Multi Evaluation of a Modified GoldNano Carb Test for Carbapenemase Detection in Clinical Isolates of Gram-Negative Bacilli

Arpasiri Srisrattakarn 1, Aroonlug Lulitanond 1, Nicha Charoensri 1, Lumyai Wonglakorn 2, Suthida Kenprom 2, Chutipapa Sukkasm 2, Waewta Kuwatjanakul 2, Sirikan Piyanapathanakul 3, Onphailin Luanphairin 4, Wichuda Phukaw 5, Kunthida Khanchai 6, Jantira Pasuram 7, Chotechana Wilailuckana 1, Juneerut Daduang 1 and Aroonwadee Chanawong 1, *

1 Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand; arpas@kkumail.com (A.S.); arolul@kku.ac.th (A.L.); nicha@kku.ac.th (N.C.); wchote@kku.ac.th (C.W.); jurpoo@kku.ac.th (J.D.)
2 Clinical Microbiology Unit, Srinagarind Hospital, Khon Kaen University, Khon Kaen 4002, Thailand; wulumya@kku.ac.th (L.W.); sutheke@kku.ac.th (S.K.); tirapi@kku.ac.th (C.S.); pwaewt@kku.ac.th (W.K.)
3 Clinical Microbiology Laboratory, The Queen Sirikit National Institute of Child Health, Bangkok 10400, Thailand; bloodmackaa@gmail.com
4 Medical Technology Laboratory, Maesot General Hospital, Tak 63110, Thailand; aonphailin.48@gmail.com
5 Clinical Microbiology Laboratory, Lerdsin Hospital, Bangkok 10500, Thailand; wichudapookaw@gmail.com
6 Clinical Microbiology Laboratory, Koh Samui Hospital, Surat Thani 84140, Thailand; k_kunthida@kkumail.com
7 Clinical Microbiology Laboratory, Buriram Hospital, Buri Ram 31000, Thailand; jantira_p@kkumail.com
* Correspondence: aroonwad@kku.ac.th; Tel.: +66-(0)-4320-2086

Abstract: Carbapenemase-producing Gram-negative bacteria have been increasingly reported. Simple and sensitive methods for carbapenemase detection are still needed. In this study, a gold nanoparticle (AuNP) solution was modified by the addition of zinc sulfate (ZnSO4) for improving the conventional GoldNano Carb (cGoldC) test, and the modified GoldC (mGoldC) test was then evaluated for phenotypic detection of carbapenemase production in Gram-negative bacilli clinical isolates. ZnSO4 was added to give final concentrations of 0.25, 0.5, 0.75, and 1 mM. The performance of the mGoldC test was evaluated in Enterobacterales, Acinetobacter spp., and Pseudomonas aeruginosa isolates from six hospitals in different regions using polymerase chain reaction (PCR) as a gold standard. The AuNP solution with 0.25 mM ZnSO4 was used for the mGoldC test. Evaluation of the mGoldC test in 495 Enterobacterales, 212 Acinetobacter spp., and 125 P. aeruginosa isolates (including 444 carbapenemase producers and 388 non-carbapenemase producers) revealed sensitivity, specificity, a positive likelihood ratio, and a negative likelihood ratio of 98.6%, 98.2%, 54.7, and 0.01, respectively. This test is fast, easy to perform, cost-effective (~0.25 USD per test), and highly sensitive and specific for routine carbapenemase detection, thus leading to effective antimicrobial therapy and infection control measures.

Keywords: Acinetobacter spp.; AuNP; carbapenem resistance; Enterobacterales; modified GoldNano Carb test; Pseudomonas aeruginosa

1. Introduction

Gram-negative bacilli (GNB) such as Enterobacterales, Pseudomonas aeruginosa, and Acinetobacter spp. with reduced susceptibility to carbapenems by carbapenemase production have been reported worldwide with increasing frequencies [1,2]. Carbapenemases commonly found in these organisms include class A Klebsiella pneumoniae carbapenemase (KPC), class B imipenemase (IMP), Verona integron-encoded metallo-β-lactamase (VIM).
and New Delhi metallo-β-lactamase (NDM), and class D oxacillinase (OXA) carbapenemases [2,3]. The prevalence rates of carbapenem-resistant GNB isolates vary significantly from one country to another (1–90%) [4,5]. According to National Antimicrobial Resistance Surveillance Center, Thailand (NARST) data, carbapenem resistance rates in *K. pneumoniae* and *Acinetobacter* spp. isolates increased from ~3% and ~45% in 2015 up to ~10 and ~70%, respectively, in 2020 [6]. In addition, the distribution of carbapenemase genes among carbapenem-resistant Enterobacterales (CRE) in Thailand from 2016 to 2018 under the national antimicrobial resistance surveillance system developed by the Thailand National Institute of Health (NIH) was 97% [7]. Thus, detection of carbapenemase production is considered important for clinical practice or infection control purposes.

Currently, various commercial carbapenemase tests that are easy, rapid, highly sensitive, and specific have been introduced into the market [8]. These include colorimetric tests such as the Rapidec Carba NP (bioMerieux Deutschland GmbH, Nürtingen, Germany) and the β-CARBA (BioRad, Marnes-la-Coquette, France) or immunochromatographic tests such as the RESIST-4 O.K.N.V. (Coris BioConcept, Gembloux, Belgium) and the CARBA-5 (CARBA-5, NG biotech, Guipry, France). However, false negatives in isolates with weak or low-level carbapenemase activities were found [8–10]. The rapid and accurate MALDI-TOF-MS-based carbapenem hydrolysis assay is also used to detect the carbapenemase activity of bacterial strains [11]. Although the costs of this measurement are low, the equipment remains expensive, thus limiting the wide application of this method in a routine laboratory [12,13]. The price of the rapid commercial kits and the instruments needed for MALDI-TOF-MS remain expensive for low-resource settings.

