters T, Nair S. Characterization of new *Salmonella* serovars by whole-genome sequencing and traditional typing techniques. J Med Microbiol 2016; 65(10): 1074–8.

[24] Pooncharoen K, Chaiwat Pulsrikarn, Sukon Khamvichai PT, Pakpoom Tadee. The feasibility study of High-Resolution Melting curve analysis and real-time PCR method for a rapid serotyping of frequently isolated *Salmonella* serovar from hospitalized patients. J Assoc Med Sci 2019; 52(1): 36-40

[25] Vugia DJ, Samuel M, Farley MM, Marcus R, Shiferaw B, Shallow S, et al. Invasive *Salmonella* Infections in the United States, FoodNet, 1996–1999: Incidence, Serotype Distribution, and Outcome. Clin Infect Dis 2004; 38(Supplement_3): S149–56.

[26] Shimoni Z, Pitlik S, Leibovici L, Samra Z, Konigsberger H, Drucker M, et al. Nontyphoid *Salmonella* Bacteremia: Age-Related Differences in Clinical Presentation, Bacteriology, and Outcome. Clin Infect Dis 1999; 28(4): 822–7.

[27] Neuert S, Nair S, Day MR, Doumith M, Ashton PM, Mellor KC, et al. Prediction of phenotypic antimicrobial resistance profiles from whole genome sequences of non-typhoidal *Salmonella* enterica. Front Microbiol 2018; 9: 1–11.

[28] Tadee P, Boonkhot P, Pornruangwong S, Patchanee P. Comparative phenotypic and genotypic characterization of *Salmonella* spp. in pig farms and slaughterhouses in two provinces in northern Thailand. PLoS One 2015; 10(2): 1–11.

[29] Huoy L, Pornruangwong S, Pulsrikarn C, Chaturongakul S. Molecular Characterization of Thai *Salmonella* enterica Serotype Typhimurium and Serotype 4,5,12:i:- Reveals Distinct Genetic Deletion Patterns. Foodborne Pathog Dis [Internet]. 2014;11(8):589–92. Available from: http://online.liebertpub.com/doi/abs/10.1089/fpd.2013.1723

[30] Yang X, Wu Q, Zhang J, Huang J, Guo W, Cai S. Prevalence and characterization of *Salmonella* serovar 1,4,[5],12:i:-of food origin in China. PLoS One 2015; 10(9): 1–10.

[31] Soyer Y, Switt a. M, Davis M a., Maurer J, McDonough PL, Schoonmaker-Bopp DJ, et al. *Salmonella enterica* serotype 4,5,12:i:-, an emerging *Salmonella* serotype that represents multiple distinct clones. J Clin Microbiol 2009; 47(11): 3546–56.

[32] Guerra B, Junker E, Miko a, Helmuth R, Mendoza MC. Characterization and Localization of Drug Resistance 2004; 10(2): 83–91.

[33] Phu Huong Lan N, Le Thi Phuong T, Nguyen Huu H, Thuy L, Mather AE, Park SE, et al. Invasive Non-typhoidal *Salmonella* Infections in Asia: Clinical Observations, Disease Outcome and Dominant Serovars from an Infectious Disease Hospital in Vietnam. PLoS Negl Trop Dis 2016; 10(8): 1–13.

[34] Noda T, Murakami K, Etoh Y, Okamoto F, Yatsuyanagi J, Sera N, et al. Increase in Resistance to Extended-Spectrum Cephalosporins in *Salmonella* Isolated from Retail Chicken Products in Japan. PLoS One [Internet]. 2015;10(2):e0116927. Available from: http://dx.plos.org/10.1371/journal.pone.0116927

[35] Zhang WH, Lin XY, Xu L, Gu XX, Yang L, Li W, et al. CTX-M-27 producing *Salmonella* enterica serotypes typhimurium and Indiana are prevalent among food-producing animals in China. Front Microbiol 2016; 7:1-11.

[36] Dallenne C, da Costa A, Decré D, Favier C, Arlet G. Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in Enterobacteriaceae. J Antimicrob Chemother 2010; 65(3): 490–5.

[37] Rahn K, Grandis A De, Clarke RC, McEwen S. A, Galin JE, Ginocchio C, et al. Amplification of *inv*A gene of *Salmonella* by polymerase chain reaction (PCR) as a specific method for detection of Salmonellae. Mol Cell Probes 1992; 6(2): 271–9

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Effectiveness of the protocol for enhancing handwriting readiness skills of preschoolers aged 4-6 years

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ABSTRACT

Background: Handwriting problem is an important issue for school-aged children and is believed to affect their ability to learn, self-confidence, and participation in school activities. In order to prevent handwriting problems, handwriting readiness training is essential to build crucial pre-writing skills in Thai students.

Objectives: The aim of this research was to examine the effectiveness of the protocol for enhancing handwriting readiness skills of preschoolers aged 4-6 years.

Materials and methods: The study was conducted with quasi-experimental design; pretest-posttest control group. In total, 40 students with low standard scores on the Beery™ VMI, 6th edition participated in this study. Participants of the experimental group (n=20) participated in a 20-session protocol, across four weeks with 4 sessions in a week, lasting 30 minutes per session, whereas the control group (n=20) students continued their normal school classes. Two students from the experimental group were withdrawn because they attended less than 16 sessions; therefore 38 participants completed the study successfully. The outcome measures included the Beery™ VMI, 6th edition full form and its supplemental Motor Coordination Test; and the BOT™-2. Data were analyzed using descriptive and inferential statistics.

Results: By using the Wilcoxon Signed-Rank Test, a comparison of VMI and motor coordination scores between before and after receiving a protocol in the experimental group showed the statistical difference of $p<0.05$, and no statically significant difference were found in fine motor skill scores ($p>0.05$) with 95% confidence. However, by using the Mann-Whitney U Test, motor coordination scores in the experimental group after post-protocol were higher than pre-protocol with a statistical significance of $p<0.05$.

Conclusion: This study indicated that the protocol for enhancing handwriting readiness skills of preschoolers aged 4-6 years could be beneficial for improving motor coordination.

Introduction

It was found that 12-33 per cent of primary students have handwriting problems.[1] The Office of Basic Education Commission reported that students in primary 1-3 have handwriting problems at 8.70, 11.20 and 7.61 per cent, respectively.[2] These problems affected their ability to learn, self-confidence, and participation in children's learning activities.[3] Early intervention on facilitating handwriting readiness skills for preschoolers is important in order to prevent failure in learning and participation of school activities. There are researches to support that children in preschool are at the appropriate age to develop handwriting readiness effectively,[4-6] through practicing skills related to handwriting.[5]

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Handwriting readiness is a development in which children are satisfactorily able to perform the skills necessary and related to handwriting.[7] In the early stage of learning to write, children learn to copy which requires the processes of perception and movement in the form of visual-motor integration and fine motor skills. These underlying skills are important and occupational therapists play a major role in developing handwriting readiness skills to decrease and prevent handwriting problems that children may have.[8] The role of the occupational therapist is evolving from that of the assessment and provision of intervention related to handwriting readiness and handwriting, which may focus on different skills related. There are many skills involved in handwriting, for example, hand and finger strength, visual perception, motor coordination, postural control, etc. However, it has been found that there are three major underlying skills, including motor coordination skill, fine motor skill,[9] and visual-motor integration skill.[10-13] Research highly confirmed their relationship to handwriting ability and that they are the most important predicting factors for handwriting in school-age children.

Motor coordination and fine motor skills are movement ability which requires the coordination of the hands and fingers. Children who have problems with these skills have decreased the ability to write; inconsistent letter size and inability to keep the letters on the lines.[14] Another important skill affecting handwriting readiness is visual-motor integration skill, which is the collaboration of visual perception and coordinated movement of the hand and fingers.[15] Children with problems in visual-motor integration skill have difficulties with copying letters with consistent size,[14] staying on the lines, and spacing between letters.[10,16-17] Numerous research studies have found that visual-motor integration skill is highly related to children's ability to write and is the most important predicting factor for legibility in handwriting for school-age children.[10,14,16-17] Moreover, the most common reason school-age children are referred for occupational therapy services is handwriting difficulty.[18] In order to prevent handwriting problems in Thai students, handwriting readiness training is essential to build the crucial pre-writing skills. Thus, we developed the protocol for enhancing handwriting readiness in preschoolers with the purpose to examine its effectiveness in preschoolers aged 4-6 years.

Materials and methods

Research design and participants

This research was designed as a quasi-experimental research, pretest-posttest control group, to study the effectiveness of a protocol for enhancing handwriting readiness in preschoolers. The samples were students who were studying in kindergarten 2 in 4 classrooms at the Dara Academy, Chiangmai province during 2017 academic year. The sample size was determined by G*power 3.1 program from Lust & Donica (2011).[19] The result was 18 samples for each group. With the 10 per cent dropout rate considered, the sample of 40 students were included. They were then divided into 2 groups of 20 through simple randomization. One group received the protocol and the other did not receive the protocol. The inclusion criteria were as followed 1) being able to communicate with fluent Thai, 2) having no cognitive or physical deficits, 3) having scores on the Beery™ VMI, 6th edition ranking in the lowest 40 out of all the samples, and 4) obtaining informed consent signed by parents before participating in the study. The data of participants who attended the protocol less than 80% were excluded from the study (less than 16 sessions).

Ethical approval for the study was obtained from the ethical review committee for research in humans, Faculty of Associated Medical Sciences, Chiang Mai University (Ref. no AMSEC-60EX-027)

Measures

Components of 3 instruments were used in data collection, the Beery-Buktenica Developmental Test of Visual-Motor Integration, Sixth Edition (Beery™ VMI, 6th edition) full form, its supplemental Motor Coordination Test, and the Fine Motor Precision and Fine Motor Integration subtests of the Bruininks-Oseretsky Test of Motor Proficiency, Second Edition (BOT™-2).

