Original Article

JHSMR Journal of Health Science and Medical Research

Prevalence of Imipenemase and Verona Integron-Encoded Metallo-β-Lactamase in Imipenem-Resistant *Pseudomonas aeruginosa*

Wanutsanun Tunyapanit, M.Sc.[1], Pornpimol Pruekprasert, M.D.[1], Kamolwish Laoprasopwattana, M.D.[1], Sureerat Chelae, M.Sc.[2]

[1]Department of Pediatrics, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand.
[2]Department of Pathology, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand.

Received 6 August 2020 ● Revised 9 December 2020 ● Accepted 21 December 2020 ● Published online 22 April 2021

Abstract:

Objective: To determine the prevalence of metallo-β-lactamase (MBL) including imipenemase (IMP) and Verona integron-encoded metallo-β-lactamase (VIM) in imipenem-resistant *Pseudomonas aeruginosa* (IRPA) isolates and investigate the *in vitro* activities of 9 antimicrobial agents against MBL-positive isolates.

Material and Methods: Seventy-eight IRPA isolates were obtained from Songklanagarind Hospital. MBL production was detected by the combined-disk test, and the bla_{IMP} and bla_{VIM} genes were determined via Polymerase Chain Reaction (PCR). The *in vitro* activities of amikacin, gentamicin, aztreonam, ceftazidime, meropenem, ciprofloxacin, colistin, piperacillin/tazobactam, and ticarcillin/clavulanate were determined using the E-test method.

Results: Of the 78 IRPA isolates, 20 (25.6%, 95% CI 17.2-36.4%) were MBL phenotype-positive, and 30 (38.5%, 95% CI 28.5-49.6%) were MBL genotype-positive (29 Imipenemase (IMP)-type and 1 Verona integron-encoded metallo-β-lactamase (VIM)-type). Ninety percent of the MBL genotype-positive isolates were MDR. Most MBL genotype- positive isolates were susceptible to colistin (susceptibility rate of 96.7% and MIC_{50} value of 1.5 μg/mL).

Conclusion: Our results showed a high prevalence of MBL-positive isolates (38.5%) in IRPAs, and the IMP-type isolates were dominant among the metallo-β-lactamase-producing imipenem-resistant *P. aeruginosa* tested. Most MBL-positive isolates were susceptible to colistin and had low MIC_{50} values.

Keywords: bla_{IMP}, bla_{VIM}, imipenem-resistant, metallo-β-lactamase, *Pseudomonas aeruginosa*

Contact: Wanutsanun Tunyapanit, M.Sc.
Department of Pediatrics, Faculty of Medicine, Prince of Songkla University,
Hat Yai, Songkhla 90110, Thailand.
E-mail: wanutsanun.t@psu.ac.th

J Health Sci Med Res 2021;39(6):459-465
doi: 10.31584/jhsmr.2021805
www.jhsmr.org

Introduction

Pseudomonas aeruginosa (P. aeruginosa) is a serious pathogen that is infamous for its multi-drug resistance (MDR). Carbapenems are potent agents for the treatment of infections caused by MDR *P. aeruginosa* (MDRPA).[1] Metallo-β-lactamases (MBLs) represent one of the most important resistance mechanisms that enables resistance to all β-lactams, including carbapenems, and β-lactamase inhibitors like clavulanic acid, sulbactam, and tazobactam, except monobactams. The infections caused by metallo-β-lactamase-producing *P. aeruginosa* (MBLPA) isolates are associated with high mortality rates.[1-4]

Imipenemase (IMP) and Verona integron-encoded metallo-β-lactamase (VIM) are important acquired MBL types. The MBL genes, including bla_{IMP} and $bla_{VIM,}$ are carried on mobile genetic elements to a location that eventually facilitates the horizontal spreading among different strains.[1,2] In addition, the IMP-type and VIM-type metallo-β-lactamases in *P. aeruginosa* isolates have been reported worldwide to associate with a clonal spread and hospital outbreaks.[1,2,5]

In Asian countries, recent studies have found a high prevalence of MBL-producing imipenem-resistant *P. aeruginosa* (IRPA) in clinical isolates from tertiary hospitals (68.0-76.0%).[6,7]

In Thailand, the prevalence of MBLPA ranges from 17.0-28.0% of isolates, and the IMP-type and VIM-type MBLs are commonly detected in *P. aeruginosa* clinically isolated from tertiary hospitals.[4,8] Therefore, the aim of this study was to determine the prevalence of IMP-type and VIM-type MBLs in IRPA that were clinically isolated at a tertiary hospital in southern Thailand as well as investigate the *in vitro* activities of 9 antipseudomonal agents against IMP-type and VIM-type MBLIRPA isolates.