The Carba NP (CNP) test and modified carbapenem inactivation method (mCIM) have been recommended by the Clinical and Laboratory Standards Institute (CLSI) and are extensively validated worldwide for Enterobacterales and *P. aeruginosa* [14–18]. The mCIM test requires neither special reagents nor equipment but takes 18–24 h. The CNP test is rapid (2 h) and mostly shows high specificity and sensitivity for detecting class A and class B carbapenemases but low sensitivities for OXA carbapenemases [15,17]. In addition, the commercial lysis buffer (B-PERII; Thermo Scientific Pierce, Rockford, IL, USA) used for β-lactamase extraction is costly. The CNP test also failed to detect metallo-β-lactamases (MBLs) (sensitivity of 94% for NDM) [15]. In our previous study, conventional GoldNano Carb (cGoldC) was developed for rapid carbapenemase identification by the use of gold nanoparticles (AuNPs) as a pH indicator for detecting acid production from imipenem hydrolysis [19]. This test is rapid (within 2 h), inexpensive (~0.25 USD per test), and convenient with no requirement of an extraction solution. It provided a sensitivity of 99% for carbapenemase detection in Enterobacterales (100%), *P. aeruginosa* (100%), and *Acinetobacter* spp. (96.7%). However, a false-negative due to very weak carbapenemase activity (OXA) was also observed by the cGoldC test (0.5%). In addition, this method has not yet been evaluated in a large number of clinical isolates of Gram-negative bacilli.

The Zn ion is required for MBL activity and play key roles in the catalytic mechanism [20]. In addition, it can induce a decrease in the inter-nanoparticle distance and electrostatic repulsion invoked by the citrate ligand adsorbed on the surface of the particles [21]. In this study, the cGoldC test was modified by the addition of zinc sulfate (named modified GoldNano Carb or mGoldC test) into the AuNP solution (AuNP-Zn solution) in order to increase its sensitivity for detecting carbapenemase production by accelerating the reaction. The mGoldC test was then evaluated for phenotypic detection of carbapenemase-producing GNB (CPGNB) isolates from various hospitals in different regions of Thailand using PCR as the gold standard.

**2. Results and Discussion**

Among the Enterobacterales isolates from the six hospitals, the NDM rates were higher than those of the OXA-48-like in four hospitals, B, D, E, and F, similar to our previous report in hospital A [22]. In the present study, the majority of OXA-48-like enzymes was observed in hospitals A and C. It was noteworthy that the NDM type was dominant...
in early surveillance of many regions in Thailand, whereas the OXA-48-like type was likely to increase over the years. Teeraputon et al. also reported that the prevalence rate of OXA-48-like in a hospital from northern Thailand during 2018–2019 was 80% [23]. This trend was also observed in Taiwan, where the OXA-48 rate among carbapenemase-producing *Klebsiella pneumoniae* increased 6-fold during 2012–2015 [24]. In addition, IMP producers of each *K. pneumoniae* and *Enterobacter* spp. were found in hospital D where NDM was prevalent in 2016 [25]. No KPC enzyme was detected in any hospital. Our results indicate that NDM and OXA-48-like are predominant carbapenemase types, whereas the KPC type has very low prevalence among CRE isolates in Thailand [18,26,27]. For the *P. aeruginosa* isolates, IMP, VIM, and NDM enzymes were found in hospitals A, B, and E, whereas only one VIM producer was reported from hospital D. Interestingly, the NDM type was more prevalent than VIM and IMP in the *P. aeruginosa* isolates from hospital B, suggesting the outbreak of a clonal NDM-producing strain in the hospital. Among the *Acinetobacter* spp. isolates, OXA-23-like, OXA-58-like, and NDM were seen in all four hospitals. This study demonstrated that VIM and IMP were major carbapenemase types in *P. aeruginosa*, whereas OXA-23-like was the most common in *Acinetobacter* spp., similar to previous studies [28–30]. In addition, the NDM type was found in various species of Enterobacterales, *Acinetobacter* spp., and *P. aeruginosa* (Supplementary Material Table S2), corresponding to the study of Wu et al. [31]. However, outbreaks of clonal strains may exist in these hospitals, suggesting further investigation. Our limitation due to the small sample size of hospital F should also be noted.

To improve its sensitivity, the cGoldC test was modified by using the AuNP-Zn solution for CPGNB detection. The detection times of the mGoldC using the AuNP-Zn solution with any concentration (0.25–1 mM) were faster than those of the cGoldC (without Zn) (Figure 1). The AuNP solution with either 0.25 or 0.5 mM ZnSO$_4$ was still clear with red-wine color (data not shown), but the solution with 0.25 mM ZnSO$_4$ had the longest shelf life, at least six months at 4°C. Thus, the optimal concentration of 0.25 mM ZnSO$_4$ was selected for the following experiments. Initial evaluation revealed that the mGoldC detected all carbapenemase producers (29/29) within 1–60 min, whereas 28 from 29 isolates were cGoldC-positive within 1–80 min (Figure 2). One false negative by the cGoldC test was OXA-23-like-producing *A. baumannii*. Therefore, the mGoldC and cGoldC tests provided sensitivity of 100.0% and 96.6%, respectively. The 37 non-CPGNB isolates were negative in both tests, thus giving 100.0% specificity (Table 1). In addition, times to positivity by the mGoldC test were markedly shorter than those by the cGoldC test (Figure 2). Positive results within 5 min by the mGold versus cGoldC were 69.0% versus 41.4% and 63.6% versus 0% for all carbapenemase and OXA producers, respectively (Supplementary Material Figure S1). The Zn ion may enhance the exchange of ions between surface negative charges of the citrate-capped AuNPs and the positive charges of acid products from the hydrolysis of imipenem by carbapenemases [21]. It is also an essential cofactor for MBLs such as NDM, IMP, and VIM, thus leading to increased enzyme activity [32,33]. With the use of Zn-supplemented AuNP solution, the sensitivity and speed of the mGoldC method were markedly increased. Girlich et al. also reported higher sensitivity of the modified Hodge test (MHT) for detecting MBL producers by using Zn-containing medium (100 µg/mL ZnSO$_4$) [34]. Recently, the immunochromatographic test for NDM showed increased sensitivity when tested using Zn-supplemented Mueller Hinton agar (50 µg/mL ZnSO$_4$) [33]. Therefore, the AuNP-Zn solution was used for the detection of CPGNB isolates by the mGoldC test in further evaluation.
Figure 1. Optimization of ZnSO₄ (final concentrations of 0, 0.25, 0.5, 0.75, and 1.0 mM) in the AuNP solution for carbapenemase detection by the modified GoldNano Carb test. The reactions were read at 10 min. Tubes A, control (without imipenem); and B, test (with 5 mg/mL imipenem). +, positive result; +w, weakly positive result; -, negative result.