Beery-Buktenica Developmental Test of Visual-Motor Integration, Sixth Edition (Beery™ VMI, 6th edition) is an assessment tool used to find a standard score to measure visual-motor integration skill related to copying shapes.[15] In this study, Beery™ VMI, 6th edition which is a full battery, was used to assess visual-motor integration skill. Moreover, the supplementary Motor Coordination Test was administered to assess motor skills by tracing within a confined space. The Beery™ VMI, 6th edition has sound psychometric properties, with high interrater (r=0.93), test–retest reliability (r=0.87), internal consistency score of 0.96, as well as, content and concurrent validity.[15]

Bruininks-Oseretsky Test of Motor Proficiency, Second Edition (BOT™-2) is a commonly used standardized test designed to measure the gross and fine motor skills of people ages 4–21 yr. This test includes four composites of two subtests each. For this study, the Fine Manual Control Composite, including Fine Motor Precision (Subtest 1) and Fine Motor Integration (Subtest 2), was administered. Fine Motor Precision measures bilateral hand skills and accuracy with folding paper, cutting, and coloring. Fine Motor Integration addresses visual–motor skills determined by copying different types of shapes. Both subtests were used to evaluate participants' skills in integrating visual perception with hand and finger motor movements. Internal consistency reliability for Fine Manual Control has been established in 4-yr-olds (=0.87) and 5-yr-olds (r=0.86). BOT–2 has published test–retest reliability for Fine Manual Control with 4- to 7-yr-olds (r=0.81).[20]

Intervention

The protocol for enhancing handwriting readiness skills of preschoolers aged 4-6 Years was developed by the researchers. The protocol consisted of two main parts: (1) indirect intervention, in which we worked with homeroom teachers through 2 ways: the classroom observation with the purpose to understand the kindergarten curriculum and classroom contexts, and the therapist-teacher meeting with the purpose to explain and discuss about the protocol and its implementation; and (2) direct intervention, in

which four occupational therapy graduate students led twenty 30-min sessions, four times a week for 5 consecutive weeks for participants in the experimental group (1 adult, 5 students), whereas the control group students continued their normal school classes. Scheduled direct interventions for the experimental group were begun with a 5-min warm-up activity, and then followed by a 25-min group activity of visual-motor integration, motor coordination and fine motor skill activities. Everyone conducting direct intervention had the training of the protocol to ensure consistency before the study began.

Data collection schedule

Pre- and posttest data were collected within 1-day timeframe immediately before and immediately after the study, by two occupational therapy graduate students who were trained in the proper administration of the battery. These two graduate students were blinded to group assignment during the assessment. They recorded raw scores during the BOT-2 (Fine Motor Precision and Fine Motor Integration subtests) administration, and administered the Beery™ VMI, 6th edition full form and its supplemental Motor Coordination Test. These graduate students were neither involved in scoring nor providing the intervention protocol Interrater reliability testing was not implemented due to its potential effect on student's performance. For the indirect intervention, the first author worked closely with the homeroom teachers throughout the intervention process. Other four occupational therapy graduate students, including the first author, administering the direct intervention were formally trained. They met with the research team daily to plan the intervention session, and review progress. Finally, without respect to the person administering the test or grouping, we checked and reviewed all data scoring to verify the accuracy of reporting and scoring.

Statistical analysis

Data were presented by descriptive statistics. Statistical comparison between pretest and posttest performance within a group was performed using the Wilcoxon Signed-Rank Test, and comparisons between the experimental and control groups were performed using the Mann-Whitney U Test. The level of significance for testing was set at 0.05. All statistical analyses were conducted using SPSS statistical package.

Results

Two students from the experimental group were withdrawn because they attended less than 16 sessions; therefore 38 participants completed the study successfully. For both groups, the samples were mostly females at the percentage of 60 and 55.56, respectively. The control group has an average age of 59.60 months, and the experimental group has an average age of 58.28 months. For both groups, the samples were mostly right-hand dominant at the percentage of 80 and 83.33, respectively. Results from the assessment via the Beery™ VMI, 6th edition 'before' receiving the protocol showed that most of the samples from both groups were ranked at 50 percent standard score, as shown in Table 1.

Table 1. Demographic data for the control group and experimental group.

	Control Group (n=20)	Experimental Group (n=18)
Gender *(n,%)*		
Male	8 (40)	8 (44.44)
Female	12 (60)	10 (55.56)
Age, months *(n,%)*		
48-59	11 (55)	11 (61.11)
60-71	9 (45)	7 (38.89)
Maximum	54	53
Minimum	66	64
Mean (SD)	59.60 (3.71)	58.28 (3.08)
Dominant hand *(n,%)*		
Right	16 (80)	15 (83.33)
Left	4 (20)	3 (16.67)
Level of VMI* performance *(n,%)*		
Below Average	1 (5.50)	1 (5)
Average	9 (50)	10 (50)
Above Average	7 (39)	4 (20)
High	1 (5.50)	5 (25)

**VMI: Visual -motor integration*

Comparison of scores on three handwriting readiness skills including visual-motor integration, motor coordination, and fine motor, 'before and after' receiving the protocol was done using the Wilcoxon Signed-Rank Test. It was found that the control group has a statistical significant difference (p<0.05) in visual-motor integration skill scores (p=0.029). No statistical significant difference (p<0.05) was found in motor coordination-skill scores (p=0.195) and fine motor scores (p=0.321). As for the experimental group, statistical significant differences (p<0.05) were found in visual-motor integration skill scores (p=0.016) and motor coordination skill scores (p=0.001). No statistical significant difference (<0.05) was found in fine motor scores (p=0.312), as shown in Table 2.

Table 2. Comparison of handwriting readiness skill scores in visual-motor integration, motor coordination, and fine motor, 'before and after' receiving the protocol between the control and experimental groups.

Handwriting readiness skills	Control Group (n=20)				Experimental Group (n=18)			
	Pretest *Mean (SD)*	Posttest *Mean (SD)*	*z*	*p*	Pretest *Mean (SD)*	Posttest *Mean (SD)*	*z*	*p*
VMI	110.15 (9.35)	114.60 (6.14)	-2.19	0.029*	107.22 (7.92)	112.44 (5.70)	-2.41	0.016*
MC	106.35 (12.80)	109.45 (10.47)	-1.30	0.195	109.28 (6.50)	117.78 (8.48)	-3.41	0.001*
FM	55.50 (7.33)	57.30 (6.71)	-0.99	0.321	54.22 (9.40)	55.89 (7.66)	-1.01	0.312

*VMI: Visual-motor integration, MC: Motor coordination, FM: Fine motor, *p<0.05*

Comparison of scores for three handwriting readiness skills including visual-motor integration, motor coordination, and fine motor, 'after' receiving the protocol between the 2 groups was analyzed using Mann-Whitney U Test. Statistical significant difference (p<0.05) was found in motor coordination skill scores (p=0.017). No statistical significant difference (p<0.05) was found in visual-motor integration skill scores (p=0.239) and fine motor scores (p=0.518), as shown in Table 3.

Table 3. Comparison of handwriting readiness skill scores in visual-motor integration, motor coordination, and fine motor, after' receiving the protocol between the control and experimental groups.

Handwriting readiness skills	Control Group (n=20)	Experimental Group (n=18)		
	Mean (SD)	*Mean (SD)*	*z*	*p*
VMI	114.60 (6.14)	112.44 (5.70)	-1.18	0.239
MC	109.45 (10.47)	117.78 (8.48)	-2.39	0.17*
FM	57.30 (6.71)	55.89 (7.66)	-0.65	0.518

*VMI: Visual-motor integration, MC: Motor coordination, FM: Fine motor, *p<0.05*

Discussion

Results from this study indicate that the protocol for enhancing handwriting readiness has potential as a promising intervention for preschoolers who were at risk of handwriting readiness, especially in motor coordination skill. Motor coordination is considered as an important skill toward handwriting readiness and is a predicting factor for the ability to write.[10,14,16] The reason for increased scores on motor coordination after receiving the protocol was the type of activities which was designed to provide repetitive practice to develop fine motor coordination skills, for example, making paper butterflies, making paper crown and tiara, paper tearing art, pegboard patterns game, making necklaces from plastic chains, using both hands to scoop and transfer pompom with ice-cream sticks, etc. These fine motor coordination activities are perfect for developing a variety of specific fine motor skills in a coordinated manner. Moreover, through the principles of sequencing and adapting tasks,[21] students in the experimental group had the intensive opportunity to repeatedly practice skills of their hands that were needed to perform delicate manipulation as well as to attain and manipulate objects. Fine motor coordination activities were designed and graded so that students successfully increased the coordination in their hands in performing more precision and delicate tasks. Such movement training is effective in enhancing children's motor learning[22] and consistent with the study by Hoy, Eagan, and Feder[23] which found that the more opportunities to practice and repeat in programs to enhance handwriting components, the more effective the intervention will be.

Increase in visual-motor integration skill scores of both groups can be explained by the rapid rate of visual-motor integration skill development of children aged 4-5 years. Learning to write in early stage through copying[24] highly relies on visual-motor integration skill.[15,25] Development of visual-motor integration skill in children this age is dynamic

and rapid. It was found that children at the age of 4 years old develop this skill very quickly and will develop fastest in the age of 5 years old.[26] Moreover, the context and learning activities in 'outdoor activity period' hosted by the school provided the opportunity for the control group students who did not receive the protocol to experience and practice visual-motor integration skills during the outdoor activity period. However, when comparing the average score of change between the two sample groups, it was found that the group who received the protocol have higher scores in visual-motor integration skill. This may be due to the direct intervention received from participating in the protocol, which specifically reinforced appropriate participation from the children.[27] All intervention sessions were also implemented in a small group format (one occupational therapy graduate student to every five students) that provided a comfortable and playful context to practice. The "just-right" challenges were provided so that children could succeed and master the tasks. Children were motivated to participate in the selected activities and consequently, expected performance outcomes were enhanced.[28]

In this study, no difference was found in fine motor skill scores both before and after receiving the protocol and between the two sample groups. It may be due to the fine motor activities were designed based on training multiple sub-skills, for example, muscle strength of the hands and fingers, in-hand manipulation (e.g., finger-to-palm translation and palm-to-finger translation), and hand dexterity. Therefore, the number of practice and repetition of each sub-skill was lessened and insufficient to induce the change in fine motor skill scores, especially translation and dexterity, which have the least proportion. These two sub-skills are predicting factors for children's handwriting ability.[29]

Our findings have some implications for occupational therapy practitioners. Occupational therapy practitioners play a key role in contributing to effective intervention that support the needs of students who are at risk of developing proper handwriting readiness skills in the classroom environment. Short-term interventions can have a significant effect on the fine motor coordination skills required for handwriting readiness in preschoolers.

Limitations and future research

There were some limitations of this study. Because this was a pilot study, the sample was small. The use of one geographic location also limits the generalizability of this study. An important future research must increase the collaboration process with classroom teachers in the intervention protocol. Furthermore, it is needed to examine whether students who receive the protocol perform better during midyear pre-handwriting tasks than a control group.

