



Original article

Detection and phenotypic characterization of vancomycin-resistant enterococci in pigs in Thailand

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ARTICLE INFO

Article history:

Received 17 April 2015

Accepted 6 February 2016

Available online 25 June 2016

Keywords:

Antimicrobial resistance

Commercial pig farm

Enterococcus

Pigs

Vancomycin resistance

ABSTRACT

The presence and characteristics were investigated of vancomycin-resistant enterococci (VRE) in pigs in Thailand. A total of 179 rectal swabs were collected aseptically from suckling pigs, fattening pigs and breeding sows on four commercial farms located in Central Thailand. VRE with minimum inhibitory concentrations ranging from 8 µg/mL to 16 µg/mL were detected in 43 of 179 pigs (an overall prevalence rate of 24%). VRE carriers were identified in 12 of 61 (19.7%) suckling pigs, 15 of 60 (25%) fattening pigs and 16 of 58 (27.6%) breeding sows, respectively. *Enterococcus gallinarum* was the most prevalent species for VRE in all age groups, followed by the detection of *Enterococcus casseliflavus*. All of the isolates were susceptible to teicoplanin. A large proportion of VRE isolates showed resistance to tetracycline (86.5%), erythromycin (61.5%), ampicillin (53.8%), chloramphenicol (34.6%) and ciprofloxacin (32.7%). Resistance to ampicillin was more prevalent in *E. gallinarum* isolates than in *E. casseliflavus* isolates. The results of this study indicate that VRE isolates of pigs are of the VanC phenotype and commonly exhibit multiple drug resistance. Different antimicrobial susceptibility is present between VanC species, while *E. gallinarum* is less susceptible than *E. casseliflavus*.

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Introduction

Enterococci are ubiquitous, Gram-positive, catalase-negative, facultative-anaerobic bacteria and while they inhabit humans and animals and present as a part of the normal intestinal flora (Noble, 1978), they are now recognized as one of the leading causes of hospital-associated infections (Hidron et al., 2008). Many infections with enterococci are life-threatening and can be difficult to treat, due to their resistance to several antimicrobials (Huycke et al., 1998). Of particular concern has been the emergence of strains with resistance to glycopeptides. Vancomycin and teicoplanin are the glycopeptide antibiotics currently in use for the treatment of Gram-positive bacterial infections (Murray, 2000). Vancomycin-resistant enterococci (VRE) are typically multidrug-resistant and treatment options are significantly limited. Moreover, certain VRE

genotypes have the potential to transfer the resistant genes to the more virulent Gram-positive pathogen, *Staphylococcus aureus* (Noble et al., 1992).

VRE were first reported in the late 1980s (Leclercq et al., 1988). Since then, VRE have been detected worldwide. In the United States, there was a 20-fold increase in VRE infection rates within a 5-year period and the percentage of VRE identified from patients in the intensive care unit with nosocomial infections was 28.5% in 2003 (National Nosocomial Infections Surveillance, 2004). In Europe, a high frequency of VRE has been reported among food animals, retail meats and non hospitalized people (Bager et al., 1997; Bonten et al., 2001). It was found that the use of avoparcin (a glycopeptide analogue) during livestock production was an important factor for the emergence of VRE (Bager et al., 1997). Due to the possible transmission of VRE and their resistant genes from farm animals to humans, a ban was enforced throughout the European Union on the use of avoparcin (Casewell et al., 2003). A study that followed the change resulting from the ban found a decrease in the prevalence of VRE in farm animals (Bager et al.,

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1999). However, this trend has not been observed in some European countries (Aarestrup, 2000). It has been hypothesized that continued use of other antibiotics in animal husbandry is one important factor for the persistence of VRE, since co-selection for resistant genes located on the same genetic element can occur (Aarestrup, 2000). In the United States, avoparcin has never been permitted for use with food animals, but VRE have been recently isolated from pigs (Donabedian et al., 2010). Therefore, avoparcin may not be an absolute factor for the development of vancomycin resistance in animals.