Material and Methods

Seventy-eight IRPA isolates were collected from clinical samples such as sputum (47), bodily fluid (9), urine (8), tissue (6), pus (4), and blood (4) from patients at Songklanagarind Hospital in Songkhla province, Thailand during the July 2012-October 2013 period. The *P. aeruginosa* isolates were identified by conventional methods, which comprised colony morphology; Gram staining; pyocyanin, pyoverdine, and pyorubin pigment production; and oxidase, citrate, cetrimide, urease, and Triple Sugar Iron fermentation tests.[9] Polymerase Chain Reaction (PCR) with specific primers (forward: 5′ATGGAAATGCTGAAATTCGGC3′, reverse: 5′CTTCTTCAGCTCGACGCGACG3′) was used to identify the *oprL* gene as described by Aghamiri et al.[10] IRPA was defined as an isolate confirmed to be resistant to imipenem. MDR was defined as an isolate that is resistant to three or more classes of antimicrobial agents.[11] The Ethics Committee of the Faculty of Medicine, Prince of Songkla University approved this study (REC 55-340-01-8-3 and REC 60-039-01-8).

All isolates were tested for MBL production using the combined-disk test as described by Franklin et al.[12] as well as for MBL-encoding genes.[10] The deoxyribonucleic acid template was prepared using the boiling method.[10] The bla_{IMP} gene was detected by PCR with bla_{IMP}-specific primers (forward: 5′GTTTGAAGAAGTTAACGGGTGG3′, reverse: 5′ATAATTTGGCGGACTTTGGC3′), and the bla_{VIM} gene was detected by PCR with bla_{VIM}-specific primers (forward: 5′TGGTGTTTGGTCGCATATCG3′, reverse: 5′GAGCAAGTCTAGACCGCCCG3′) as described by Aghamiri et al.[10] IMP-15-producing *P. aeruginosa* and VIM-producing *P. aeruginosa* Department of Medical Sciences Thailand 41883 were used as positive controls.

The antimicrobial susceptibilities and the minimum inhibitory concentrations (MICs) of amikacin, gentamicin, aztreonam, ceftazidime, meropenem, ciprofloxacin, colistin, piperacillin/tazobactam, and ticarcillin/clavulanate (Liofilchem, Roseto degli Abruzzi, Italy) were determined using the E-test, and the results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[13] *P. aeruginosa* ATCC 27853 was used as the control strain.

The R software 3.5.2 (R Foundation for Statistical Computing, Vienna, Austria) was used for the statistical analysis. The normality test was carried out via the Shapiro-Wilk test. The Wilcoxon rank-sum test was employed for continuous variables, and either Pearson's chi-squared test or Fisher's exact test was used to analyze categorical variables where appropriate. A p-value of <0.050 was considered to indicate statistical significance.

Results

Of the 78 IRPA isolates, 20 (25.6%, 95% CI 17.2-36.4%) resulted MBL phenotype-positive using the combined-disk test, and 30 (38.5%, 95% CI 28.5-49.6%) were found to be MBL genotype-positive using the PCR method. The bla_{IMP} and bla_{VIM} genes were detected in 29 (96.7%) and 1 (3.3%) of the MBL-positive isolates, respectively (Table 1 and Table 2). In addition, 27 (90.0%) of the 30 MBL-positive isolates were MDR. The IMP-type isolates were obtained mainly from patients with a history of respiratory tract infection (44.8%), and the majority of these isolates were from patients who were admitted in the Respiratory Care Unit (27.6%).

The susceptibility percentages of amikacin, gentamicin, ceftazidime, meropenem, aztreonam, ciprofloxacin, piperacillin/tazobactam, and ticarcillin/clavulanate, except for that of colistin, against MBL-positive isolates were significantly lower than those against MBL-negative isolates (Table 1).