Conclusion

On the basis of the current findings, the study results proved that the protocol for enhancing handwriting readiness skills designed especially for preschoolers aged 4-6 years is effective in developing motor coordination skills of children at risk of handwriting readiness problems.

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References

[1] Office of the Basic Education Commission. Annual report 2015 office of the Basic Education Commission. Bangkok: The Agicultural Cooperative Federation of Thailand. Limited; 2015.

[2] Overvelde A, Bommel-Rutgers I, Bosgra-Stork I, Cauteren MV, Halfwerk, B, Smits-Engelsman BC. KNGF evidence statement motorische schrijf-problemen bij kinderen. Ned Tijdschr Fysiother 2011; 121: 1-65.

[3] Ratzon NZ, Efraim D, Bart O. A short-term graphomotor program for improving writing readiness skills of first-grade students. Am J Occup Ther 2007; 61: 399-405.

[4] Heckman JJ, Stixrud J, Urzua S. The effects of cognitive and non-cognitive abilities on labor market outcomes and social behavior. J Labor Econ 2006; 24: 411-82.

[5] Ohl AM, Graze H, Weber K, Kenny S, Salvatore C, Wagreich S. Effectiveness of a 10-week tier-1 response to intervention program in improving fine motor and visual–motor skills in general education kindergarten students. Am J Occup Ther 2013; 67: 507-14.

[6] Puranik CS, Lonigan CJ. Emergent writing in preschoolers: preliminary evidence for a theoretical framework. Read Res Q 2014; 49: 453-67.

[7] Marr D, Cermark S, Cohn ES, Henderson A. Fine motor activities in Head Start and kindergarten class room, Am J Occup Ther 2003; 57: 550-7.

[8] Van Hartingsveldt MJ, Groot IJ, M D, Aarts PBM, Nijhuis-Van der Sanden MWG. Standardized tests of handwriting readiness: a systematic review of the literature. Dev Med Child Neurol 2011; 5: 506-15.

[9] Grissmer D, Grimm KJ, Aiyer SM, Murrah WM, Steele JS. Fine motor skills and early comprehension of the world: Two new school readiness indicators. Dev Psychol 2010; 46: 1008-17.

[10] Shen I, Lee T, Chen C. Handwriting performance and underlying factors in children with attention deficit hyperactivity disorder. Res Dev Disabil 2012; 33: 1301-09.

[11] Barnhardt C, Borsring E, Deland P, Pham N, Vu T. Relationship between visual-motor integration and spatial organization of written language and math. Optom Vis Sci 2005; 82: 138-43.

[12] Volman MJ, van Schendel BM, Jongmans MJ. Handwriting difficulties in primary school children: A search for underlying mechanisms. Am J Occup Ther 2006; 60: 451-60.

[13] Kushki A, Chau T, Anagnostou E. Handwriting difficulties in children with autism spectrum disorders: A scoping review. J Autism Dev Disord 2011; 41: 1706-16.

[14] Racine MB, Majnemer A, Shevell M, Snider L. Handwriting performance in children with attention deficit hyperactivity disorder (ADHD). J Child Neurol 2008; 23: 399-406.

[15] Beery KE, Buktenica NA, Beery NA. The Beery-Buktenica Developmental Test of Visual-Motor Integration, 6th ed. MN: NCS Pearson; 2010.

[16] Brossad-Racine M, Majnamer A, Shevell M, Snider L, Belanger SA. Handwriting capacity in children newly diagnosed with Attention Deficit Hyperactivity Disorder. Res Dev Disabil 2011; 32: 2927-34.

[17] Lee TI, Howe TH, Chen HL, Wang TN. Predicting handwriting legibility in Taiwanese elementary school children. Am J Occup Ther 2016; 70: 1-9.

[18] Schneck CM, Amundson SJ. Prewriting and handwriting skills. In: Case-Smith J, O'Brien JC, editors. In Occupational therapy for children. 6th ed. Maryland Heights, MO: Mosby Elsevier; 2010. p. 555-580.

[19] Lust C, Donica D. Effectiveness of a handwriting readiness program in head start: A two- group controlled trial. Am J Occup Ther 2011; 65: 560-8.

[20] Bruininks R, Bruinink B. Bruininks-Oseretsky Test of Motor Proficiency, 2nd ed. Minneapolis: NCS Pearson; 2005.

[21] O'Brien J. Application of motor control/motor learning to practice. In: Case-Smith J, O'Brien J, editors. Occupational Therapy for Children and Adolescents. 7th ed. St. Louis: Mosby; 2015. p. 193-219.

[22] Schmidt RA, Lee TD. Motor Control and Learning: A Behavioral Emphasis. 4th. IL: Human Kinetics; 2005.

[23] Hoy MMP, Egan MY, Feder KP. A systematic review of interventions to improve handwriting. Can J Occup Ther 2011; 78: 13-25.

[24] Marr D, Cermark S, Cohn ES, Henderson A. Fine motor activities in Head Start and kindergarten class room, Am J Occup Ther 2003;57:550-7.

[25] Benbow M. Principle and practice of teaching handwriting. In: Herderson A, Pehoski C, editors. Hand function in the child: Foundations for remediation. 2nd ed. St. Louis: Mosby; 2006.

[26] Fang Y, Wang J, Zhang Y, Qin J. The relationship of motor coordination, visual perception, and executive function to the development of 4-6-year-old Chinese preschooler's visual-motor integration skills. Biomed Res Int 2017: 1-8.

[27] Case-Smith J, Frolek Clark GJ, Schlabach TL. Systematic review of interventions used in occupational therapy to promote motor performance for children ages birth–5 years. Am J Occup Ther 2013; 67: 413-24.

[28] Case-Smith J. Effects of occupational therapy services on fine motor and functional performance in preschool children. Am J Occup Ther 2000; 54: 372-80.

[29] Cornhill H, Case-Smith J. Factors that relate to good and poor handwriting. Am J Occup Ther 1996; 50: 732-9.

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Assessment of CareStart™ G6PD RDT for G6PD deficiency screening in newborns and malaria diagnosed subjects in the Northern Thailand

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ABSTRACT

Background: Glucose-6-phosphate dehydrogenase (G6PD) deficiency is an inherited enzymatic disorder associated with severe neonatal hyperbilirubinemia and acute hemolysis after exposure to certain drugs or infections. The most common test for screening G6PD deficiency is fluorescent spot test which is rapid and convenient. However, fluorescent spot test needs tools and skills in diagnosis and interpretation of the results. CareStart™ G6PD Rapid Diagnosis Test (RDT) is an enzyme chromatographic strip test based on reduction of colorless nitro blue tetrazolium into formazan which gives purple color and the results could be read within 10 minutes.

Objectives: This study aimed to analyze the sensitivity, specificity and accuracy of CareStart™ G6PD RDT for screening of G6PD deficiency in newborn and subjects with malaria diagnosis.

Materials and methods: This study was conducted from December 2017 to February 2018. G6PD diagnostic tests including fluorescent spot test and Carestart™ G6PD RDT were performed in 196 newborns aged varied from 1 day to 12 years and 48 subjects with malaria diagnosis. The efficiency of CareStart™ G6PD RDT was analyzed by comparing with the reference method, fluorescent spot test.

Results: CareStart™ G6PD RDT demonstrated 100% sensitivity, 100% specificity and 100% accuracy for screening of G6PD deficiency in malarial diagnosed samples. However, it presented invalid results up to 27.04% in newborns. Therefore, sensitivity, specificity and accuracy were not applicable in this group.

Conclusion: CareStart™ G6PD RDT could be used for preliminary screening of G6PD deficiency in malarial diagnosed samples. However, it should be improved to reduce the invalid results in newborns.

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Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common enzyme deficiency worldwide and the best described red blood cell (RBC) enzymopathy. It is an X-linked recessive disorder affecting about 400 million people globally with a high prevalence in Sub-Saharan Africa, Asia, Middle East, Latin America, and the Mediterranean.[1,2] In Thailand, G6PD deficiency can be found in 16.9% of the total population with 11.1% in men and 5.8% in women.[3] In addition, the G6PD deficiency occurs most frequently in areas where malaria has been endemic, presumably the result of natural selection due to some degree of protection from malaria infection.[1,4]

Most of G6PD deficient individuals are asymptomatic throughout their lives until or following triggering by agents such as oxidative drugs, some infections or ingestion of fava beans.[5,6] G6PD deficiency was discovered half a century ago. Severe hemolytic anemia may occur in some G6PD deficient individuals who are treated with anti-malarial drug in group of 8-aminoquinolines such as primaquine, which is the only available for radical cure of *Plasmodium vivax*.[7] The risk of primaquine-induced hemolysis in G6PD deficient individuals is an essential public health concern in malaria endemic countries, especially in tropical areas.[8] Even with treatment, severe malaria has a mortality rate of approximately 10-40% depending upon the time between initial symptoms and effective treatment and hospital facilities for the management of its complications.[9] In addition, there was a previous report of recurrent parasitemia of *P. vivax* within 6 months in 18.3% patients treated with primaquine (15 mg/day for 14 days).[10] At this dose, malaria-infected patients with G6PD deficiency could not stand the effect of primaquine, which lead to hemolysis on G6PD deficient RBCs and eventually forced the patients to stop taking medicine. Unfortunately, these series of events result in drug resistance of *P. vivax*. Subsequently, World Health Organization (WHO) had suggested 30 mg/day of primaquine treatment for 14 days to gain effective cure against *P. vivax*.[11] Thus, the diagnostic test for G6PD deficiency is necessary for a malaria-infected patient to prevent the clinical manifestation and mortality. Moreover, in 2015, WHO recommended screening for G6PD deficiency before primaquine treatment.[12]

The most common screening test for G6PD deficiency is the fluorescent spot test (FST) which had 100% sensitivity and 98% specificity of detection.[13] It was also recommended by International Committee for Standardization in Hematology (ICSH) as the most acceptable and reliable screening test for G6PD deficiency.[14] This method is easy to perform and gives a rapid result. However, a source of electricity for an ultraviolet (UV) light is required. To increase the efficiency of the G6PD screening test, the Access Bio has developed a Carestart™ G6PD Qualitative Rapid Diagnostic Test (RDT) which is based on the reduction of colorless nitro blue tetrazolium to dark purple color formazan.[15] It has been used for screening G6PD deficiency in malaria endemic areas, including Cambodia,[15] Haiti,[16] Philippines[10] or Southwestern Uganda.[17] Thus, the aim of this study is to analyze the sensitivity, specificity and accuracy of CareStart™ G6PD RDT for preliminary screening of G6PD deficiency in newborn and malaria diagnosed subjects.