In Thailand, VRE have been isolated from patients, companion animals, frozen foods and environmental water (Chalermchaikit et al., 2005; Tansuphasiri et al., 2006; Thongkoom et al., 2012). In addition, in the 1990s, contamination of VRE in the poultry products exported to Japan was first detected (Ike et al., 1999) and resulted in severe economic losses. In order to implement an effective prevention and control program for VRE, all potential sources should be investigated. Although antimicrobials are commonly used in swine production, there has been no report of VRE detection from pig farms in Thailand. The goals of this study were: 1) to isolate VRE from pigs in Central Thailand; 2) to identify the species; and 3) to characterize antimicrobial susceptibility profiles.

Materials and methods

Pig farms, antimicrobial use and collection of samples

Sample collection was performed during 2011 in four commercial pig farms, located in Lopburi, Saraburi, Suphanburi and Ratchaburi provinces, Central Thailand. The farm in each province had a sow herd size of about 500, 400, 1200 and 4000 breeding sows, respectively. Data on antimicrobial use in the pig farms were also collected. In all farms, antimicrobial agents were used as feed supplements for swine disease control. The use of antimicrobials was under strict veterinary supervision and the specific types of antimicrobials used were penicillin (amoxicillin), tetracycline (chlortetracycline), macrolide (tilmicosin and valosin), polypeptides (colistin), pleuromutilin (tiamulin) and phosphonic acid (fosfomycin).

Rectal swabs were collected aseptically from randomly sampled pigs of different age groups: suckling pigs, fattening pigs and breeding sows. Fecal samples were preserved in Cary-Blair medium and transported to the laboratory on ice and processed within 18 h.

Isolation of vancomycin-resistant enterococci from fecal samples

The protocol for screening VRE in pigs has been described elsewhere (Pimarn et al., 2011). Fecal samples (approximately 0.5 g) were subjected to a 10-fold serial dilution with buffered peptone water. Diluted fecal slurries were then inoculated onto bile-esculin azide (BEA) agars (Becton Dickinson and Company; Franklin Lakes, NJ, USA) supplemented with 6 µg/mL vancomycin (Sigma–Aldrich; St. Louis, MO, USA). BEA with vancomycin (BEAV) was used as the VRE selective agar, as recommended by Clinical and Laboratory Standards Institute (2011). After incubation at 37 °C for 48 h, culture plates were examined for typical enterococcal colonies. From each sample, colonies with a brown to black halo from the BEAV plates were isolated and then tested for catalase and Gram stain. An isolate demonstrating Gram-positive cocci and negative for catalase was prepared as stock culture in Luria-Bertani broth with 20% glycerol and stored at –80 °C until subsequent identification and characterization of VRE.

Identification of vancomycin-resistant enterococci isolates

The genus identity of presumptive VRE isolates was confirmed by testing on a bile-esculin reaction, 6.5% salt-tolerance test, medium for growth at 45 °C, L-pyrrolidonyl-β-naphthylamide (PYR) and leucine-β-naphthylamide (LAP) tests (Facklam and Elliott, 1995). The PYR and LAP tests were performed, according to the manufacturer's instructions (Oxoid Ltd.; Hampshire, UK). Identification to the species level followed the procedures and biochemical key for *Enterococcus* spp., as previously described (Facklam and Elliott, 1995; Teixeira et al., 2007). Any isolates with ambiguous identification were confirmed by species-specific polymerase chain reaction (PCR) analysis (Jackson et al., 2004). *Enterococcus faecalis* ATCC 14506, *Enterococcus gallinarum* ATCC 49573 and *Enterococcus casseliflavus* ATCC 25788 were included as reference strains.

Antimicrobial susceptibility testing

Levels of vancomycin resistance were determined using minimal inhibitory concentration (MIC) testing with the agar dilution technique (Clinical and Laboratory Standards Institute, 2011). Briefly, the MIC testing was carried out on Mueller-Hinton agars (Neogen Corporation; Lansing, MI, USA) containing serial two-fold dilutions (1–128 µg/mL) of vancomycin. The agar plates were then spot inoculated with 1×10^4 colony-forming units per spot using a microplanter and incubated at 37 °C for 24 h. *E. faecalis* ATCC 29212 and 51299 were used as sensitive and resistant controls, respectively. The Clinical and Laboratory Standards Institute (2011) breakpoints for vancomycin susceptibility are 4 µg/mL or less for susceptible, 8–16 µg/mL for intermediate resistance and 32 µg/mL and above for resistant. Strains with intermediate resistance are also clinically significant, so in this paper, isolates with vancomycin MICs of 8 µg/mL or more were regarded as VRE.