Of the 30 MBL-positive isolates, colistin showed the highest percentages of susceptibility against MBL-positive isolates (96.7%), and its MIC_{50} value was 1.5 μg/mL. Meropenem and ticarcillin/clavulanate showed the highest

Table 1 Antimicrobial susceptibilities of 78 imipenem-resistant *Pseudomonas aeruginosa* isolates

Antimicrobial agent	Imipenem-resistant *P. aeruginosa* (n=78)				p-value
	MIC_{50} (μg/mL)		Susceptible (%)		
	MBL-positive (n=30)	MBL-negative (n=48)	MBL-positive (n=30)	MBL-negative (n=48)	
Amikacin	32.0	3.0	13 (43.3)	36 (75.0)	<0.001
Gentamicin	96.0	1.5	4 (13.3)	33 (68.8)	<0.001
Ceftazidime	>256.0	8.0	4 (13.3)	25 (52.1)	<0.001
Meropenem	>32.0	8.0	1 (3.3)	6 (12.5)	0.019
Aztreonam	16.0	12.0	3 (10.0)	23 (47.9)	<0.001
Ciprofloxacin	>32.0	0.1	4 (13.3)	32 (66.7)	<0.001
Colistin	1.5	1.5	29 (96.7)	46 (95.8)	1.000
Piperacillin/tazobactam	32.0	16.0	4 (13.3)	24 (50.0)	0.004
Ticarcillin/clavulanate	>256.0	>256.0	1 (3.3)	10 (20.8)	0.007

MIC breakpoints of amikacin: susceptible ≤16 μg/mL; aztreonam: susceptible ≤8 μg/mL; ceftazidime: susceptible ≤8 μg/mL; ciprofloxacin: susceptible ≤1 μg/mL; colistin: susceptible ≤2 μg/mL; gentamicin: susceptible ≤4 μg/mL; meropenem: susceptible ≤2 μg/mL; piperacillin/tazobactam: susceptible ≤16/4 μg/mL; and ticarcillin/clavulanate: susceptible ≤16/2 μg/mL (CLSI, 2017)
MIC: minimum inhibitory concentration, difference was considered significant at a p-value of <0.050

Table 2 Antimicrobial susceptibilities of 30 metallo-β-lactamase-producing imipenem-resistant *Pseudomonas aeruginosa* isolates

Antimicrobial agent	MBL-positive (n=30)			
	IMP-type (n=29)		VIM-type (n=1)	
	MIC_{50} (μg/mL)	S (%)	MIC (μg/mL)	S (%)
Amikacin	32.0	13 (44.8)	96.0	0 (0.0)
Gentamicin	16.0	4 (13.8)	512.0	0 (0.0)
Ceftazidime	>256.0	4 (13.8)	96.0	0 (0.0)
Meropenem	>32.0	1 (3.4)	>32.0	0 (0.0)
Aztreonam	16.0	3 (10.3)	16.0	0 (0.0)
Ciprofloxacin	>32.0	4 (13.8)	>32.0	0 (0.0)
Colistin	1.5	28 (96.5)	1.0	1 (100)
Piperacillin/tazobactam	32.0	4 (13.8)	>256.0	0 (0.0)
Ticarcillin/clavulanate	>256.0	1 (3.4)	>256.0	0 (0.0)

MIC breakpoints of amikacin: susceptible ≤16 μg/mL; aztreonam: susceptible ≤8 μg/mL; ceftazidime: susceptible ≤8 μg/mL; ciprofloxacin: susceptible ≤1 μg/mL; colistin: susceptible ≤2 μg/mL; gentamicin: susceptible ≤4 μg/mL; meropenem: susceptible ≤2 μg/mL; piperacillin/tazobactam: susceptible ≤16/4 μg/mL; and ticarcillin/clavulanate: susceptible ≤16/2 μg/mL (CLSI, 2017)
S: susceptible, MIC: minimum inhibitory concentration

percentages of resistance against MBL-positive isolates (96.7 and 96.7%, respectively), and their MIC_{50} values were >32 μg/mL and >256 μg/mL, respectively (Table 1). Concerning the IMP-type isolates, 28 of 29 isolates (96.5%) were susceptible to colistin (MIC_{50}=1.5 μg/mL); 1 isolate (3.5%) was non-susceptible to colistin (MIC=3 μg/mL). All other antimicrobials tested were active against ≤44.8% of the 29 IMP-type isolates, and their MIC_{50} values were higher than the CLSI susceptibility breakpoint (Table 2). In addition, 1 VIM-type isolate exhibited resistance against all antimicrobial agents; the MIC values of all antimicrobial agents were higher than the CLSI susceptibility breakpoint, except for colistin (MIC=1 μg/mL) (Table 2).