Materials and methods

Study sites and populations

The study was conducted from December 2017 to February 2018 in Chiang Mai and Mae Hong Son Provinces, Thailand. Briefly, the 196 EDTA blood samples of newborn with ages varied from 1 day to 12 years were obtained from five hospitals (Maharaj Nakorn Chiang Mai Hospital, Chiang Mai Health Promoting Hospital, Jomthong Hospital, Hangdong Hospital and Sanpathong Hospital) in Chiang Mai, Thailand. In addition, the 48 EDTA blood samples of subjects with malarial diagnosis were obtained from Pai Hospital and Malaria clinic of Mae Sariang, Mae Hong Son, Thailand. The study protocol was reviewed and approved by Ethics Committee of the Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand (AMSEC-60EX-014).

Fluorescent spot test

The test was performed according to the ICSH with a minor modification.[18] Briefly, 5 µL blood samples were transferred into 100 µL of G6PD screening reagent that was prepared immediately before using. The reactions were then incubated at 25°C and spotted onto a Whatman filter paper after incubating for 5, 10 and 15 min. The spots were air-dried and examined under UV light with a wavelength of 340-365 nm. The peripheral bloods from G6PD non-deficient and deficient control samples were included in each assay. Samples were interpreted as G6PD deficiency when opaque blood spots were observed under UV light whereas samples with strong fluorescence on all three spots of 5, 10 and 15 min for incubation periods were interpreted as G6PD normal. In addition, samples with a weak fluorescence on three spots were interpreted as G6PD intermediate.

CareStart™ G6PD RDT

CareStart™ G6PD RDT was provided by the manufacturer (AccessBio, New Jersey, USA). It is a qualitative enzyme chromatographic test, based on the reduction of colorless nitro blue tetrazolium dye to purple colored formazan. Following the manufacturer's instruction, 2 µL of EDTA blood samples were transferred into the sample well and then two drops of provided buffer were immediately added into the buffer well. The results were read visually after 10 min. The purple color appeared on the reading window in samples with G6PD normal while no color change was observed in samples with G6PD deficiency. If red color appeared on the reading window or the blood sample flowed poorly in the lateral flow, it could not be interpreted and was an invalid result. The samples with G6PD deficiency and non-deficiency samples were used as a control when analyzing patient samples.

Statistical analysis

Data were presented as median and interquartile range (IQR). Statistical analyses were performed using SPSS software package (Statistical Package for the Social Sciences 11.0, Chicago, IL, USA). In addition, sensitivity [true positive/(true positive+false negative)], specificity [true negative/(true negative+false positive)], positive predictive value

[true positive/(true positives+false positive)], negative predictive value [true negative/(true negative+false negative)] and accuracy [(true positive+true negative)/n] of CareStart™ G6PD RDT were analyzed by using fluorescence spot tests as the reference method.

Results

Performances of CareStart™ G6PD RDT in newborns

A total of 196 newborns were recruited in this group, 102 males and 94 females. Median age was 4 days (IQR 4-6 days). The test results and efficiency performances of CareStart™ G6PD RDT compared to fluorescent spot test are shown in Table 1. Fluorescent spot test showed 168 of G6PD non-deficiency, 24 deficiency and 4 intermediate samples while Carestart™ G6PD RDT showed 107 of G6PD non-deficiency, 36 deficiency and also presented the invalid results up to 27.04% (53 samples). Therefore, the sensitivity, specificity and accuracy of the CareStart™ G6PD RDT were not applicable. Prevalence of G6PD deficiency analyzed by using fluorescent spot test was 12.24% (24 of 196 subjects; 19 males and 5 females) while 4 samples were diagnosed as G6PD intermediate and all of them were female.

Table 1. Performance of the Carestart™ G6PD RDT compared to fluorescent spot test in newborns.

	Fluorescent spot test					
Carestart™ RDT	Normal	Deficiency	Intermediate	Total	Performances	
Normal	107	0	0	107	Sensitivity	*NA
Deficiency	17	16	3	36	Specificity	*NA
Invalid	44	8	1	53	Accuracy	*NA
Total	168	24	4	196	Positive predictive value	*NA
Prevalence of G6PD deficiency (%)				12.24	Negative predictive value	*NA

**NA: Not applicable*

Performances of CareStart™ G6PD RDT in subjects with malaria diagnosis

A total of 48 subjects including 28 males and 20 females were used for analyzing. The median age was 30 years (IQR 13.5-41.0 years). The prevalence of G6PD deficiency in this group was 4.17% (2 of 48 subjects). The results of Carestart™ G6PD RDT was similar to those of fluorescent spot test, including 46 subjects with G6PD non-deficiency (27 males and 19 females) and 2 subjects with G6PD deficiency (1 male and 1 female). Moreover, the invalid result of Carestart™ G6PD RDT was not found in this group. Thus, sensitivity, specificity and accuracy of the CareStart™ G6PD RDT were 100% (Table 2).

Table 2. Performance of the Carestart™ G6PD RDT compared to fluorescent spot test in Subjects with malaria diagnosis.

	Fluorescent spot test					
Carestart™ RDT	Normal	Deficiency	Intermediate	Total	Performances	
Normal	46	0	0	46	Sensitivity	100%
Deficiency	0	2	0	2	Specificity	100%
Invalid	0	0	0	0	Accuracy	100%
Total	46	2	0	48	Positive predictive value	100%
Prevalence of G6PD deficiency (%)				4.17	Negative predictive value	100%

Discussion

Many malaria-endemic countries around tropical or sub-tropical areas are participating in malaria eradication by 8-aminoquinolines-based drug treatment for transmission blocking or radical cure from the hypnozoite forms that reside in the liver which is the cause of malaria relapse. Therefore, prior to treatment with 8-aminoquinolines-based drugs, the status of the G6PD deficiency should be considered to minimize adverse effects such as drug-induced hemolysis from primaquine in G6PD deficient patients or kernicterus in newborns. Currently, the reliable G6PD assays require laboratory equipment and skilled technicians. Therefore, to assay the G6PD diagnosis of individuals quickly in remote area without requiring a high level of clinical laboratory equipment, point-of-care assay, Carestart™ G6PD RDT, plays an important role to determine which individual can be treated with 8-aminoquinolines-based drug. Furthermore, the cost per test of Carestart™ G6PD RDT and Fluorescent spot test was comparable ($1.5 vs $1.1 USD, respectively).[16] In this study, Carestart™ G6PD RDT had 100% of sensitivity, specificity and accuracy for screening of G6PD deficiency in malaria diagnosed subjects while those in newborn was not applicable because the invalid results were observed in 27.04% of samples. Thus, it is possible that Carestart™ G6PD RDT is an appropriate and effective tool to determine the G6PD status in endemic rural area of malaria but not in

newborns. The current study is consistency with the previous studies showing that Carestart™ G6PD RDT had a high sensitivity in determining G6PD deficiency and that it also could be used for point-of-care testing in malaria endemic areas.[10,19,20] Nevertheless, the newborns showed invalid results and that might be due to several causes such as hematocrit levels or in cases of heterozygous G6PD deficiency in females. Generally, the hematocrit of newborns is higher than those of adults. Thus, their blood samples could not easily flow through the filter paper and that might be a cause for difficulty in differentiating between the red color of hemoglobin and the purple color of reduced formazan. In addition, a relatively poor diagnostic performance among females due to mosaic G6PD phenotype had been also reported previously by Satyagraha et al.[21]

The present study has some limitations. First the hematological parameters including hematocrit especially in newborns were not measured thus a correlation between hematocrit and invalid results cannot be verified. Second, the assessment of G6PD enzyme activity which is the gold standard for identified G6PD deficiency is not performed. Therefore, G6PD enzyme concentration which is sufficient to generate purple color of reduced formazan cannot be analyzed especially in mild forms of G6PD deficiency such as in heterozygous females and also in invalid samples.

In conclusion, CareStart™ G6PD RDT is relatively inexpensive, easy to use and requires significantly less-time. Thus, it can be used in malaria endemic areas before administering 8-aminoquinoline-based drug to substantially reduce the risk of adverse treatment outcomes. However, it should be improved to reduce the invalid results in newborns.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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References

[1] Beutler E. G6PD deficiency. Blood. 1994;84(11): 3613-36.

[2] Nkhoma ET, Poole C, Vannappagari V, Hall SA, Beutler E. The global prevalence of glucose-6-phosphate dehydrogenase deficiency: a systematic review and meta-analysis. Blood Cells Mol Dis 2009; 42(3): 267-78.

[3] Nuchprayoon I, Sanpavat S, Nuchprayoon S. Glucose-6-phosphate dehydrogenase (G6PD) mutations in Thailand: G6PD Viangchan (871G>A) is the most common deficiency variant in the Thai population. Hum Mutat 2002; 19(2): 185.

[4] Cappellini MD, Fiorelli G. Glucose-6-phosphate dehydrogenase deficiency. Lancet 2008; 371(9606): 64-74.

[5] Guindo A, Fairhurst RM, Doumbo OK, Wellems TE, Diallo DA. X-linked G6PD deficiency protects hemizygous males but not heterozygous females against severe malaria. PLoS Med 2007; 4(3): e66.

[6] Mehta A, Mason PJ, Vulliamy TJ. Glucose-6-phosphate dehydrogenase deficiency. Baillieres Best Pract Res Clin Haematol 2000; 13(1): 21-38.

[7] Watson J, Taylor WR, Menard D, Kheng S, White NJ. Modelling primaquine-induced haemolysis in G6PD deficiency. Elife. 2017; 6.

[8] Ley B, Luter N, Espino FE, Devine A, Kalnoky M, Lubell Y, et al. The challenges of introducing routine G6PD testing into radical cure: a workshop report. Malar J 2015; 14: 377.

[9] World Health Organization CDC. Severe falciparum malaria. Trans R Soc Trop Med Hyg 2000; 94(Suppl 1): S1-90.

[10] Espino FE, Bibit JA, Sornillo JB, Tan A, von Seidlein L, Ley B. Comparison of Three Screening Test Kits for G6PD Enzyme Deficiency: Implications for Its Use in the Radical Cure of Vivax Malaria in Remote and Resource-Poor Areas in the Philippines. PLoS One 2016; 11(2): e0148172.

[11] WHO. Guidelines for the treatment of malaria. Third ed. Geneva2015. 316 p.

[12] MPAC. Point—of—care G6PD testing to support safe use of primaquine for the treatment of vivax malaria, WHO. Geneva, Switzerland:. 2015.