VRE isolates were tested for susceptibility to a selection of antimicrobials using the Kirby–Bauer disc diffusion method (Bauer et al., 1966). The antimicrobials consisted of 13 antimicrobial agent discs (Oxoid Ltd.; Hampshire, UK) of teicoplanin (30 µg), ampicillin (10 µg), tetracycline (30 µg), ciprofloxacin (5 µg), nitrofurantoin (300 µg), rifampin (5 µg), chloramphenicol (30 µg), quinupristin–dalfopristin (15 µg), linezolid (30 µg), fosfomycin (200 µg), streptomycin (300 µg), gentamicin (120 µg) and erythromycin (15 µg). *Staphylococcus aureus* ATCC 25923 was used as a control strain and inhibition zones were evaluated following the guidelines of Clinical and Laboratory Standards Institute (2011). Isolates were classified as multidrug resistant based on the occurrence of resistance to more than two antibiotics.

Statistical analysis

Fisher's exact and Student's t tests were undertaken using the QuickCals Program (GraphPad Software Inc.; La Jolla, CA, USA). The results were deemed statistically significant if $p < 0.05$.

Results

Species distribution and prevalence rates of vancomycin-resistant enterococci in pigs of different age groups

In total, 179 fecal samples were collected from 61 suckling pigs, 60 fattening pigs and 58 breeding sows. Using isolation with the VRE screening agars and identification to the genus level, 71 presumptive isolates were detected. These isolates were each identified to the species level and the levels of vancomycin resistance were determined by the MIC method. With biochemical phenotyping and PCR analysis, 44 isolates were identified as *E. gallinarum*,

25 were *E. casseliflavus* and 1 each were *E. faecalis* and *Enterococcus phoeniculicola*. *E. gallinarum* and *E. casseliflavus* isolates exhibited vancomycin MICs ranging from 2 to 16 µg/mL and 4–16 µg/mL, respectively. The mean MIC in *E. gallinarum* (8.3) was higher than in *E. casseliflavus* (6.5) isolates, with statistical significance ($p < 0.05$). In total, 52 isolates with MICs ranging from 8 to 16 µg/mL were classified as VRE and further characterized.

The VRE carriers were identified in 12 of 61 (19.7%) suckling pigs, 15 of 60 (25%) fattening pigs and 16 of 58 (27.6%) breeding sows, as shown in Table 1. VRE was detected in 43 of the 179 pigs, accounting for an overall prevalence rate of 24%. The occurrence of VRE between age groups was not statistically significant. When the VRE detection rates were categorized by species, 17.9% of pigs were found to harbor vancomycin-resistant *E. gallinarum* (VREg) and 5% were positive for vancomycin-resistant *E. casseliflavus* (VREc). VRE carriers of the two different species were detected in 2 (1.1%) breeding sows. VREg was more prevalent in pigs than VREc ($p = 0.0002$). This trend was observed in all age groups.

Antimicrobial resistance rates of vancomycin-resistant enterococci

The activities of 13 different antimicrobials were tested on VRE. All isolates were susceptible to teicoplanin. Table 2 shows the rates of antimicrobial resistance in the 52 VRE isolates. One (1.9%) and two (3.8%) isolates displayed resistance to nitrofurantoin and fosfomicin, respectively. Seven (13.5%) isolates were resistant to quinupristin–dalfopristin, 11.5% were resistant to linezolid and 9.6% were resistant to rifampin. For susceptibility to aminoglycosides, eight (15.4%) isolates exhibited high-level resistance to gentamicin and 6 (11.5%) exhibited high-level resistance to streptomycin. A large proportion of VRE isolates showed resistance to tetracycline (86.5%), erythromycin (61.5%), ampicillin (53.8%), chloramphenicol (34.6%) and ciprofloxacin (32.7%).

The antimicrobial resistance frequency of VREg and VREc was also analyzed. Surprisingly, resistance to ampicillin was more prevalent in VREg (65%) than in VREc (16.7%) isolates ($p = 0.0066$). Forty percent of VREg isolates were resistant to ciprofloxacin, and prevalence rates of 8.3% were found among VREc isolates. Statistical analysis of these ciprofloxacin-resistant rates showed no significant differences.

