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Application of the multifactor dimensionality reduction method in evaluation of the roles of multiple genes/enzymes in multidrug-resistant acquisition in *Pseudomonas aeruginosa* strains

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SUMMARY

Multidrug-resistant *Pseudomonas aeruginosa* (MDRPA) infections are major threats to healthcare-associated infection control and the intrinsic molecular mechanisms of MDRPA are also unclear. We examined 348 isolates of *P. aeruginosa*, including 188 MDRPA and 160 non-MDRPA, obtained from five tertiary-care hospitals in Guangzhou, China. Significant correlations were found between gene/enzyme carriage and increased rates of antimicrobial resistance ($P < 0{\cdot}01$). *gyrA* mutation, OprD loss and metallo-β-lactamase (MBL) presence were identified as crucial molecular risk factors for MDRPA acquisition by a combination of univariate logistic regression and a multifactor dimensionality reduction approach. The MDRPA rate was also elevated with the increase in positive numbers of those three determinants ($P < 0{\cdot}001$). Thus, *gyrA* mutation, OprD loss and MBL presence may serve as predictors for early screening of MDRPA infections in clinical settings.

Key words: Genes/enzymes, multidrug-resistant, multifactor dimensionality reduction, *Pseudomonas aeruginosa*.

INTRODUCTION

Pseudomonas aeruginosa is among the most commonly isolated pathogens in healthcare-associated settings worldwide [1–3] often causing serious infections in various body sites, including sputum, urine and wounds [4]. Furthermore, *P. aeruginosa* readily acquires resistance to multiple antimicrobial agents and the rate of multidrug-resistant *P. aeruginosa* (MDRPA) healthcare-associated infections has risen sharply over past decades [5]. The infections caused by MDRPA are problematic not only because they restrict therapeutic options, but also because they may result in higher mortality rates and longer hospital stay compared to non-MDRPA [6].

The mechanisms of resistance against antibacterial agents in *P. aeruginosa* are complex and include the loss of the outer membrane channel protein OprD [7], presence of β-lactamase genes and expression of

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β-lactamase enzyme genes [8, 9], aminoglycoside-modifying enzymes (AMEs) [10], presence of integrons [11, 12], and mutations of quinolone resistance-determining regions (QRDRs) in *gyrA* and *parC* genes [13]. The application of conventional statistical methods, such as logistic regression models and stratified analysis to analyse the contribution of various factors in the acquisition of antimicrobial drug resistance has obvious limitations in identification of high-order interactions of multiple factors. However, the multifactor dimensionality reduction approach offers some resolution of this difficult issue and has been widely applied to the evaluation of the effect of high-level gene–gene and/or gene–environment interactions on susceptibilities of complex diseases [14, 15].

In this study, we collected *P. aeruginosa* isolates from five tertiary-care hospitals in Guangzhou, China and determined the possible relationships between various potential molecular resistance determinants and antimicrobial susceptibility. Subsequently, we used a multifactor dimensionality reduction approach to detect the optimal combination of determinants as predictors of MDRPA infections in clinical settings.

MATERIALS AND METHODS

Setting and study design

The study was carried out from July 2008 to December 2012 in five tertiary-care hospitals in Guangzhou, China, and the subjects were *P. aeruginosa*-infected patients.

The study consisted of three parts. First, was surveillance of the relationships between genes/enzymes and susceptibility of isolates to antibiotics tested. Second, a combination of univariate logistic regression analysis and multifactor dimensionality reduction analysis was used to explore the underlying predictors of MDRPA. Finally, a descriptive study was used to identify the relationship between MDRPA rate and positive number of screened factors.

Microbiological experiments

P. aeruginosa isolates were confirmed by the Vitek 32 microbial identification system (bioMérieux, France), and tested for antimicrobial susceptibility by the Kirby–Bauer disk diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI 2012) [16]. Isolates were tested for susceptibility to seven antimicrobial classes: cephalosporins (ceftazidime and cefepime), penicillins (piperacillin and ticarcillin), β-lactamase inhibitors (piperacillin-tazobactam), aminoglycosides (gentamicin, tobramycin and amikacin), fluoroquinolones (levofloxacin and ciprofloxacin), carbapenems (imipenem and meropenem), and monobactams (aztreonam). All resistant and intermediate susceptible isolates were classified as the resistant-strain group and patients were categorized as being in the MDRPA group if their strains were non-susceptible to $\geqslant 1$ agents in $\geqslant 3$ antimicrobial classes; other *P. aeruginosa*-positive patients were categorized in the non-MDRPA group.