[13] Kaplan M, Leiter C, Hammerman C, Rudensky B. Comparison of commercial screening tests for glucose-6-phosphate dehydrogenase deficiency in the neonatal period. Clin Chem 1997; 43(7): 1236-7.

[14] Beutler E, Blume KG, Kaplan JC, Lohr GW, Ramot B, Valentine WN. International Committee for Standardization in Haematology: recommended screening test for glucose-6-phosphate dehydrogenase (G-6-PD) deficiency. Br J Haematol 1979; 43(3): 465-7.

[15] Kim S, Nguon C, Guillard B, Duong S, Chy S, Sum S, et al. Performance of the CareStart G6PD deficiency screening test, a point-of-care diagnostic for primaquine therapy screening. PLoS One 2011; 6(12): e28357.

[16] von Fricken ME, Weppelmann TA, Eaton WT, Masse R, Beau de Rochars MV, Okech BA. Performance of the CareStart glucose-6-phosphate dehydrogenase (G6PD) rapid diagnostic test in Gressier, Haiti. Am J Trop Med Hyg 2014; 91(1): 77-80.

[17] Roh ME, Oyet C, Orikiriza P, Wade M, Mwanga-Amumpaire J, Boum Y, 2nd, et al. Screening for Glucose-6-Phosphate Dehydrogenase Deficiency Using Three Detection Methods: A Cross-Sectional Survey in Southwestern Uganda. Am J Trop Med Hyg 2016; 95(5): 1094-9.

[18] Tripatara A. Diagnosis of G6PD deficiency. In: Fucharoen G, Sanchaisuriya E, editors. Laboratory tests for red blood cell abnormalities. 3rd ed. Division of Clinical Microscopy, Faculty of Medical Technology, Khon Kaen University, Khon Kaen; 2003: 105-13.

[19] Adu-Gyasi D, Asante KP, Newton S, Dosoo D, Amoako S, Adjei G, et al. Evaluation of the diagnostic accuracy of CareStart G6PD deficiency Rapid Diagnostic Test (RDT) in a malaria endemic area in Ghana, Africa. PLoS One 2015; 10(4): e0125796.

[20] Burka ER. Infectious disease: a cause of hemolytic anemia in glucose-6 phosphate dehydrogenase deficiency. Ann Intern Med.1969; 70(1): 222-5.

[21] Satyagraha AW, Sadhewa A, Elvira R, Elyazar I, Feriandika D, Antonjaya U, et al. Assessment of Point-of-Care Diagnostics for G6PD Deficiency in Malaria Endemic Rural Eastern Indonesia. PLoS Negl Trop Dis 2016; 10(2): e0004457.

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Relationship between clinical features of dizziness and self-perceived dizziness handicap

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ABSTRACT

Background: Dizziness symptoms have a negative impact on daily activities and quality of life. The relationship between clinical features of dizziness and self-perceived dizziness handicap would gain a better understanding of impact of dizziness.

Objectives: To investigate the correlations of the clinical features of dizziness symptoms and the self-perceived dizziness handicap.

Materials and methods: Fifty participants (13 men and 37 women), aged between 18-65 years old, were recruited from the Otolaryngology clinic. All participants had experienced dizziness at least one month. The clinical features of dizziness measured were intensity (a 10-cm Visual Analogue Scale), frequency (times/week), duration (minutes each episode), and time of onset (months). The self-perceived level of handicap was measured using the Thai version of Dizziness Handicap Inventory (DHI-TH).

Results: The total score of DHI-TH was positively correlated with dizziness intensity and frequency (r=0.65 and 0.48, respectively, $p<0.01$). The subscale scores (physical, emotional and functional) were positively correlated with dizziness intensity and frequency (r ranged from 0.37 to 0.59, $p<0.01$). There were no correlations between the total and subscale DHI scores and duration and time of onset ($p>0.05$).

Conclusion: Self-perceived dizziness handicap as measured by the DHI-TH had a positive correlation with intensity and frequency of dizziness.

Introduction

Dizziness is one of the most common symptoms which bring patients to medical consultations. The prevalence of dizziness is approximately 20-30% of persons in the general population.[1] Dizziness has a tendency to increase with age and is more prevalent in women than men.[2,3] Dizziness symptoms can arise from various etiologies such as benign paroxysmal postural vertigo, vestibular neuritis, Ménière's disease, cardiovascular diseases, neurological disorders, psychological disorders, and chronic nonspecific dizziness.[4] Previous studies demonstrated that patients with either acute or chronic dizziness had diminished their daily activities and quality of life.[5-8] Severity of dizziness is often quantified by its intensity and frequency of dizziness attacks. However, severity is a multidimensional concept which may not be fully explained by only intensity and frequency of symptoms.

Dizziness Handicap Inventory (DHI) is a widely used questionnaire designed to evaluate self-perceived handicaps due to dizziness.[9] The original DHI was developed in English version and subsequently translated into many languages including Thai.[10-12] Original and translated versions of the DHI have been shown to have good validity and reliability.[9-1] DHI of Thai version was also shown to have good validity (Cronbach's alpha ranged from 0.75 to 0.92).[10] The DHI consists of three subscales identifying physical, emotional, and functional aspects. The total DHI scores reflect

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dizziness-related handicap perceived by the patients and help therapist in setting intervention goals and evaluating treatment or rehabilitation programs. Research evidence suggests that the DHI used in conjunction with physical examination and history taking might help quantifying the benefits of medical and rehabilitative treatments.[13]

Relationship of the DHI scores and subjective perception of dizziness has been investigated in previous studies.[14-16] Grigol et al.[14] found that the DHI had a positive correlation with a 10-cm visual vertigo analog scale in patients with vestibular dysfunctions. Likewise, Son et al.[16] found that the DHI was significantly correlated with a 10-cm vertigo visual analog scale and disability scale. Perez et al.[15] also demonstrated that the DHI was related to vestibular handicap, vestibular disability and visuo-vestibular disability. However, whether the clinical features of dizziness symptoms reflected the magnitude of self-perceived dizziness handicaps still remain unclear. The dizziness symptoms may not necessarily be correlated with the level of self-perceived handicaps due to dizziness. The relationship between clinical features of dizziness and self-perceived dizziness handicap will provide a better understanding of impact of dizziness. Thus the purpose of this study was to investigate the correlations of the clinical features of dizziness symptoms and self-perceived dizziness handicap, as measured by the DHI.

Materials and methods

Participants

Fifty participants were recruited from the Otolaryngology clinic, Chiang Mai University Hospital. Participants were aged between 18-65 years old and complained of dizziness at least one month. Participants were excluded if they had dizziness due to cardiovascular or neurological diseases (i.e. Stroke, Parkinson's disease, and multiple sclerosis), a history of head trauma, somatic illness, diagnosis of dementia and cognitive impairment, and psychological disorders.

The study was approved by the research ethics committee for research in humans, Faculty of Medicine, Chiang Mai University (No. NONE-2561-05250). All participants signed written informed consent forms before the commencement of the study.

Questionnaires

All participants were asked to complete a questionnaire designed to collect demographic data and clinical features of dizziness. The participants also completed the Dizziness Handicap Inventory-Thai version (DHI-TH) questionnaire to evaluate self-perceived level of handicap due to dizziness.

Clinical features of dizziness

For this study, the clinical features of dizziness are intensity, frequency, duration and time of onset. A Visual Analogue Scale (VAS) was used to determine intensity of dizziness. The VAS is a 10-cm long horizontal line labeled "no dizziness" at one end and "maximum dizziness" on the other end. The participants were asked to indicate their average dizziness intensity over the past week by marking a point on the VAS. The VAS score was subsequently classified into groups as mild intensity (VAS<4.0), moderate intensity (VAS 4.0-6.9), severe intensity (VAS≥7.0).[14] Frequency was defined as the average number of dizziness attack per week (times/week). Duration was defined as the length of time each episode of dizziness lasted (minutes). Time of onset was referred to the length of time that the participants had experienced dizziness symptoms (months).

Self-perceived level of handicap

Self-perceived level of handicap was measured using the DHI-TH. The DHI-TH has 25 items, divided into 3 subscales: emotional (9 items), functional (9 items), and physical (7 items). Each item has 3 response options "yes, sometimes and no", scored as "4, 2 and 0", respectively. The possible total DHI-TH score ranges from 0 (no handicap) to 100 (maximum handicap), with higher score indicates greater perceived handicap. DHI-TH scores were then classified into 3 groups of self-perceived level of handicap with a total score of 0-30 indicating mild, 31-60 moderate, and 61-100 severe.[17] The reliability of the DHI-TH for this study was shown to be excellent (ICC=0.90-0.96).

Statistical analysis

Sample size for the study was determined according to Roscoe.[18] The minimum number of cases required for correlation analysis should be at least 50. Descriptive statistics were used to describe demographic data and all outcome measures. Kolmogrov Smirnov test was used to test the assumption of normality. The DHI-TH scores (both total and its subscales) and intensity of dizziness data were normally distributed whereas the normality of the frequency, duration and time of onset of dizziness was not met. Pearson's correlation coefficient was then used to determine the relationship between the DHI-TH scores and intensity of dizziness and Spearman's correlation to determine the relationships between the DHI-TH scores and the frequency, duration and time of onset of dizziness. The correlation coefficient of 0.00-0.10 was considered as negligible, 0.10-0.39 weak, 0.40-0.69 moderate, 0.70-0.89 strong and 0.90-1.0 very strong.[19] Chi-square was used to examine the correlation of severity classification (mild, moderate and severe) between the DHI and VAS. A significance level was set at 0.05.

Results

Of the 50 participants, 70% were diagnosed with benign paroxysmal positional vertigo. The intensity of dizziness as measured by VAS was regarded as moderate (mean=4.99±1.95). Total DHI-TH score indicated a moderate level of self-perception handicap (mean=47.64±18.30, the scores range between 20 and 86). The scores ranged from 4 to 28 for the physical subscale, 2 to 30 for the emotional subscale, and 6 to 36 for the functional subscale. Demographic data, clinical features and the DHI-TH scores of the participants are presented in Table 1.

Table 1 Demographic and clinical data of the participants.