Figure 2. Boxplot of time to positivity by the modified GoldNano Carb test compared with the conventional GoldNano Carb test among 29 carbapenemase producers (all producers) and 11 OXA producers.

The mGoldC test provided overall sensitivity, specificity, positive likelihood ratio (LR+), and negative likelihood ratio (LR-) values of 98.6%, 98.2%, 54.7, and 0.01, respectively (Table 2). Its performance for CPE detection in each hospital showed 94.9–100.0% sensitivity. The mGoldC test failed to detect three NDM-producing *K. pneumoniae* isolates.
and one OX-A-48-like-producing *K. pneumoniae* isolate. Tijet et al. suggested that false negative results of the Carba NP test were associated with strains presenting mucoid colonies [35]. The mGoldC test had excellent sensitivity of 100.0% for the *P. aeruginosa* isolates from the six hospitals. It also provided good performance for detecting carbapenemases in the *Acinetobacter* spp. isolates (96.6–100.0% sensitivity). This test could not detect two *A. baumannii* isolates with either OXA-23-like or OXA-58-like. This is in accordance with the fact that class D carbapenemases have weak carbapenemase activity [16,36,37]. Poirel and Nordmann also reported misidentification of OXA-23-producing *A. baumannii* isolates by the Rapidec Carba NP test [38]. In addition, the false negative by the mGoldC may be due to the small inoculum of 1 µL [19]. Therefore, five loops (1 µL loop) of bacterial colonies were used in this study. Tijet et al. also reported that increasing the bacterial inoculum yielded more positive Carba NP results, particularly in isolates producing OXA carbapenemases [35]. Therefore, the amount of bacteria is critical for phenotypic carbapenemase tests. For the best results of the mGoldC test, we recommend using 5 µL inoculum of bacterial colonies.

**Table 1.** Comparison between conventional and modified GoldNano Carb tests for detecting carbapenemase-producing Gram-negative bacilli using PCR as the gold standard.

| β-Lactamase Classes and Types (n) | Species (n) | Specified β-Lactamases | No. of Isolates Tested by a |
|-------------------------------|-------------|------------------------|---------------------------|
|                               |             |                        | cGoldC | mGoldC |
|                               |             |                        | + (+w) | -      | + (+w) | -  |
| **Carbapenemase producers (29)** |
| **Ambler class A (1)**  | *K. pneumoniae* ATCC BAA-1705 (1) | KPC-2 | 1 | 1 |
| KPC-type (1)  | | | | |
| **Ambler class B (17)** | *K. pneumoniae* (4) | NDM | 4 | 4 |
| NDM-type (7)  | *E. coli* (2) | NDM | 2 | 2 |
| *A. pittii* (1) | NDM-1 | 1 | 1 |
| **IMP-type (7)** | *P. aeruginosa* (2) | IMP-14a | 2 | 2 |
| *K. pneumoniae* (2) | IMP-14a | 2 | 2 |
| *P. aeruginosa* (1) | IMP-1 | 1 | 1 |
| *P. aeruginosa* (1) | IMP-9 | 1 | 1 |
| **VIM-type (3)** | *P. aeruginosa* (3) | VIM-2 | 3 | 3 |
| **Ambler class D (11)** | *K. pneumoniae* (2) | OXA-48-like | 2 | 2 |
| OXA-type (11) | *E. coli* (1) | OXA-48-like | 1 | 1 |
| *A. baumannii* (5) | OXA-181 | 1 | 1 |
| *A. baumannii* (1) | OXA-23-like | 4(3) | 1 | 5(1) |
| *Acinetobacter* spp. (1) | OXA-72 | 1 | 1 |
| **Non-carbapenemase producers (13)** |
| ESBL (3) | *K. pneumoniae* (1) | CTX-M-1-like, SHV | 1 | 1 |
| E. coli (1) | | | 1 | 1 |
| *K. pneumoniae* (1) | | | 1 | 1 |
| AmpC (1) | *E. coli* (1) | CTX-M-1-like, TEM-1 | 1 | 1 |
| Enterobacter spp. (1) | | | 1 | 1 |
| pAmpC (4) | *E. coli* (1) | CMY-2 | 1 | 1 |
| E. coli J53 (pSLK54) (1) | ACC-1 | 1 | 1 |
| E. coli J53 (pMG251) (1) | ACT-1 | 1 | 1 |
| *K. pneumoniae* (1) | | | 1 | 1 |
| ESBL & pAmpC (4) | *E. coli* (1) | VEB-like, CMY-8b | 1 | 1 |
| E. coli (1) | | | 1 | 1 |
| *K. pneumoniae* (1) | | | 1 | 1 |
| E. coli (1) | | | 1 | 1 |
| ESBL & pAmpC (4) | *K. pneumoniae* ATCC BAA-1706 (1) | | 1 | 1 |