Discussion

Our study found that most IRPA isolates from the clinical samples tested were MBL-negative isolates (61.5%), which exhibited a high susceptibility level to colistin, amikacin, gentamicin, ciprofloxacin, ceftazidime, and piperacillin/tazobactam (50.0-95.8% susceptibility rates and low MIC_{50} values), indicating that these antimicrobial agents could be useful in the treatment of infections caused by MBL-negative organisms.

We found that 38.5% of our 78 IRPA isolates were MBL genotype-positive. Furthermore, we observed that 90.0% of the 30 MBL-positive isolates were MDR and exhibited high percentages of drug resistance to most antimicrobials tested. Only colistin had good activity against MBL-positive isolates (susceptibility=96.7% and MIC_{50}=1.5 μg/mL). Likewise, Huang et al.[14] found that 24.0% of their 72 IRPA isolates were MBL-positive, and colistin was an effective antimicrobial based on *in vitro* testing. Acharya et al.[7] reported that 69.0% of their 35 IRPA isolates were MBL-positive and MDR. In addition, their MBL-positive isolates were highly susceptible to colistin (susceptibility=100% and MICs ≤2 μg/mL).

Our results suggest that MBL-negative isolates remained susceptible to commonly used antimicrobial

agents, whereas MBL-positive isolates were susceptible to only colistin.

A previous study also observed that MBL-positive isolates were significantly more resistant to routinely prescribed antimicrobial agents than were MBL-negative isolates.[15,16]

In addition, we found that 25.6% of the 78 IRPA isolates were MBL phenotype-positive using the combined-disk test, whereas 38.5% were MBL genotype-positive. Based on this finding, the underestimation of MBL prevalence in the clinical setting is a real possibility. Isolates harboring MBL-encoding genes might act as silent reservoirs of such resistance determinants with an ability to spread, since MBL-encoding genes are often carried by mobile genetic elements[1,2], and this might lead to a possible therapeutic failure in infections caused by MBLPA isolates. Therefore, the PCR confirmation for the detection of MBL-producing isolates should be pursued.

Regarding MBLIRPA isolates, previous studies have reported their prevalence among clinical isolates from tertiary hospitals to range from 17.0-76.0%.[6-8] In this study, 30 isolates were MBLIRPA (38.5%), and the IMP-type isolates were predominant among the MBLIRPAs (96.7%). Similarly, another study from Thailand by Piyakul et al.[8] reported that 92.0% of their 13 MBLIRPA isolates were IMP-type, and the remaining 8.0% were VIM-type. On the other hand, a study from Nepal by Acharya et al.[7] reported that 25.0% of their 24 MBLIRPA isolates were IMP-type, and the rest (75.0%) were VIM-type. We suspect that the reason for these difference in the prevalence of MBLIRPA isolates may be associated with differences in geographic locations, specimen sources, clinical settings, and broad-spectrum antimicrobial consumption.[17]

A previous study reported that IMP-type isolates were more commonly isolated from respiratory samples (36.7%), and that the use of mechanical ventilation was associated significantly with the presence of MBL-positive isolates.[18] In this study, the IMP-type isolates were obtained mainly from patients with a history of respiratory tract infection (44.8%), and the majority of those isolates were from patients who were admitted in the Respiratory Care Unit (27.6%). This finding indicated an alarming spread of IMP-type MBL-positive isolates in the Respiratory Care Unit of our hospital, which makes the implementation of effective infection-control strategies crucial for the prevention of the spread of MBLPAs.

Regarding the IMP-type and VIM-type isolates in this study, we found that 89.6% of the 29 IMP-type isolates and 1 VIM-type isolate were MDR; all of them exhibited high percentages of resistance to most antimicrobials tested, indicating limited treatment options. These findings show colistin to be a viable therapeutic option for infections caused by IMP-type and VIM-type bacteria. However, colistin has been associated with nephrotoxicity, especially in patients with a history of chronic renal failure, neurotoxicity, and colistin resistance.[19-21] Therefore, close monitoring and caution are often required during colistin therapy.