Demographic data	
Gender (Male: Female)	13:37
Age (years)	46.80±13.60
Clinical features of dizziness	
Intensity (0-10 VAS)	4.99±1.95
Frequency (times per week)	13.88±9.51
Duration (minutes each episode)	15.80±21.53
Time of onset (months)	10.76±34.27
Diagnosis of dizziness	
Benign Paroxysmal Positional Vertigo (n, %)	35 (70%)
Vestibular neuritis (n, %)	7 (14%)
Ménière's disease (n, %)	3 (6%)
No vestibular disease (n, %)	3 (6%)
Unknown (n, %)	2 (4%)
Medication used for dizziness (n, %)	42 (84%)
Dizziness Handicap Inventory score	
DHI total score	47.64±18.30
Physical subscale	16.36±5.72
Emotional subscale	11.40±7.00
Functional subscale	19.88±7.94

VAS: Visual Analogue Scale, Data are expressed as mean±standard deviation, otherwise as indicated.

Correlations between the DHI-TH scores and clinical features of dizziness

The total, physical, functional subscale scores of the DHI-TH were moderately correlated with both intensity and frequency of dizziness symptoms (r ranged from 0.47-0.65, $p<0.01$). The emotional subscale score had a weak correlation with the dizziness frequency (r=0.37, $p<0.01$) (Table 2). There was no correlation between the total DHI-TH score and any subscale scores with the dizziness duration and time of onset ($p>0.05$).

Table 2 Correlations between Dizziness Handicap Inventory scores and clinical features of dizziness.

Clinical features of dizziness	DHI - TH scores			
	Total	Physical	Emotional	Functional
Dizziness intensity (0-10 VAS) [a]	0.65*	0.57*	0.55*	0.59*
Dizziness frequency (times per week)[b]	0.48*	0.50*	0.37*	0.47*
Dizziness duration (minutes each episode)[b]	0.10	-0.01	0.10	0.12
Time of onset (months)[b]	-0.11	-0.13	-0.02	-0.12

**p<0.01, a data were analysed using Pearson's correlation, b data were analysed using Spearman's correlation, VAS: Visual Analogue Scale, DHI-TH: Dizziness Handicap Inventory-Thai version*

The frequency distribution of level of dizziness severity between the VAS and DHI-TH scores is presented in Table 3. Agreement in the severity classification of dizziness between the VAS and DHI-TH was 50% (mild =12%, moderate = 20%, severe = 18%). The Chi-square analysis revealed a moderate correlation between the severity level of handicap classified by the VAS and by the DHI-TH (r=0.50, $p<0.01$).

Table 3 Frequency distribution of the VAS and DHI regarding the level of handicap due to dizziness.

	DHI - TH	DHI -TH	DHI - TH	Total
	Mild	Moderate	Severe	
VAS - Mild	6 (12%)	7 (14%)	0 (0%)	13 (26%)
VAS - Moderate	7 (14%)	10 (20%)	7 (14%)	24 (48%)
VAS - Severe	1 (2%)	3 (6%)	9 (18%)	13 (26%)
Total	14 (28%)	20 (40%)	16 (32%)	50 (100%)

VAS: Visual Analogue Scale, DHI-TH: Dizziness Handicap Inventory-Thai version, Data are presented as number (%)

Discussion

The results of this study demonstrated the moderate correlations between the total DHI-TH scores and dizziness intensity and frequency. Higher intensity and frequency of dizziness were associated with greater experience self-perceived handicap. The results are in agreement with the findings of previous studies regardless of etiologies and stages of dizziness.[14-16, 20] Grigol et al.[14] found a significant moderate correlation between the total DHI and VAS scores in patients with dizziness cause by vestibular dysfunction (r=0.54). Likewise, Caldara et al.[20] reported that the total DHI score was moderately correlated with the VAS (r=0.59) in patients with vertigo, dizziness or unsteadiness over prolonged periods. Son et al.[16] also found similar results in the acute phase of unilateral vestibulopathy.

In addition, the physical and functional subscale scores of the DHI-TH were moderately correlated with intensity and frequency of dizziness. The emotional subscale, however, had a relatively weak correlation with dizziness frequency. The results are similar with previous studies which demonstrated the relationships between the subscale DHI scores and intensity and frequency of dizziness.[15, 20] Caldara et al.[20] showed that VAS score was moderately correlated with all subscale scores of the DHI in patients with dizziness caused by vestibulopathy (r ranged from 0.54-0.55). Likewise, Perez et al.[15] demonstrated frequency of dizziness was correlated with all the subscales of the DHI (r ranged from 0.56-0.63). No correlations between the DHI-TH (both total and subscale scores) and dizziness duration and time of onset were found in this study. This may be explained by differences in the nature of dizziness symptoms. Additionally, patients may adapt and self-modify their daily activities even in the absence of the dizziness.[9] Medication use may also influence characteristics of dizziness.

The total DHI-TH score in this study was considered as moderate intensity, indicating impact of dizziness on quality of life. The functional subscale presented the highest score, whereas the physical subscale presented intermediate score and the emotional subscale displayed the lowest score. These results support the findings of previous studies demonstrating more marked disability in the physical and functional aspects in patients with dizziness.[21-23] An explanation for these results may be that the functional aspect of the DHI measures the interference of dizziness with certain movements of the eyes, head and body for participation in social and leisure activities and the physical subscale measures the aggravation of dizziness due to the movements of eyes, head and body.[22, 23] Alternatively, the emotional subscale measures the impact of dizziness to patient's emotional well-being such as feeling frustration, fear of going out or staying at home alone, and anxiety.[22, 24] It was noted that the participants participating in this study were middle-age population. Thus, dizziness may affect objectively the participant's ability to resume work or play, but they are still able to manage dizziness when under stress.

Furthermore, the result of this study suggested a moderate correlation of the level of handicap classified by the VAS and DHI-TH, which is consistent with the findings of a previous study.[14] About 50% of agreement were found between the VAS and DHI-TH regarding to the dizziness severity classification. However, the use of one in place of the other is not recommended. Although the VAS is simpler than the DHI, it does not provide information addressing influence of dizziness on physical, emotional and functional aspects of the patients. Besides, the VAS is more related to patient's subjective perception and experience. Thus, using only the VAS to assess the intensity of the dizziness symptom does not adequately provide the information about the impact of dizziness symptoms. The VAS and DHI should be used together when assessing patient's condition and impact of dizziness on everyday life as well as early management is also recommended.

This study has some limitations which have to be pointed out. Recall bias may occur when the participants were asked for their clinical features of dizziness symptoms. Also, the majority of our participants had dizziness caused by benign paroxysmal positional vertigo. Thus this may limit the generalization of the findings.

Conclusion

The study demonstrated a positive correlation between the intensity and frequency of dizziness and self-perceived dizziness handicap. Dizziness had substantial impact on quality of life measured with the Dizziness Handicap Inventory and its impact was more on functional and physical aspects than emotional aspect.

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References

[1] Karatas M. Central vertigo and dizziness: epidemiology, differential diagnosis, and common causes. Neurologist 2008; 14: 355-64. doi: 10.1097/NRL.0b013e31817533a3.

[2] Chang J, Hwang SY, Park SK, Kim JH, Kim HJ, Chae SW, et al. Prevalence of Dizziness and Associated Factors in South Korea: A Cross-Sectional Survey From 2010 to 2012. J Epidemiol 2018; 28: 176-84. doi: 10.2188/jea.JE20160113.

[3] Colledge NR, Wilson JA, Macintyre CC, MacLennan WJ. The prevalence and characteristics of dizziness in an elderly community. Age Ageing 1994; 23: 117-20.

[4] Chan Y. Differential diagnosis of dizziness. Curr Opin Otolaryngol Head Neck Surg 2009; 17: 200-3. doi: 10.1097/MOO.0b013e32832b2594.

[5] Bronstein AM, Golding JF, Gresty MA, Mandala M, Nuti D, Shetye A, et al. The social impact of dizziness in London and Siena. J Neurol 2010; 257: 183-90. doi: 10.1007/s00415-009-5287-z.

[6] Koo JW, Chang MY, Woo S, Kim S, Cho YS. Prevalence of vestibular dysfunction and associated factors in South Korea. BMJ open 2015; 5: e008224.

[7] Pluijm SM, Smit JH, Tromp EA, Stel VS, Deeg DJ, Bouter LM, et al. A risk profile for identifying community-dwelling elderly with a high risk of recurrent falling: results of a 3-year prospective study. Osteoporos Int 2006; 17: 417-25. doi: 10.1007/s00198-005-0002-0.

[8] Yardley L, Putman J. Quantitative analysis of factors contributing to handicap and distress in vertiginous patients: a questionnaire study. Clin Otolaryngol Allied Sci 1992; 17: 231-6.

[9] Jacobson GP, Newman CW. The development of the Dizziness Handicap Inventory. Arch Otolaryngol Head Neck Surg 1990; 116: 424-7.

[10] Emasithi A, Kosiyaporn N, Prapawanang Z. Translation and validity of the Thai version of the Dizziness Handicap Inventory. J Vestib Res 2016:202

[11] Jafarzadeh S, Bahrami E, Pourbakht A, Jalaie S, Daneshi A. Validity and reliability of the Persian version of the dizziness handicap inventory. J Res Med Sci 2014; 19: 769-75.

[12] Kurre A, van Gool CJ, Bastiaenen CH, Gloor-Juzi T, Straumann D, de Bruin ED. Translation, cross-cultural adaptation and reliability of the german version of the dizziness handicap inventory. Otol Neurotol 2009; 30: 359-67. doi: 10.1097/MAO.0b013e3181977e09.

[13] Jacobson GP, Newman CW, Hunter L, Balzer GK. Balance function test correlates of the Dizziness Handicap Inventory. J Am Acad Audiol 1991; 2: 253-60.

[14] Grigol TA, Silva AM, Ferreira MM, Manso A, Gananca MM, Caovilla HH. Dizziness Handicap Inventory and Visual Vertigo Analog Scale in Vestibular Dysfunction. Int Arch Otorhinolaryngol 2016; 20: 241-3. doi: 10.1055/s-0035-1567808.

[15] Perez N, Garmendia I, Garcia-Granero M, Martin E, Garcia-Tapia R. Factor analysis and correlation between Dizziness Handicap Inventory and Dizziness Characteristics and Impact on Quality of Life scales. Acta Otolaryngol Suppl 2001; 545: 145-54.

[16] Son EJ, Lee DH, Oh JH, Seo JH, Jeon EJ. Correlation between the dizziness handicap inventory and balance performance during the acute phase of unilateral vestibulopathy. Am J Otolaryngol 2015; 36: 823-7. doi: 10.1016/j.amjoto.2015.07.011.

[17] Whitney SL, Wrisley DM, Brown KE, Furman JM. Is perception of handicap related to functional performance in persons with vestibular dysfunction? Otol Neurotol 2004; 25: 139-43.