**a** cGoldC, conventional GoldNano Carb test; mGoldC, modified GoldNano Carb test; +, positive result; +w, weakly positive result; and -, negative result; **b** Positive by double-disc synergy and boronic acid combined disc tests for ESBL and pAmpC producers, respectively.
## Table 2. Diagnostic performance of the modified GoldNano Carb test in Gram-negative bacilli from the six different hospitals.

| Hospitals/Organisms | No. Test Isolates with PCR | % (95% CI) | No. of Isolates Giving | False Negative (Species, Types) | False Positive (Species) |
|----------------------|---------------------------|------------|------------------------|---------------------------------|--------------------------|
|                      |                           | Positive   | Negative   | Sensitivity | Specificity | LR+ | LR– | False Negative (Species, Types) | False Positive (Species) |
| **A**                |                           |            |            |             |             |     |     |                                  |                          |
| Enterobacterales     | 105                       | 26         | 79         | 100.0 (84.0–100.0) | 100.0 (94.2–100.0) | In (NaN-In) | 0 | (0-NaN)  | -                              | -                        |
| A. baumannii         | 63                        | 30         | 33         | 100.0 (85.9–100.0) | 97.0 (82.5–99.8) | 33.0 | 0   | (0-NaN)  | -                              | 1 (A. baumannii)          |
| P. aeruginosa        | 59                        | 9          | 50         | 100.0 (62.9–100.0) | 100.0 (91.1–100.0) | In (NaN-In) | 0 | (0-NaN)  | -                              | -                        |
| **Total**            | 227                       | 65         | 162        | 100.0 (93.0–100.0) | 99.4 (96.1–99.9) | (22.95–1143.1) | 0 | (0-NaN)  | -                              | -                        |
| **B**                |                           |            |            |             |             |     |     |                                  |                          |
| Enterobacterales     | 124                       | 59         | 65         | 94.9 (84.9–98.7) | 100.0 (93.0–100.0) | In (NaN-In) | 0.05 | (0.02–0.15) | 3 (K. pneumoniae, NDM) | -                        |
| A. baumannii         | 44                        | 38         | 6          | 100.0 (88.6–100.0) | 83.3 (36.5–99.1) | (1.0–35.9) | 0 | (0-NaN)  | -                              | 1 (A. baumannii)          |
| P. aeruginosa        | 21                        | 14         | 7          | 100.0 (73.2–100.0) | 100.0 (56.1–100.0) | In (NaN-In) | 0 | (0-NaN)  | -                              | -                        |
| **Total**            | 189                       | 111        | 78         | 97.3 (91.7–99.3) | 98.7 (92.1–99.9) | (7.5–532.1) | 0.03 | (0.009–0.08) | -                              | -                        |
| **C**                |                           |            |            |             |             |     |     |                                  |                          |
| Enterobacterales     | 94                        | 71         | 23         | 100.0 (93.6–100.0) | 95.7 (76.0–99.8) | 23 (3.4–156.4) | 0 | (0-NaN)  | -                              | 1 (E. coli)               |
| **D**                |                           |            |            |             |             |     |     |                                  |                          |
| Enterobacterales     | 72                        | 20         | 52         | 100.0 (79.9–100.0) | 100.0 (91.4–100.0) | In (NaN-In) | 0 | (0-NaN)  | -                              | -                        |
| A. baumannii         | 67                        | 58         | 9          | 96.6 (87.0–99.4) | 88.9 (90.7–99.4) | (1.4–55.2) | 0.04 | (0.01–0.16) | 1 (A. baumannii, OXA-23); 1 (A. baumannii, OXA-58) | -                        |
| A. haemolyticus      | 1                         | 1          | 0          | 100.0 (5.5–100.0) | NaN (NaN-NaN) (NaN-NaN) (NaN-NaN) | In (NaN-In) | 0 | (0-NaN)  | -                              | -                        |
| P. aeruginosa        | 24                        | 1          | 23         | 100.0 (5.5–100.0) | 100.0 (82.2–100.0) | In (NaN-In) | 0 | (0-NaN)  | -                              | -                        |
| **Total**            | 164                       | 80         | 84         | 97.5 (90.4–99.6) | 98.8 (92.6–99.9) | (11.7–574.8) | 0.03 | (0.006–0.1) | -                              | -                        |
| Hospitals/Organisms | No. of Isolates | No. of Test Isolates | % (95% CI) | No. of Isolates Giving | False Negative (Species, Types) | False Positive (Species) |
|---------------------|-----------------|----------------------|------------|------------------------|--------------------------------|-------------------------|
|                     |                 | Positive | Negative | Sensitivity | Specificity | LR+ | LR− |                      |                           |
| **E**               |                 | 100.0   | 90.0     | (87.0–100.0) | (66.9–98.2) | 10  | 0   | (0-NaN)               | -                          |
| Enterobacterales    | 53              | 33      | 20       | 100.0       | 100.0       | In  | 0   | (0-NaN)               | -                          |
| A. baumannii        | 37              | 36      | 1        | 100.0       | (5.5–100.0) | NaN-In | 0 | (0-NaN)               | -                          |
| P. aeruginosa       | 21              | 20      | 1        | 100.0       | (5.5–100.0) | NaN-In | 0 | (0-NaN)               | -                          |
| **Total**           | 111             | 89      | 22       | 100.0       | (69.4–98.4) | 11  | 0   | (0-NaN)               | 2 (E. coli)                |
| **F**               |                 | 96.4    | 94.7     | (79.8–99.8) | (71.9–99.7) | 18.3 | 0.04 | (0.005–0.3) | 1 (K. pneumoniae, OXA-48-like) |
| Enterobacterales    | 47              | 28      | 19       | 98.3        | (95.4–99.5) | 63.4 | 0.02 | (0.006–0.05) | 4                          |
| Acinetobacter spp.  | 212             | 163     | 49       | 98.8        | (82.1–98.4) | 16.1 | 0.01 | (0.003–0.05) | 2                          |
| P. aeruginosa       | 125             | 44      | 81       | 98.4        | (94.4–100.0) | 0   | 0   | (0-NaN)               | 3                          |
| **Total**           | 832             | 444     | 388      | 98.6        | (96.9–99.4) | 54.7 | 0.01 | (0.006–0.03) | 6                          |