Generally, MBLs are susceptible to aztreonam.[1,2] In this study, 26 (89.6%) of the 29 IMP-type isolates and the 1 VIM-type isolate (100%) were resistant to aztreonam. Similarly, the study of Abaza et al.[18] demonstrated that 73.3% of their 30 MBLPA isolates were resistant to aztreonam. Furthermore, the study of Barros et al.[22] reported the IMP-type MBLPA isolates to be resistant to aztreonam. Notably, we observed that all of our aztreonam-resistant isolates were MDR. We speculate that the resistance mechanisms of the aztreonam-resistant isolates in this study may involve AmpC-type β-lactamases, Extended Spectrum Beta-Lactamases, and the MexAB-OrpM efflux pump.[7,22,23]

The limitations of this study were the small number of isolates and lack of clonal relatedness information. Therefore, repetitive clones might have been present. In addition, apart from MBL production, the other resistance

mechanisms associated with IRPA isolates were not evaluated. To clarify these other resistance mechanisms, further investigations involving larger numbers of isolates are needed.

Conclusion

Our results showed a high prevalence of MBL-positive isolates (38.5%) in IRPAs, and IMP-type was dominant among the MBLIRPAs tested. The majority of MBL-negative and MBL-positive isolates were susceptible to colistin and had low MIC_{50} values.

Acknowledgement

This work was supported by a grant from the Faculty of Medicine, Prince of Songkla University. We thank all of the patients for the samples provided. We are also grateful to Dr. Chanwit Tribuddharat for providing the IMP-type MBLPA strain and the Department of Medical Sciences for providing the VIM-type MBLPA strain. Our gratitude also goes to Mrs. Lamy Kaewjungwad for her assistance with the laboratory facilities and Mr. Edmond Subashi for correcting the English manuscript.

Conflict of interest

No conflicts of interests are declared.

References

1. Hong DJ, Bae IK, Jang IH, Jeong SH, Kang HK, Lee K. Epidemiology and characteristics of metallo-β-lactamase-producing Pseudomonas aeruginosa. Infect Chemother 2015;47:81-97.
2. Hirakata Y, Yamaguchi T, Nakano M, Izumikawa K, Mine M, Aoki S, et al. Clinical and bacteriological characteristics of IMP-type metallo-β-lactamase-producing Pseudomonas aeruginosa. Clin Infect Dis 2003;37:26-32.
3. Carmeli Y, Akova M, Cornaglia G, Daikos GL, Garau J, Harbarth S, et al. Controlling the spread of carbapenemase-producing gram-negatives: therapeutic approach and infection control. Clin Microbiol Infect 2010;16:102-11.
4. Khuntayaporn P, Montakantikul P, Santanirand P, Kiratisin P, Chomnawang MT. Molecular investigation of carbapenem resistance among multidrug-resistant *Pseudomonas aeruginosa* isolated clinically in Thailand. Microbiol Immunol 2013;57:170-8.
5. Cornaglia G, Giamarellou H, Rossolini GM. Metallo-β-lactamase: a last frontier for β-lactams? Lancet Infect Dis 2011;11:381-93.
6. Biradar S, Roopa C. Prevalence of metallo-beta-lactamase producing Pseudomonas aeruginosa and its antibiogram in a tertiary care centre. Int J Curr Microbiol App Sci 2015;4:952-6.
7. Acharya M, Joshi PR, Thapa K, Aryal R, Kakshapati T, Sharma S. Detection of metallo-β-lactamases-encoding genes among clinical isolates of Pseudomonas aeruginosa in a tertiary care hospital, Kathmandu, Nepal. BMC Res Notes 2017;10:718.
8. Piyakul C, Tiyawisutsri R, Boonbumrung K. Emergence of metallo-β-lactamase IMP-14 and VIM-2 in *Pseudomonas aeruginosa* clinical isolates from a tertiary-level hospital in Thailand. Epidemiol Infect 2012;140:539-41.
9. Giligan PH. Pseudomonas and Burlkholder. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH, editors. Manual of clinical microbiology. 6th ed. Washington, DC: ASM Press; 1995;p.509-19.
10. Aghamiri S, Amirmozafari N, Mehrabadi JF, Fouladtan B, Kafil HS. Antibiotic resistance pattern and evaluation of metallo-beta lactamase genes including bla-IMP and bla-VIM types in *Pseudomonas aeruginosa* isolated from patients in Tehran hospitals. ISRN Microbiol [serial on the Internet]. 2014 [cited 2019 Jun 6]. Available from: http://dx.doi.org/10.1155/2014/941507
11. Adjei CB, Govinden U, Moodley K, Essack SY. Molecular characterisation of multidrug-resistant *Pseudomonas aeruginosa* from a private hospital in Durban, South Africa. South Afr J Infect Dis 2018;33:38-41.
12. Franklin C, Liolios L, Peleg AY. Phenotypic detection of carbapenem-susceptible metallo-β-lactamase-producing gram-negative bacilli in the clinical laboratory. J Clin Microbiol 2006;44:3139-44.
13. Jean B, Melvin P, George M, Stephen G, James S, Brandi L, et al. Performance standards for antimicrobial susceptibility testing. 27th ed. Pennsylvania: Clinical and Laboratory Standards Institute; 2017;p.42-5.
14. Huang YT, Chang SC, Lauderdale TL, Yang AJ, Wang JT. Molecular epidemiology of carbapenem-resistant *Pseudomonas aeruginosa* carrying metallo-β-lactamase genes in Taiwan. Diagn Microbiol Infect Dis 2007;59:211-6.