[18] Roscoe JT. Fundamental Research Statistics for the Behavioral Sciences. New York: Holt, Rinehart and Winston; 1975.

[19] Mukaka MM. A guide to appropriate use of Correlation coefficient in medical research. Malawi Med J 2012; 24: 69-71.

[20] Caldara B, Asenzo AI, Paglia GB, Ferreri E, Gomez RS, Laiz MM, et al. Cross-cultural adaptation and validation of the Dizziness Handicap Inventory: Argentine version. Acta Otorrinolaringol Esp 2012; 63: 106-14. doi: 10.1016/j.otorri.2011.09.006.

[21] Castro ASO, Gazzola JM, Natour J, Ganança FF. Versão brasileira do Dizziness Handicap Inventory. Audiol Commun Res 2007; 19: 97-104.

[22] Handa PR, Kuhn AM, Cunha F, Schaffleln R, Gananca FF. Quality of life in patients with benign paroxysmal positional vertigo and/or Meniere's disease. Braz J Otorhinolaryngol 2005; 71: 776-82.

[23] Ten Voorde M, Zaag-Loonen HJ, Leeuwen RB. Dizziness impairs health-related quality of life. Qual Life Res 2012; 21: 961-6. doi: 10.1007/s11136-011-0001-x.

[24] Grimby A, Rosenhall U. Health-related quality of life and dizziness in old age. Gerontology 1995; 41: 286-98. doi: 10.1159/000213696.

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A prototype of automatic mattress turning device for pressure ulcer prevention

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ABSTRACT

Background: Pressure ulcer is a major health care problem in bed ridden patients. One of preventive measures is repositioning every 2 hours which is a burden workload on a caregiver.

Objectives: The study aimed to develop and test a laboratory prototype of the automatic mattress turning device (AMTD) whether it could turn one side of the mattress and reposition a patient in a hospital bed as suggesed by the National Pressure Ulcer Advisory Panel (NPUAP).

Materials and methods: A laboratory prototype of AMTD consisted of a set of air bellows and controller. Air bellows were placed under a mattress. An air pump and solenoid valve are controlled by a microcontroller to inflate and deflate the air bellows for turning the mattress, thus reposition from supine to a 30° side-lying or lateral turn position as recommended by NPUAP. Duration for each position can be adjusted from 0 - 120 minutes. A 3-day test was conducted with different duration of turn with bed head tilted at 0° and 30°.

Results: The test revealed that AMTD can provide accurate turning positions and durations of each position. In addition, it worked efficiently when head of bed was lifted up at 30°.

Conclusion: AMTD could turn a hospital mattress and assist with patient repositioning. Further development for commercial use would lead to a new device for pressure ulcer prevention.

Introduction

Pressure ulcer is a serious health problem in bed bound patients. Identifying risk factors and eliminating such factors have been recommended as a preventive measure of pressure injury. So far, many devices such as low-air-loss mattresses, a pressure redistribution device and an Ergo Nurse device, have been used in developed countries.[1] In practice, nurses or caregivers reposition bed-bound patients regularly every 2 hours as recommended by the National Pressure Ulcer Advisory Panel (NPUAP)[2]. In addition, it is advised to reposition patients from supine to side-lying or lateral turn/tilt position of 30°, limit the head tilt up to less than 30°, bend the knees 5° to 10° and lift heels up a little to prevent heel ulcer.[2] Repositioning every 2 hours is not a complicated task, but it becomes a significant workload as it has to be done consistently, especially for paralyzed patients from spinal cord injury (SCI). One to two assistants require approximately 3.5 minutes to turn a patient each time and this must be repeated every 2 hours as long as the patient is at the hospital.[3]

Prolonged lay on a bed causes pressure ulcer which is a life-threatening complication and expensive to treat. An estimated cost of pressure ulcer treatment in UK in 2000 increased substantially to 1.4-2.1 billion dollars,[4] while in USA in 2012 was approximately 6-15 billion dollars.[5] In addition, pressure ulcer in elderly is also related to a high

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mortality rate of 60% within a year after discharged from a hospital.[6] The study by Kammuang-lue and Kovindha in 2007-2009 found that 20% of the SCI patients admitted at rehabilitation ward, Maharaj Nakorn Chiang Mai Hospital were due to pressure ulcers. Some patients had further complications and had to be admitted between 38-380 days with the average treatment cost of 2,000 Baht per day.[7] Another study which collected data for 11 years found that 73.4% of 742 patients had pressure ulcer at least once, and it was common among those with complete loss of motor control and ability to walk.[8] For those lying in bed for an extended period, pressure ulcers commonly developed at the sacrum, buttock, and heels with occurrence rates of 33.9%, 27.3%, and 25.9% respectively.[9]

In developed countries, specialized beds for pressure ulcer prevention are available at hospitals. However, such beds (rotation bed and Freedom BedTM) are very expensive and they are not available in developing countries.[10, 11] As the solution, an automatic mattress turning device (AMTD) was developed and tested the efficiency in turning the hospital mattress to a required lateral tilt angle of 30°.

Materials and methods

Researchers from the Faculty of Medicine and Faculty of Engineering, Chiang Mai University, worked together and developed a prototype of AMTD to assist with repositioning as recommended by NPUAP. It is controlled by a microprocessor so that 3 different positions – supine, 30° left and right lateral turn positions could be obtained. The duration for each position would not exceed 120 minutes. Laboratory prototype test was carried out using a dummy weighted 47 kg, 147 cm tall and BMI of 21.76 kg/m^2.

AMTD consisted of a set of air bellows which was placed under a Lundal Corporation mattress on a Liberty neo bed. Twenty-four hours continuous experiment was recorded for 3 days using slope angle measuring device (© Radislav 2015) to measure the turn/lateral tilt angle at the hip level of the dummy. The test was set as followed:

Day 1: started from a 30° right lateral turn for 30 minutes, then a supine position for 30 minutes and a 30° left lateral turn for 30 minutes

Day 2: started from a supine position for 120 minutes, then a 30° left lateral turn for 120 minutes, and 30° right lateral turn for 120 minutes

Day 3: started from a 30° right lateral turn for 60 minutes, then a 30° left lateral turn for 30 minutes. To be noted, at this stage, the bed was set in a 30° head tilt.

Results

AMTD consisted of 2 main parts – a set of air bellows and a controller as illustrated in Figure 1. Air bellows were made of thermoplastic polyurethane coated nylon and have the dimension of 190 x 90 cm. The set comprised of 2 large 190x35 cm air bellows placed 15 cm apart, and 2 small 190x25 cm ones stitched on top of the large ones as illustrated in Figure 2. Once fully inflated, the height of large and small air bellows would be 25 cm and 15 cm respectively. Each air bellow was connected to controller by pneumatic lines.

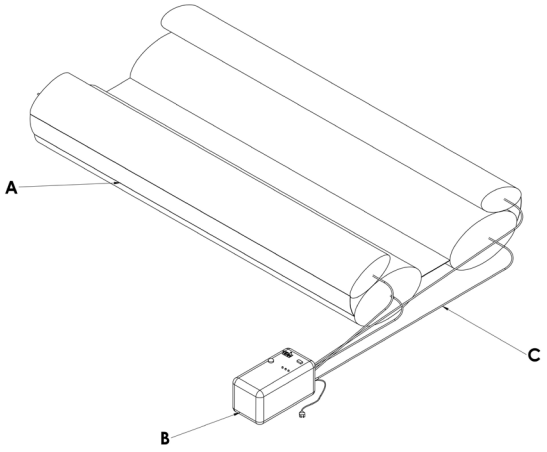


Figure 1 *Structure of AMTD consisted of a set of air bellows (A), a controller (B) and pneumatic line (C)*

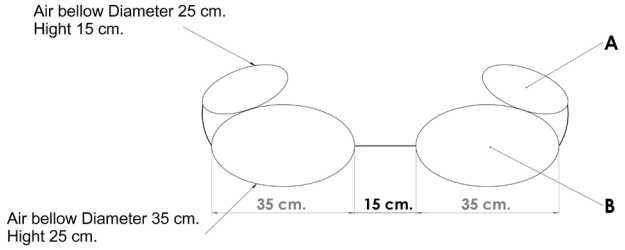


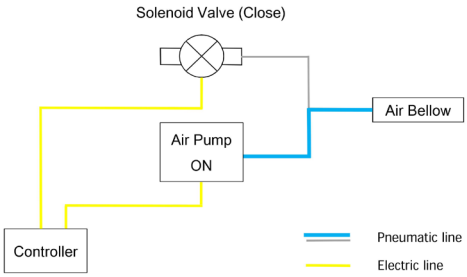
Figure 2 *Cross-sectional shape and size of small air bellows (A) and large air bellows (B)*

The controller comprised of a micro-controller, an air pump, and a solenoid valve to pump, retain, and release air in/out of air bellows. It is based on 220 V power supply with on/off switch, 3 position setting switches, including supine position, left lateral turn, and right lateral turn, and a time setting button with 5 minutes interval adjustment, and a display. Users could select the positions by switching at least 2 positions switches up; set the position changing time, position duration, and check the display. After a dummy was placed in the middle of mattress, start switch was turned on. Thereafter, AMTD started changing the positions automatically according to the setting - a right 30° lateral turn, supine and left 30o lateral turn.

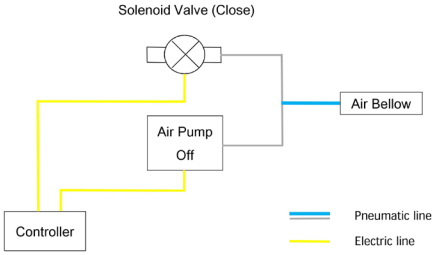
Pump controller controlled air input and output of the air bellows via pump and solenoid valve. Microcontroller controlled pump to push air into air bellows and close solenoid valve. Once air bellows were fully inflated, micro-controller stopped pump and kept solenoid valve closed in order to hold air inside air bellows for a set period of time. When time expired, solenoid valve opened to deflate the air bellows. Figure 3 and Figure 4 show the method of inflation, air retention, and deflation, pushing one side of the mattress up and creating a curvature to reposition the dummy. For the 30° left lateral turn, air was pumped into left small air bellow for 5 minutes and right large bellow

for 15 minutes simultaneously to prevent dummy from sliding to the left and slowly turn dummy to the left. The process was continued in this position for the set duration. Similarly, turning dummy to the right 30° lateral turn position, air was pumped into the right small air bellow and the left large one simultaneously. The process took about 15 minutes and dummy remained at lateral right turn for the set duration. Supine position was achieved by deflating all air bellows. Deflation took about 10 minutes and the dummy rested in this position for a set duration. To be noted, AMTD worked automatically in a cycle starting from left lateral turn, supine and right later turn positions. The cycle was repeated as set. If one position was not selected, the device would skip the unselected position and alternate between 2 selected positions, each for a set period of time.