LR+, positive likelihood ratio; LR−, negative likelihood ratio; In, infinity; NaN, the calculation cannot be performed because the values entered include one or more instances of zero. A & B, Northeast; C & D, Central; E, West; F, South.
Among the six hospitals, the mGoldC test had 90.0–100.0%, 100%, and 83.3–100.0% specificity for all Enterobacterales, \textit{P. aeruginosa}, and \textit{Acinetobacter} spp. isolates, respectively. False positives were found in seven isolates (three \textit{A. baumannii}, three \textit{E. coli}, and one \textit{K. pneumoniae}) (Table 2) with the detection times of 5–120 min. These may be due to the very weak carbapenemase activity of ESBLs or pAmpCs with or without porin loss, similar to those observed by the Carba NP method [16,39]. Recently, Nordmann et al. reported false positive results by the NitroSpeed-Carba NP test in ACC- or CMY-type AmpC producers [40]. Whitley et al. also observed a false positive of \textit{A. baumannii} with the BD Phoenix CPO Detect test [41]. However, the false positive isolates may contain other known (e.g., SPM, SIM, GIM, GES) or novel carbapenemases. Further investigation by whole-genome sequencing is needed. In addition, the lower specificity for the \textit{Acinetobacter} spp. isolates may be due to the limited numbers of non-carbapenemase producers of these organisms (Table 2). Therefore, evaluation of the mGoldC test in larger samples of \textit{Acinetobacter} spp. is still needed.

3. Materials and Methods

3.1. Bacterial Collection

A total of 832 non-repetitive GNB clinical isolates, 495 Enterobacterales, 212 \textit{Acinetobacter} spp., and 125 \textit{P. aeruginosa} isolates (444 carbapenemase producers and 388 non-carbapenemase producers), collected from six hospitals in Thailand between February 2019 and August 2020, were included in this study (Supplementary Material Table S1). The six hospitals were as follows: 1670 beds (A) and 900 beds (B) in the Northeast, 500 beds (C) and 435 beds (D) in the central region, 317 beds (E) in the West, and 167 beds (F) in the South.

All isolates were identified by conventional biochemical tests and screened for carbapenemase production by either disc diffusion or minimum inhibitory concentration (MIC) determination methods using criteria as follows: zone diameters of <19 mm in response to either 10 \(\mu\)g imipenem or 10 \(\mu\)g meropenem disks for \textit{P. aeruginosa}, <22 and <18 mm in response to imipenem and meropenem disks, respectively, for \textit{Acinetobacter} spp. according to the CLSI guideline [14], and those of <25 mm in response to meropenem and/or ertapenem disks for Enterobacterales according to the EUCAST (https://eucast.org/clinical_breakpoints/) (accessed on 8 November 2016) guidelines as suggested by Huang et al. [42]. Carbapenem MICs were determined by broth microdilution, and the results were interpreted using the CLSI criteria [14].

This study was approved by the Khon Kaen University Ethics Committee (project number HE561476).

3.2. Molecular Detection of Carbapenemase Genes

All isolates were characterized for the presence of carbapenemase genes by the conventional multiplex PCR assays [43,44]. The target genes included \texttt{blaKPC}, \texttt{blaNDM}, \texttt{blaOXA-48-like}, \texttt{blaVIM}, and \texttt{blaIMP} for Enterobacterales; \texttt{blaNDM}, \texttt{blaVIM}, and \texttt{blaIMP} for \textit{P. aeruginosa}; and \texttt{blaNDM}, \texttt{blaOXA-23}, \texttt{blaOXA-24}, \texttt{blaOXA-51}, and \texttt{blaOXA-58} for \textit{Acinetobacter} spp.

3.3. Modification of AuNP Solution

A colloidal suspension of 13 nm AuNP was prepared by using a citrate-reduction method described by Hill and Mirkin [45] with some modifications according to our previous report [19]. The ZnSO\(_4\) concentrations (0.25, 0.5, 0.75, and 1 mM) in the AuNP solution were optimized, and 0.25 mM ZnSO\(_4\) was selected for further experiments (AuNP-Zn solution).

3.4. Optimization of the mGoldC

The performance of the cGoldC and mGoldC was compared using the strains previously confirmed for their \(\beta\)-lactamas by molecular methods [19]. The bacterial strains included 29 carbapenemase producers (eleven OXA, seven NDM, seven IMP, three VIM, three
and one KPC-2) and 13 non-carbapenemase producers (four pAmpC, four ESBL & pAmpC, three ESBL, one AmpC, and one non-ESBL and non-AmpC) (Table 1).

The cGoldC test was performed and interpreted as previously described [19] with slight modifications. Briefly, bacterial colonies grown on Mueller–Hinton agar (MHA) (Oxoid, Basingstoke, UK) were scraped by a 1 µL loopful for five times (approximately 5 µL inoculum size), and the reactions were read immediately and at 5, 10, 15, 30, 60, 90, and 120 min. For the mGoldC test, the clinical isolates were tested with the same procedure as the cGoldC test except that the AuNP-Zn solution was used.