Detection of relevant genes and enzymes

All *P. aeruginosa* isolates were screened for outer membrane channel protein OprD, β-lactamases [extended spectrum β-lactamases (ESBLs), metallo-β-lactamases (MBLs) and AmpC enzyme], β-lactamase genes (bla_{IMP}, bla_{VIM}, $bla_{\mathrm{OXA\text{-}10}}$, bla_{TEM} and bla_{PER}), integrons, AME genes [*aac(6′)* and *ant(3″)*] and mutations in QRDR (*gyrA* and *parC*). The presence of OprD, bla_{IMP}, bla_{VIM}, $bla_{\mathrm{OXA\text{-}10}}$, bla_{TEM}, bla_{PER}, *aac(6′)* and *ant(3″)* was confirmed by conventional polymerase chain reaction (PCR) amplification with previously reported primers and conditions [17–20]. The production of ESBLs, MBLs and AmpC was determined by the combined disk test method [21], the imipenem-EDTA double-disk synergy test (DDST) method [22] and the modified three-dimensional test [23], respectively. The mutation of QRDR in *gyrA* and *parC* genes along with the presence of integrons were determined by PCR–restriction fragment-length polymorphism (RFLP) analysis [24], and sequencing of amplification products.

Statistical analysis

The antimicrobial susceptibility profiles were displayed as resistant rates together with their 95% confidential intervals (CIs) as error bar plots. The χ^2 test for gender and Mann–Whitney U test for age were used to test demographical differences between MDRPA patients and non-MDRPA patients. The univariate logistic regression analysis was applied to detect potentially significant genes/enzymes that may impact on MDRPA acquisition. Those genes/enzymes with $P < 0{\cdot}2$ in the logistic regression model were entered into Multifactor Dimensionality Reduction software (v. 2.0, Beta 8.1; Computational Genetics Laboratory, USA) to explore high-order interactions and other analyses were perfprmed using Stata software v. 13.1 (StataCorp., USA).

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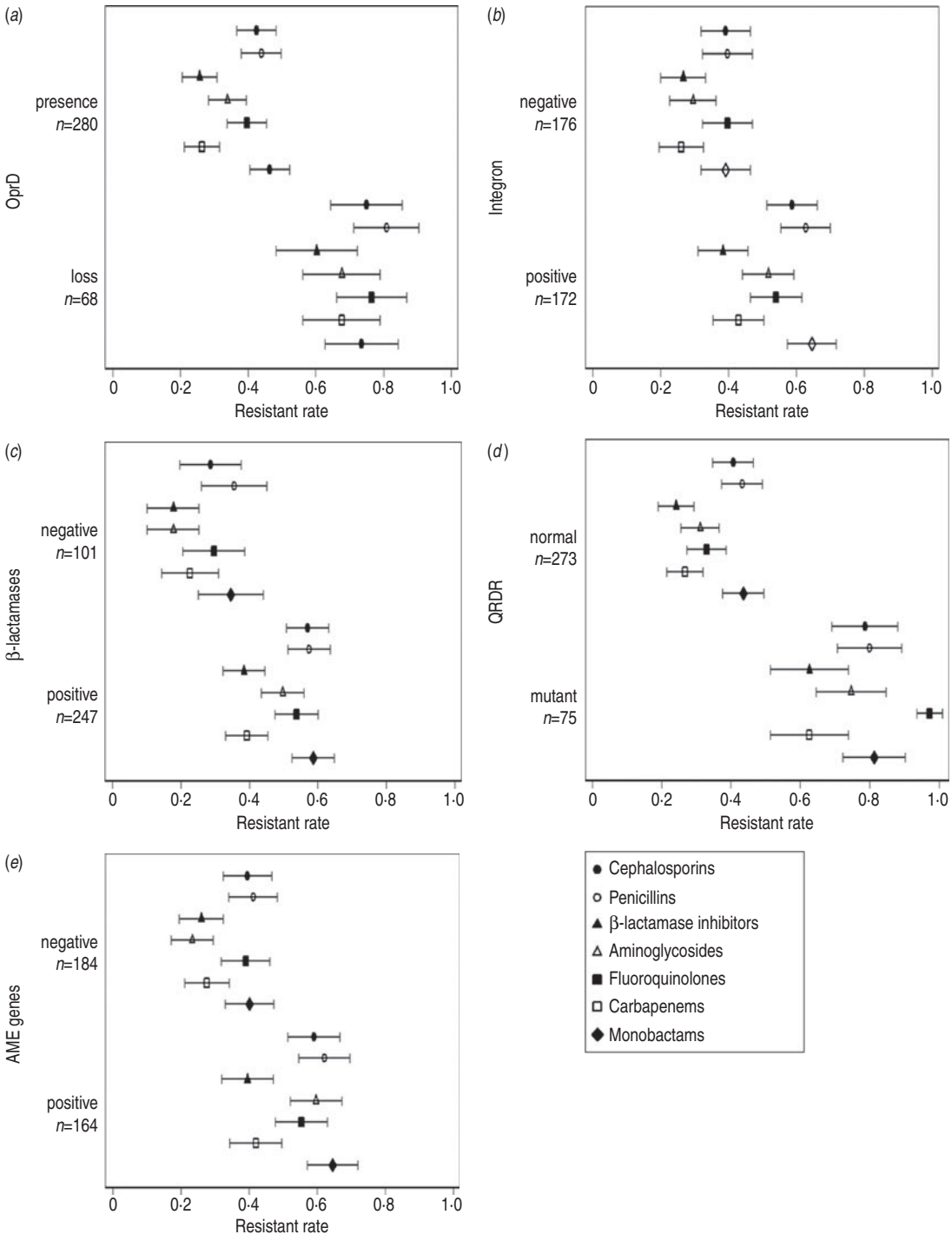

Fig. 1. Effects of determinants on antimicrobial susceptibility stratified by (*a*) loss of OprD; (*b*) presence of integrons; (*c*) presence of *β*-lactamase-related markers; (*d*) mutation of quinolone resistance determining regions (QRDRs); (*e*) presence of aminoglycoside modifying enzyme (AME) genes.