Antimicrobial resistance profiles of vancomycin-resistant enterococci

Table 3 shows the antimicrobial resistance patterns of VRE and their fecal samples of origin. There was only one VRE isolate susceptible to all the antimicrobials tested. A degree of resistance with 2–5 antimicrobials was found in the majority of VRE isolates. The detection rates of multidrug-resistant VRE in suckling pigs, fattening pigs and breeding sows were 84.6%, 94.1% and 90.9%, respectively. Multiple resistant strains were detected in 47 (90.4%) of the 52 isolates.

Table 1
Species distribution and prevalence rates of vancomycin-resistant enterococci (VRE) in pigs of different age groups.

Species distribution pattern	Number (n) of pigs positive for VRE (%)			Total (n = 179)
	Suckling pig (n = 61)	Fattening pig (n = 60)	Breeding sow (n = 58)	
<i>E. gallinarum</i>	9	12	11	32 (17.9) ^a
<i>E. casseliflavus</i>	3	3	3	9 (5) ^a
<i>E. gallinarum</i> and <i>E. casseliflavus</i>	0	0	2	2 (1.1)
Total	12 (19.7)	15 (25)	16 (27.6)	43 (24)

^a Significantly different (Fisher's exact test, $p = 0.0002$).

Table 2
Prevalence of antimicrobial resistance in vancomycin-resistant *Enterococcus gallinarum* (VREg) and *E. casseliflavus* (VREc) isolated from pigs.

Antimicrobial agent	Number (n) of isolates resistant to antimicrobials (%)		Total (n = 52)
	VREg (n = 40)	VREc (n = 12)	
Tetracycline	36 (90)	9 (75)	45 (86.5)
Erythromycin	24 (60)	8 (66.7)	32 (61.5)
Ampicillin	26 (65) ^a	2 (16.7) ^a	28 (53.8)
Chloramphenicol	13 (32.5)	5 (41.7)	18 (34.6)
Ciprofloxacin	16 (40)	1 (8.3)	17 (32.7)
Gentamicin	7 (17.5)	1 (8.3)	8 (15.4)
Quinupristin–dalfopristin	6 (15)	1 (8.3)	7 (13.5)
Linezolid	4 (10)	2 (16.7)	6 (11.5)
Streptomycin	5 (12.5)	1 (8.3)	6 (11.5)
Rifampin	2 (5)	3 (25)	5 (9.6)
Fosfomicin	1 (2.5)	1 (8.3)	2 (3.8)
Nitrofurantoin	1 (2.5)	0	1 (1.9)

^a Significantly different (Fisher's exact test, $p = 0.0066$).

When antimicrobial resistant patterns were analyzed, correlations among resistant phenotypes were found. All strains with streptomycin resistance were also resistant to erythromycin. In addition, the combination of resistant characters, TE–E–AMP–CIP–C, was common in isolates with multiple resistance to 5 or more antimicrobials and detected in 8 of the 12 isolates. The resistant phenotypes, TE–E, TE–AMP, TE–E–AMP and TE–E–C, were found in 28, 25, 16 and 15 isolates, respectively.

Discussion

Vancomycin resistance in enterococci can be acquired or can be intrinsic. Two main types of acquired vancomycin resistance, VanA and VanB, have been described (Courvalin, 2005). The VanA phenotype is characterized by high-level (MIC, ≥ 64 µg/mL), transferable resistance to both vancomycin and teicoplanin, while variable levels of resistance to vancomycin and susceptibility to teicoplanin are the characteristics of the VanB phenotype. The VanB phenotype is also considered to be transferable. The VanC phenotype appears to be an intrinsic property of *E. gallinarum* and *E. casseliflavus* and is a low level of vancomycin resistance (Courvalin, 2005). VanA, VanB and VanC phenotypes are mediated by *vanA*, *vanB* and *vanC* gene clusters, respectively. Since Van phenotypes are different in their transferability and susceptibility, the status of the Van type in clinical settings and more broadly is important epidemiologic data for VRE control.

In this study, the prevalence of VRE colonization in pigs and their microbiological characteristics were reported. Forty-three out of 179 (24%) pigs were found to harbor VRE. This VRE detection rate is higher than previously reported by Bustamante et al. (2003) in Costa Rica where a similar protocol for VRE isolation was used and showed a VRE prevalence rate of 11%. Identical vancomycin concentrations but different media in the current study protocol were used and this might explain the different detection rates. The direct

Table 3
Antimicrobial resistant patterns of vancomycin-resistant enterococci (VRE) and their fecal samples of origin.