3.5. Evaluation of the mGoldC Test for Carbapenemase Detection in Six Hospitals

The mGoldC test kits were submitted to the six hospitals. The bacterial colonies of carbapenemase screening-positive isolates from MHA plates were tested following the mGoldC protocol as mentioned above, and the results were recorded. All isolates were submitted to our laboratory for confirmation of carbapenemase genes by medical technologists without any information of the mGoldC results.

The diagnostic parameters of the sensitivity, specificity, an LR+ and LR- of the mGoldC test were calculated by the free software vassarStats (http://vassarstats.net/ (accessed on 11 May 2022) using the PCR method as the gold standard [46].

4. Conclusions

The mGoldC test is inexpensive, user-friendly by using bacterial colonies and imipenem/cilastatin powder directly without an extraction buffer, and easy to interpret with a fast and strong positive reaction. The AuNP-Zn solution also has a long shelf-life. Therefore, it could be used as an alternative method for rapid identification of CPGNB.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics11050684/s1, Table S1: Detail of Gram-negative bacilli clinical isolates collected from six hospitals; Table S2: Carbapenemase genes in clinical isolates collected from the six hospitals, Figure S1: Time to positivity by the modified GoldNano Carb test compared with the conventional GoldNano Carb test among 29 carbapenemase producers and 11 OXA producers.

Author Contributions: Conceptualization, A.C. and A.S.; methodology, A.C., A.S., L.W., S.K., C.S., W.K., S.P., O.L., W.P., K.K. and J.P.; formal analysis, A.C., A.S., A.L., N.C., C.W. and J.D.; investigation, A.C. and A.S.; resources, L.W., S.K., C.S., W.K., S.P., O.L., W.P., K.K. and J.P.; data curation, A.C. and A.S.; writing—original draft preparation, A.C. and A.S.; writing—review and editing, A.C., A.S., A.L., L.W., S.K., C.S., W.K., S.P., O.L., W.P., K.K., J.P., N.C., C.W. and J.D.; supervision, A.C.; project administration, A.C. and A.S.; funding acquisition, A.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by a research grant from Khon Kaen University (project number 6200016).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We thank the Centre for Research and Development in Medical Diagnostic Laboratory (CMDL), Faculty of Associated Medical Sciences, Khon Kaen University. We are grateful to David Livermore, Antibiotic Resistance Monitoring & Reference Laboratory, HPA Microbiology Services-Colindale Unit, London, UK; Professor George A Jacoby, Lahey Clinic, Burlington, Massachusetts, USA, DMST Culture Collection, National Institute of Health, Thailand for kindly providing the reference strains; and the staff of Clinical Microbiology laboratories in Srinagarind Hospital, Buriram Hospital, Lerdsin Hospital, The Queen Sirikit National Institute of Child Health (QSNICH), Koh Samui Hospital, and Maesot General Hospital for collecting the clinical isolates. We also extend our gratitude to Kamonwan Lunha, Phatsarawadee Hemwaranon, Chutima Mophuebg, Nadach Onura, Jindaphorn Suttayamuk, Pattaramon Siripan, Phantitra Sudadech, Angsuda Lekklung, and Wittaya Kongkaew for testing carbapenemase genes by the PCR assays.
Conflicts of Interest: The authors declare no conflict of interest.