15. Laupland KB, Parkins MD, Church DL, Gregson DB, Louie TJ, Conly JM, et al. Population-based epidemiological study of infections caused by carbapenem-resistant *Pseudomonas aeruginosa* in the Calgary health region: importance of metallo-β-lactamase (MBL)-producing strains. J Infect Dis 2005; 192:1606-12.
16. Ranjan S, Banashankari GS, Sreenivasa Babu PR. Comparison of epidemiological and antibiotic susceptibility pattern of metallo-beta-lactamase-positive and metallo-beta-lactamase-negative strains of Pseudomonas aeruginosa. J Lab Physicians 2014; 6:109-13.
17. Khorvash F, Yazdani M, Shabani S, Soudi A. *Pseudomonas aeruginosa* producing metallo-β-lactamases (VIM, IMP, SME, and AIM) in the clinical isolates of intensive care units, a university hospital in Isfahan, Iran. Adv Biomed Res 2017;6: 147.
18. Abaza AF, El Shazly SA, Selim HSA, Aly GSA. Metallo-beta-lactamase producing Pseudomonas aeruginosa in a health-care setting in Alexandria, Egypt. Pol J Microbiol 2017;66:297-308.
19. Leung CH, Wang NY, Liu CP, Weng LC, Hsieh FC, Lee CM. Antimicrobial therapy and control of multidrug-resistant *Pseudomonas aeruginosa* bacteremia in a teaching hospital in Taiwan. J Microbiol Immunol Infect 2008;41:491-8.
20. Dalfino L, Puntillo F, Ondok MJ, Mosca A, Monno R, Coppolecchia S, et al. Colistin-associated acute kidney injury in severely Ill patients: a step toward a better renal care? a prospective cohort study. Clin Infect Dis 2015;61:1771-7.
21. Memar MY, Pormehrali R, Alizadeh N, Ghotaslou R, Baghi HB. Colistin, an option for treatment of multiple drug resistant *Pseudomonas aeruginosa*. Physiol Pharmacol 2016;20:130-6.
22. Barros EMLR, Morais VMS, Castro KCB, Orsi AR, Maranhao FCA, Silva ZP, et al. First detection of metallo-β-lactamases in nosocomial isolates of *Pseudomonas aeruginosa* in Alagoas, Brazil. J Bras Patol Med Lab 2015;51: 291-5.
23. Rostami S, Sheikh AF, Shoja S, Farahani A, Tabatabaiefar MA, Jolodar A, et al. Investigating of four main carbapenem-resistance mechanisms in high-level carbapenem resistant *Pseudomonas aeruginosa* isolated from burn patients. J Chin Med Assoc 2018; 81:127-32.