Degree of antimicrobial resistance	Number (n) of VRE resistant to antimicrobials			Total n = 52	Resistant patterns (number of isolates > 1)
	Suckling pig (n = 13)	Fattening pig (n = 17)	Breeding sow (n = 22)		
1	1	2	1	4	TE (2), RD, AMP
2	3	4	5	12	TE-E (3), TE-AMP (4), TE-CN (2), TE-C, TE-LZD, E-S
3	5	4	3	12	TE-E-AMP (2), TE-E-C, TE-E-CN (2), TE-E-QD (2), TE-AMP-CIP, TE-AMP-LZD, TE-AMP-QD, TE-CIP-CN, E-C-CIP,
4	1	3	7	11	TE-E-AMP-C (2), TE-E-AMP-S, TE-E-AMP-CIP, TE-E-C-FOS, TE-E-C-RD, TE-AMP-C-RD, TE-AMP-CIP-RD, TE-CIP-LZD-FOS, E-AMP-CIP-QD, E-AMP-S-QD
5	1	2	5	8	TE-E-AMP-C-CIP (4), TE-E-AMP-CN-S, TE-E-C-RD-LZD, TE-E-C-CIP-CN, TE-E-AMP-CIP-S
6	0	2	0	2	TE-E-AMP-C-CIP-LZD, TE-E-AMP-C-CIP-QD
7	1	0	0	1	TE-E-AMP-C-CIP-CN-S
8	0	1	0	1	TE-E-AMP-C-CIP-F-QD-LZD
Total multidrug-resistant isolates (%)	11 (84.6)	16 (94.1)	20 (90.9)	47 (90.4)	

TE = tetracycline; E = erythromycin; AMP = ampicillin; C = chloramphenicol; CIP = ciprofloxacin; CN = gentamicin; S = streptomycin; LZD = linezolid; QD = quinupristin-dalfopristin; RD = rifampin; FOS = fosfomicin; and F = nitrofurantoin.

plating technique used in the current study has been recommended by [Clinical and Laboratory Standards Institute \(2011\)](#) and is commonly used in laboratories as a method of VRE screening from human stool specimens ([Gambarotto et al., 2000](#)). The current results indicate that the method is applicable to swine fecal samples.

E. gallinarum and *E. casseliflavus* were the two species identified among VRE isolates. These VRE strains exhibited vancomycin resistance with MICs of 8–16 µg/mL and neither were resistant to teicoplanin. Accordingly, the VRE isolates of pigs were classified to the VanC phenotype. However, a strain of *E. gallinarum* carrying *vanB* genotype has been reported ([Mahony et al., 2010](#)). The current study also investigated the presence of the *vanB* gene using PCR analysis (data not shown). No *vanB* *E. gallinarum* strains were detected. Intrinsic resistance is widely accepted as having a minimal potential for horizontal transfer. The possibility of dissemination of vancomycin resistance from pigs via the genetic spread is thus low. In addition, there was a significant difference in species distribution, in which VREg was more prevalent in pigs than VREc. Consistent with this finding, the same pattern was found in the studies of humans ([Gambarotto et al., 2000](#)) and of chickens ([Jung et al., 2007](#)). This could be explained by the fact that *E. casseliflavus* is generally regarded as one of the plant-associated enterococci that can colonize animal intestines and vancomycin resistance is uncommon ([Müller et al., 2001](#)).

The phenotype of VRE of pigs in many European countries is mainly VanA and the emergence of this acquired-resistant type has been linked to the use of avoparcin ([Bager et al., 1997](#)). Avoparcin has been prohibited in farm animals in Thailand and its discontinued use may be an explanation for the absence of acquired vancomycin resistance in the current study. A predominance of VanC strains among VRE from pigs has also been reported in a Korean study ([Seo et al., 2005](#)). The study found that 56 out of 274 enterococcal isolates from pigs were VRE (MIC: 4–8 µg/mL) and all of them were identified as *E. gallinarum* and *E. casseliflavus*. However, a higher MIC level was observed among the isolates in the current study.