References

1. Brink, A.J. Epidemiology of carbapenem-resistant Gram-negative infections globally. *Curr. Opin. Infect. Dis.* 2019, 32, 609–616. [CrossRef] [PubMed]
2. Nordmann, P.; Poirel, L. Epidemiology and diagnostics of carbapenem resistance in Gram-negative bacteria. *Clin. Infect. Dis.* 2019, 69, S521–S528. [CrossRef] [PubMed]
3. Bonomo, R.A.; Burd, E.M.; Conly, J.; Limbago, B.M.; Poirel, L.; Segre, J.A.; Westblade, L.F. Carbapenemase-producing organisms: A global scourge. *Clin. Infect. Dis.* 2018, 66, 1290–1297. [CrossRef] [PubMed]
4. Nordmann, P. Carbapenemase-producing Enterobacteriaceae: Overview of a major public health challenge. *Med. Et Mal. Infect.* 2014, 44, 51–56. [CrossRef] [PubMed]
5. Hansen, G.T. Continuous evolution: Perspective on the epidemiology of carbapenem resistance among Enterobacterales and other Gram-negative bacteria. *Infect. Dis. Ther.* 2021, 10, 75–92. [CrossRef]
6. National Antimicrobial Resistance Surveillance Center, Thailand. Antimicrobial Resistance 2000–2020. Available online: http://narst.dmsc.moph.go.th/ (accessed on 2 April 2022).
7. Paveenkittiporn, W.; Lyman, M.; Biedron, C.; Chea, N.; Bunthi, C.; Kolwaite, A.; Janejai, N. Molecular epidemiology of carbapenem-resistant Enterobacteriaceae in Thailand, 2016–2018. *Antimicrob. Resist. Infect. Control.* 2021, 10, 88. [CrossRef] [PubMed]
8. Baeeza, L.L.; Pfennigwerth, N.; Greissl, C.; Götting, S.; Saleh, A.; Stelzer, Y.; Gatermann, S.G.; Hamprecht, A. Comparison of five methods for detection of carbapenemases in Enterobacteriaceae with proposal of a new algorithm. *Clin. Microbiol. Infect.* 2019, 25, 1286.e9–1286.e15. [CrossRef]
9. Hombach, M.; von Gunten, B.; Castelberg, C.; Bloember, G.V. Evaluation of the Rapidec Carba NP test for detection of carbapenemases in Enterobacteriaceae. *J. Clin. Microbiol.* 2015, 53, 3828–3833. [CrossRef]
10. Bir, R.; Mohapatra, S.; Kumar, A.; Tyagi, S.; Sood, S.; Das, B.K.; Kapil, A. Comparative evaluation of in-house Carba NP test with other phenotypic tests for rapid detection of carbapenem-resistant Enterobacteriales. *J. Clin. Lab. Anal.* 2019, 33, e22652. [CrossRef]
11. Yu, J.; Liu, J.; Li, Y.; Yu, J.; Zhu, W.; Liu, Y.; Shen, L. Rapid detection of carbapenemase activity of Enterobacteriaceae isolated from positive blood cultures by MALDI-TOF MS. *Ann. Clin. Microbiol. Antimicrob.* 2018, 17, 22. [CrossRef]
12. Cui, X.; Zhang, H.; Du, H. Carbapenemases in Enterobacteriaceae: Detection and antimicrobial therapy. *Front Microbiol.* 2019, 10, 1823. [CrossRef] [PubMed]
13. Lasserre, C.; De Saint Martin, L.; Cuzon, G.; Bogaerts, P.; Lamar, E.; Glucpynzyski, Y.; Naas, T.; Tandé, D. Efficient detection of carbapenemase activity in Enterobacteriaceae by matrix-assisted laser desorption ionization-time of flight mass spectrometry in less than 30 minutes. *J. Clin. Microbiol.* 2015, 53, 2163–2171. [CrossRef] [PubMed]
14. Clinical and Laboratory Standards Institute (CLSI). *Performance Standards for Antimicrobial Susceptibility Testing*. CLSI Guideline M100; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2019.
15. Pragasam, A.K.; Sahni, R.D.; Anandan, S.; Sharma, A.; Gopi, R.; Hadibasha, N.; Gunasekaran, P.; Veeraraghavan, B. A pilot study on carbapenemase detection: Do we see the same level of agreement as with the CLSI observations. *J. Clin. Diagn. Res.* 2016, 10, DC09–DC13. [CrossRef] [PubMed]
16. Cunningham, S.A.; Limbago, B.; Traczewski, M.; Anderson, K.; Hackel, M.; hindler, J.; Sahm, D.; Alyanak, E.; Lawsin, A.; Gulvik, C.A.; et al. Multicenter performance assessment of Carba NP test. *J. Clin. Microbiol.* 2017, 55, 1954–1960. [CrossRef]
17. Gallagher, L.C.; Roundtree, S.S.; Lancaster, D.P.; Rudin, S.D.; Bard, J.D.; Roberts, A.L.; Marshall, S.H.; Bonomo, R.A.; Sullivan, K.V. Performance of the CLSI Carba NP and the Rosco Carb Screen assays using North American carbapenemase-producing Enterobacteriaceae and *Pseudomonas aeruginosa* isolates. *J. Clin. Microbiol.* 2015, 53, 3370–3373. [CrossRef]
18. Laolerd, W.; Akeda, Y.; Preeyanon, L.; Ratthawongjirakul, P.; Santanirand, P. Carbapenemase-producing carbapenem-resistant Enterobacteriaceae from Bangkok, Thailand, and their detection by the Carba NP and modified carbapenem inactivation method tests. *Microb. Drug Resist.* 2018, 24, 1006–1011. [CrossRef]
19. Srisratkarn, A.; Lulitanond, A.; Wilailuckana, C.; Charoenri, N.; Daduang, J.; Chanawong, A. A novel GoldNanocarb test for rapid phenotypic detection of carbapenemases, particularly OXA type, in Enterobacteriaceae, *Pseudomonas aeruginosa* and *Acinetobacter* spp. *J. Antimicrob. Chemother.* 2017, 72, 2519–2527. [CrossRef]
20. Karsisiotis, A.I.; Damblon, C.F.; Roberts, G.C. A variety of roles for versatile zinc in metallo-β-lactamases. *Metallomics* 2014, 6, 1181–1197. [CrossRef]
21. Bothra, S.; Kumar, R.; Sahoo, S.K. Pyridoxal derivative functionalized gold nanoparticles for colorimetric determination of zinc (ii) and aluminium (iii). *RSC Adv.* 2015, 5, 97690–97695. [CrossRef]
22. Rimrang, B.; Chanawong, A.; Lulitanond, A.; Wilailuckana, C.; Charoenri, N.; Sribenjalux, P.; Plumsrikaw, W.; Wongklakorn, L.; Kerdzin, A.; Chetchotisakd, P. Emergence of NDM-1- and IMP-14a-producing Enterobacteriaceae in Thailand. *J. Antimicrob. Chemother.* 2012, 67, 2626–2630. [CrossRef]
23. Teeraputon, S.; Wimonjariyaboon, J.; Pengkong, P.; Talan, P. Prevalence of carbapenemase genes in carbapenem non-susceptible *Klebsiella pneumoniae* isolated from clinical specimens in Buddhachinaraj Phitsanulok Hospital. *J. Med. Tech. Assoc. Thailand* 2021, 49, 7616–7623. (In Thai)
24. Chiu, S.K.; Ma, L.; Chan, M.C.; Lin, Y.T.; Fung, C.P.; Wu, T.L.; Chuang, Y.C.; Lu, P.L.; Wang, J.T.; Lin, J.C.; et al. Carbapenem nonsusceptible Klebsiella pneumoniae in Taiwan: Dissemination and increasing resistance of carbapenemase producers during 2012-2015. Sci. Rep. 2018, 8, 8468. [CrossRef] [PubMed]

25. Srisrattakarn, A.; Lulitanond, A.; Wilailuckana, C.; Charoensri, N.; Wonglakorn, L.; Piyapatthanakul, S.; Supajeen, A.; Chanawong, A. Modification and evaluation of the Carba NP test by use of paper strip for simple and rapid detection of carbapenemase-producing Enterobacteriaceae. World J. Microbiol. Biotechnol. 2016, 32, 117. [CrossRef] [PubMed]