1 Air bellow inflation



2 Air retention



3 Air bellow deflation

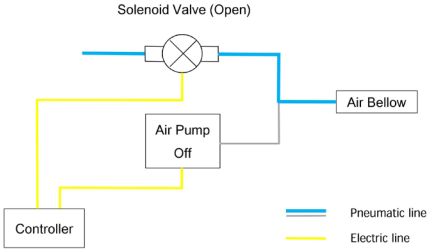


***Figure* 3** *Demonstration of controller and air bellow work process for air bellow inflation, air retention, and air bellow deflation.*

Finally, AMTD passed the 3-day test with accurate turning times and the mattress turning position. The test dummy achieved left and right 30o lateral turn; and in a 30° head tilt, the air bellows still functioned normally and were not damaged.

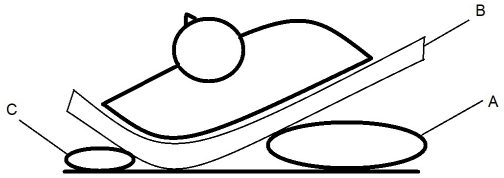


***Figure* 4** *Demonstration of inflated air bellows a large one on right (A) and the a small one on the left (C) lifting the right side of the mattress up (B) and turning dummy to the left*

Discussion

Prototype of AMTD was designed to reduce the need for manual turning. The 3-day test proved it worked automatically in turning the mattress and repositioning the dummy into a cycle of 3 positions recommended by the NPUAP.2 It was able to maintain dummy in a lateral turn position with a maximum duration for each position of 2 hours. When head of bed was tilted up at least 30°, it still functioned efficiently and air bellows were not damaged.

According to the design, when large air bellow is fully inflated, it had the height of 25 cm, which was adequate for turning dummy to a 30° lateral turn position and reducing interface pressure over scapulars and sacrum. Simultaneously, small air bellow on the opposite side is inflated to prevent dummy from sliding, and therefore minimize the shear force. In addition, there should not be pressure force on both heels.[1] Therefore, during the test, we implemented a customized 45x10x10 cm foam cushion with two 10 cm wide and 6 cm deep circular cutouts located 17 cm apart from one another to lift heels up. Another foam cushion with a dimension of 45x20x3 cm was placed under calves to bend knees at 5°, allowing blood flow in popliteal vein and preventing deep vein thrombosis (DVT)[2].

The design of this AMTD also took into account the ease of use. Users only switch on the required positions and select duration for each position, from 5 minutes to 120 minutes since being in the same position for more than 2 hours increases risk of pressure injury. Once the position and duration are set, air pump and solenoid valve will work according to the instruction programmed into microcontroller. In addition, a distinct feature of this AMTD is a slow inflation time of 15 minutes and deflation times of 10 minutes to prevent sleep interruption from position changes. The aim of inventing AMTD was to help turn immobilized patients in bed without the need for human assistance and reduce pressure injury. Researchers the conducted subsequent studies, comparing an interface pressure over bony prominences of patients with cervical cord injury and inability to change body positions by themselves, whether it could prevent

pressure injury, and whether it would work efficienty with other types of hospital mattresses. The results of the subsequent studies will be reported in due course.

To be noted, cost of this laboratory prototype was about 88,000 Baht. Moreover, the industrial design patent filling in Thailand with the application number 1802000554 and the petty patent application number 1803000364 are pending progress.

Conclusion

This automatic mattress turning device controlled by a microcontroller allows users to select required 30° lateral turn and supine positions and duration for each position. It also works efficiently when the head of the bed is tilted up 30°.

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References

[1] Cooper K. Evidence based prevention of pressure ulcers in the Intensive Care Unit. Crit Care Nurse 2013; 33(6).

[2] National pressure ulcer advisory panel (NPUAP). Prevention and treatment of pressure ulcers: quick reference guide [Internet]. Osborne Park, Western Australia; 2014 [cited 2017 Dec 17]. Available from: https://www.npuap.org/wp-content/uploads/2014/08/Updated-10-16-14-Quick-Reference-Guide-DIGITAL-NPUAP-EPUAP-PPPIA-16Oct2014.pdf

[3] Xakellis GC, Frantz R, Lewis A. Cost of pressure ulcer prevention in long-term care. J Am Geriatr Soc 1995; 43(5): 496-501.

[4] Bennett G, Dealey C, Posnett J. The cost of pressure ulcers in the UK. Age Ageing 2004; 33: 230-5.

[5] Markova A, Mostow EN. US skin disease assessment: ulcer and wound care. Dermatol Clin 2012; 30(1): 107-11.

[6] Thomas DR, Goode PS, Tarquine PH, Allman RM. Hospital-acquired pressure ulcers and risk of death. J Am Geriatr Soc 1996; 44: 1435-40.

[7] Kammuang-lue P, Kovindha A. A 3-year retrospective study on total admission charge of spinal cord injured patients with pressure ulcer at rehabilitation ward, Maharaj nakorn Chiang mai hospital. J Thai Rehabil Med 2012; 22(2): 58-63 (in Thai).

[8] Le Fort M, Espagnacq M, Perrouin-Verbe B, Ravaud JF. Risk analyses of pressure ulcer in tetraplegic spinal cord-injured persons: A French long-term survey. Arch Phys Med Rehabil 2017; 98(9): 1782-91.

[9] Stevenson R, Collinson M, Henderson V, Wilson L, Dealey C, McGinnis E, et al. The prevalence of pressure ulcers in community settings: an observational study. Int J Nurs Stud 2013; 50(11): 1550-7.

[10] Russell T, Logsdon A. Pressure ulcers and lateral rotation beds: A case study. J WOCN 2003; 30: 143-5.

[11] Marini I. The freedom bedTM-comparative costs [Internet]. Abbotsford, British Columbia Canada; 2016. [cited 2017 Nov 28]. Available from: https://www.pro-bed.com/files/File/probed_Comparative-Costs-vs-Alternatives.pdf

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- Pachori P, Gothalwal R, Gandhi P. Emergence of antibiotic resistance Pseudomonas aeruginosa in intensive care unit; a critical review. Genes Dis. 2019; 6(2): 109-19. doi: 10.1016/j.gendis.2019.04.001.
- Hung Kn G, Fong KN. Effects of telerehabilitation in occupational therapy practice: A systematic review. Hong Kong J Occup Ther. 2019; 32(1): 3-21. doi: 10.1177/1569186119849119.
- Wijesooriya K, Liyanage NK, Kaluarachchi M, Sawkey D. Part II: Verification of the TrueBeam head shielding model in Varian VirtuaLinac via out-of-field doses. Med Phys. 2019; 46(2): 877-884. doi: 10.1002/mp.13263.
- Velayati F, Ayatollahi H, Hemmat M. A systematic review of the effectiveness of telerehabilitation interventions for therapeutic purposes in the elderly. Methods Inf Med. 2020; 59(2-03): 104-109. doi: 10.1055/s-0040-1713398.
- Junmee C, Siriwachirachai P, Chompoonimit A, Chanavirut R, Thaweewannakij T, Nualnetr N. Health status of patients with stroke in Ubolratana District, Khon Kaen Province: International Classification of Functioning, Disability and Health-based assessments. Thai J Phys Ther. 2021; 43(1): 45-63 (in Thai).

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- Grove SK, Cipher DJ. Statistics for Nursing Research: A Workbook for Evidence-Based Practice. 3rd Ed. St. Louis, Missouri: Elsevier; 2019.
- Perrin DH. The evaluation process in rehabilitation. In: Prentice WE, editor. Rehabilitation techniques in sports medicine. 2nd Ed. St Louis, Mo: Mosby Year Book; 1994: 253–276.

E-book

- Dehkharghani S, editor. Stroke [Internet]. Brisbane (AU): Exon Publications; 2021 [cited 2021 Jul 31]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK572004/ doi: 10.36255/exonpublications.stroke.2021.
- Tran K, Mierzwinski-Urban M. Serial X-Ray Radiography for the Diagnosis of Osteomyelitis: A Review of Diagnostic Accuracy, Clinical Utility, Cost-Effectiveness, and Guidelines [Internet]. Ottawa (ON): Canadian Agency for Drugs and Technologies in Health; 2020 [cited 2021 Jul 31]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK562943/

Dissertation/Thesis

- Borkowski MM. Infant sleep and feeding: a telephone survey of Hispanic Americans [Dissertation]. Mount Pleasant (MI): Central Michigan University; 2002.
- On-Takrai J. Production of monoclonal antibody specific to recombinant gp41 of HIV-1 subtype E [Term paper]. Faculty of Associated Medical Sciences: Chiang Mai University; 2001 [in Thai].

Conference Proceedings

- Lake M, Isherwood J, Clansey. Determining initial knee joint loading during a single limb drop landing: reducing soft tissue errors. Proceedings of 34th International Conference of Biomechanics in Sport; 2016 Jul 18-22; Tsukuba, Japan, 2016. Available from: https://ojs.ub.uni-konstanz.de/cpa/article/view/7126.
- Ellis MD, Carmona C, Drogos J, Traxel S, Dewald JP. Progressive abduction loading therapy targeting flexion synergy to regain reaching function in chronic stroke: preliminary results from an RCT. Proceedings of the 38th Annual International Conference of the IEEE Engineering in Medicine and Biology Society; 2016: 5837-40. doi: 10.1109/EMBC.2016.7592055.

Government Organization Document

- Australian Government, Department of Health. Physical activity and exercise guidelines for all Australian. 2021 [updated 2021 May 7; cited 15 Jul 2021]. Available from: https://www.health.gov.au/health-topics/physical-activity-and-exercise/physical-activity-and-exercise-guidelines-for-all-australians.
- Department of Health. Situation survey on policy and implementation of physical activity promotion in schools for first year 2005. (in Thai). Nonthaburi: Ministry of Public Health; 2005.
- Department of Local Administration, Ministry of Interior Affairs. Standard of Sports Promotion. (in Thai). Bangkok. 2015:7–9.
- World Health Organization. WHO guidelines on physical activity and sedentary behaviour. Geneva: World Health Organization; 2020. Licence: CC BY-NC-SA 3.0 IGO.

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