Multi-drug resistance among vancomycin-resistant *Enterococcus faecium* isolates has been well described ([García-Migura et al., 2005](#)). However, there is limited data on antimicrobial resistance among VREg and VREc strains. In the VRE isolates of pigs,

multiple resistances were common. Multi-drug resistant VRE were detected in high percentages in all age groups. In addition, the majority of isolates was resistant to tetracycline, erythromycin, ampicillin, chloramphenicol and ciprofloxacin. The occurrence and emergence of antimicrobial resistance in a population are strongly correlated with antimicrobial usage ([van den Bogaard et al., 2000](#)). Although additional studies will be needed to clarify the relationship between antimicrobial usage and resistance, the occurrence of tetracycline and ampicillin resistances might be due to the wide use of chlortetracycline and amoxicillin in pig farms. The use of tylosin has been associated with the high prevalence of resistance to erythromycin ([van den Bogaard et al., 2000](#)). Tylosin has not been used in pigs and it is possible that the use of other macrolides is responsible for the occurrence of erythromycin resistance. The observed high prevalence of resistances to chloramphenicol and ciprofloxacin could not be explained by a high usage, since chloramphenicol and ciprofloxacin are no longer used in pig farms. However, it could not be excluded that antimicrobial usage in the past has resulted in persistence of resistance. Another explanation might be as a result of genetic linkage and cross selection. In this study, 94% of chloramphenicol-resistant VRE isolates were concurrently resistant to tetracycline. This correlated with the finding that the genes encoding chloramphenicol resistance are often located on the same plasmid as the genes for tetracycline resistance ([Nijsten et al., 1996](#)).

In addition to vancomycin resistance, ampicillin resistance was more prevalent in *E. gallinarum* isolates than in *E. casseliflavus* isolates. Distinction between the antimicrobial susceptibility of *E. faecium* and *E. faecalis* has been documented ([Butaye et al., 2001](#)). However, data on the antimicrobial susceptibility among VanC species are limited. In the study of chicken isolates, *E. gallinarum* strains were more frequently resistant to antimicrobials than *E. casseliflavus* strains ([Jung et al., 2007](#)). The current data from pig isolates was consistent with this finding.

Most human VRE infections have been caused by *E. faecium* and *E. faecalis* which commonly display the VanA and VanB phenotypes ([Mazuski, 2008](#)). Although VanC species have been found to infect humans sporadically, causes of serious invasive infections, including endocarditis and meningitis, have been reported ([Toye et al., 1997](#)). The epidemiology of VRE infections in Thailand has

been described as the predominant phenotype of VanB (Nilgate et al., 2003; Thongkoom et al., 2012). In the current study, no VRE strains of *E. faecium* and *E. faecalis* were recovered in pigs. Based on the current findings, a link between the presence of VRE in pigs and human infections could not be proposed. However, the importance of pigs as a possible reservoir for the transfer of VanC-type enterococci to healthy humans via the food chain could not be excluded. A survey sampling pork in Central Thailand detected contamination of VRE, in which *E. gallinarum* accounted for the majority (67%) of isolated species (Chalermchaikit et al., 2008). In addition, *E. gallinarum* and *E. casseliflavus* have been found as commensals of the human intestinal tract (Toye et al., 1997). A study in France showed a predominance of VanC strains in meat products, including pork, correlated with the occurrence of VRE in non hospitalized people (Gambarotto et al., 2001). Therefore, additional research on healthy subjects will help to determine the relevance of pigs as a potential VRE reservoir for human colonization. Furthermore, the resistant characters, TE–E–AMP–C–CIP, TE–E–C and TE–E–AMP, may be used as a predominantly resistant phenotype of pigs.

In summary, VRE isolates of pigs were characterized as the VanC phenotype. These strains are multidrug resistant and show species differences in antimicrobial susceptibility. Although the potential for horizontal gene transfer is low, the transmission risk of the VanC strains to humans could not be excluded.

Conflict of interest

There is no conflict of interest in this paper.

Acknowledgements

The authors gratefully acknowledge the Kasetsart University Research and Development Institute (KURDI) (Grant No. 76.53) for financial support. We also thank Ms Wanida Passadurak, Mrs Onwarang Narunat and Ms Orawan Limsivilai for technical assistance.

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