26. Kerdsin, A.; Deekae, S.; Chayangsu, S.; Hatrongjit, R.; Chompit, P.; Takeuchi, D.; Akeda, Y.; Tomono, K.; Hamada, S. Genomic characterization of an emerging blqmp65 gene in Thailand. Sci. Rep. 2019, 9, 18521. [CrossRef]

27. Moalic, C.; Anne, C.; Lamotte-Brasseur, J.; Devreese, B.; Van Beeumen, J.; Galleni, M.; Frère, J.M. Analysis of the importance of the metallo-β-lactamase active site loop in substrate binding and catalysis. Chem. Biol. 2003, 10, 319–329. [CrossRef]

28. Thet, K.T.; Lunha, K.; Srisrattakarn, A.; Lulitanond, A.; Tavichakorntrakool, R.; Kuwatjanakul, W.; Charoensri, N.; Chanawong, A. Modulation of the expression of blaKPC-2 in Enterobacteriaceae. World J. Microbiol. Biotechnol. 2019, 35, 1081–1087. [PubMed]

29. Thirapanmethee, K.; Srisiri-A-Nun, T.; Houngsaitong, J.; Montakantikul, P.; Khuntayaporn, P.; Chomnawang, M.; Tulpule, A.; Denys, G.A. Evaluation of the BD Phoenix CPO Detect test for detection and classification of carbapenemase-producing Enterobacteriaceae in clinical isolates. J. Clin. Microbiol. 2019, 57, 386–390. [PubMed]

30. Thirapanmethee, K.; Srisrattakarn, A.; Lulitanond, A.; Tavichakorntrakool, R.; Kuwatjanakul, W.; Charoensri, N.; Chanawong, A. Modification and evaluation of the Carba NP test by use of paper strip for simple and rapid detection of carbapenemase-producing Enterobacteriaceae in clinical isolates in Thailand. Antibiotics 2020, 9, 864. [CrossRef]

31. Wu, W.; Feng, Y.; Tang, G.; Qiao, F.; McNally, A.; Zong, Z. NDM metallo-β-lactamases and their bacterial producers in health care settings. Clin. Microbiol. Rev. 2019, 32, e00115–e00118. [CrossRef]

32. Moalic, C.; Anne, C.; Lamotte-Brasseur, J.; Grosambert, S.; Devreese, B.; Van Beeumen, J.; Galleni, M.; Frère, J.M. Analysis of the importance of the metallo-β-lactamase active site loop in substrate binding and catalysis. Chem. Biol. 2003, 10, 319–329. [CrossRef]

33. Moore, J.; Pujol, C.; Sislin, L.; Papadopoulos, A.; Richter, T.; Daverio, M. Clinical impact of resistance to ertapenem in highly endemic settings. J. Clin. Microbiol. 2020, 58, 3924–3927. [CrossRef]

34. Girlich, D.; Poirel, L.; Nordmann, P. Value of the modified Hodge test for detection of emerging carbapenemases in Enterobacteriaceae. J. Clin. Microbiol. 2012, 50, 477–479. [CrossRef]

35. Tijet, N.; Boyd, D.; Patel, S.N.; Mulvey, M.R.; Melano, M.T. Prevalence of OXA-type β-lactamase genes among carbapenem-resistant Acinetobacter baumannii clinical isolates in Thailand. Antibiotics 2020, 9, 864. [CrossRef]

36. Netikul, T.; Kiratisin, P. Genetic Characterization of carbapenem-resistant Enterobacteriaceae and the spread of carbapenem-resistant Klebsiella pneumoniae ST340 at a University Hospital in Thailand. PLoS ONE 2015, 10, e0139116. [CrossRef]

37. Bush, K.; Bradford, P.A. Epidemiology of β-lactamase-producing pathogens. Clin. Microbiol. Rev. 2015, 28, e00047-19. [CrossRef]

38. Khuntayaporn, P.; Yamprayoonwat, W.; Yasawong, M.; Chomnawang, M.T. Dissemination of carbapenem-resistance among multidrug resistant Pseudomonas aeruginosa carrying metallo-β-lactamase genes, including the novel blqmp65 gene in Thailand. Infect. Chemother. 2019, 51, 107–118. [CrossRef]

39. Girlich, D.; Poirel, L.; Nordmann, P. Value of the modified Hodge test for detection of emerging carbapenemases in Enterobacteriaceae. J. Clin. Microbiol. 2012, 50, 477–479. [CrossRef]

40. Nordmann, P.; Sadek, M.; Demord, A.; Poirel, L. NitroSpeed-Carba NP test for rapid detection and differentiation between clinical isolates in Thailand. Antibiotics 2020, 9, 864. [CrossRef]

41. Whitley, V.; Kircher, S.; Gill, T.; Hindler, J.A.; O’Rourke, S.; Cooper, C.; Tulpule, A.; Denys, G.A. Multicenter evaluation of the BD Phoenix CPO Detect test for detection and classification of carbapenemase-producing organisms in clinical isolates. J. Clin. Microbiol. 2020, 58, e01752-19. [CrossRef] [PubMed]

42. Thet, K.T.; Lunha, K.; Srisrattakarn, A.; Lulitanond, A.; Tavichakorntrakool, R.; Kuwatjanakul, W.; Charoensri, N.; Chanawong, A. Colistin heteroresistance in carbapenem-resistant Acinetobacter baumannii clinical isolates from a Thai university hospital. World J. Microbiol. Biotechnol. 2020, 36, 102. [CrossRef] [PubMed]

43. Hill, H.D.; Mirkin, C.A. The bio-barcode assay for the detection of protein and nucleic acid targets using DTT-induced ligand exchange. Nat. Protoc. 2006, 1, 324–336. [CrossRef] [PubMed]

44. VassarStats: Website for Statistical Computation. Available online: http://vassarstats.net/ (accessed on 4 May 2022).