RESULTS

Characteristics of study subjects

During the study period, a total of 348 *P. aeruginosa* subjects, including 188 MDRPA patients and 160 non-MDRPA patients, were eligible for inclusion. The majority of patients in both groups were male (MDRPA: 116/188, 70·6%; non-MDRPA: 113/160; 61·7%) ($\chi^2 = 3{\cdot}06$, $P = 0{\cdot}08$). The median age for both groups was 73 years and there was no difference

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Table 1. *Univariate logistic regression analysis of 14 genes/enzymes for multidrug-resistant* Pseudomonas aeruginosa *(MDRPA)*

	MDRPA ($N = 188$), *n* (%)	Non-MDRPA ($N = 160$), *n* (%)	OR (95% CI)	*P*
Integron(+)	113 (60·11)	59 (36·88)	2·58 (1·67–3·98)	<0·001
OprD loss	59 (31·38)	9 (5·63)	7·67 (3·66–16·08)	<0·001
gyrA mutation	59 (31·38)	7 (4·38)	10·00 (4·41–22·65)	<0·001
parC mutation	28 (14·89)	0 (0·00)	39·49 (6·91–∞)*	<0·001
bla_{TEM}(+)	95 (50·53)	56 (35·00)	1·90 (1·23–2·92)	0·004
bla_{PER}(+)	45 (23·94)	34 (21·25)	1·17 (0·70–1·93)	0·551
bla_{VIM-2}(+)	16 (8·51)	0 (0·00)	20·81 (3·50–∞)*	<0·001
bla_{IMP-1}(+)	36 (19·15)	17 (10·63)	1·99 (1·07–3·70)	0·029
MBLs(+)	37 (19·68)	2 (1·25)	19·36 (4·59–81·72)	<0·001
AmpC(+)	33 (17·55)	12 (7·50)	2·63 (1·31–5·28)	0·007
ESBLs(+)	35 (18·62)	7 (4·38)	5·00 (2·15–11·60)	<0·001
bla_{OXA-10}(+)	64 (34·04)	17 (10·63)	4·34 (2·42–7·80)	<0·001
aac(6′)(+)	69 (36·70)	17 (10·63)	4·88 (2·72–8·74)	<0·001
ant(3″)(+)	76 (40·43)	36 (22·50)	2·34 (1·46–3·75)	<0·001

OR, Odds ratio; CI, confidence interval; MBLs, metallo-β-lactamases; ESBLs, extended spectrum β-lactamases.
* Results of exact logistic regression analysis.

in age distribution between the two groups ($z = -0·24$, $P = 0·81$).

Resistance determinants and antimicrobial susceptibility profiles

The 14 genes/enzymes were divided into five determinant groups: (i) loss of OprD, (ii) presence of integrons, (iii) *gyrA* and *parC* mutations, (iv) presence of *aac (6′)* and *ant (3″)* genes, and (v) presence of β-lactamase-related markers (ESBLs, MBLs, AmpC, bla_{IMP-1}, bla_{VIM-2}, bla_{OXA-10}, bla_{TEM} and bla_{PER}). A strain was considered positive for the determinants group if it tested positive for at least one determinant in the group. The resistant rates and 95% CIs of seven antibiotic classes are shown by stratification of each respective determinant group (Fig. 1). By χ^2 test, each determinant group significantly enhanced the resistant rates of all agent categories ($P < 0·01$).

Univariate logistic regression analysis

The results of the univariate logistic regression analysis on the 14 molecular factors that possibly influenced the occurrence of MDRPA, revealed that all of the molecular factors, except bla_{PER}, were positively associated with multidrug-resistant acquisition (Table 1).

Multifactor dimensionality reduction analysis

Thirteen factors with a significance value of $P < 0·2$ in univariate logistic regression analysis were analysed by Multifactor Dimensionality Reduction software (Table 2). The three factors of OprD loss, *gyrA* mutation and presence of MBLs proved to be the most accurate model, with a testing balanced accuracy of 0·7612 and a cross-validation consistency of 10/10 (permutation $P = 0·0016$). This interaction model was associated with a 14·83-fold increased risk for MDRPA acquisition (95% CI 8·18–26·88, $P < 0·0001$).

Table 2. *Gene/enzyme interaction model by multifactor dimensionality reduction analysis*

Best model	TBA	CVC	*P* value
gyrA mutation	0·5958	6/10	0·1664
OprD loss, *gyrA* mutation	0·7191	10/10	0·0062
OprD loss, *gyrA* mutation, MBL(+)	0·7612	10/10	0·0016
OprD loss, *gyrA* mutation, MBL (+), AmpC (+)	0·7462	5/10	0·0037

TBA, Testing balanced accuracy; CVC, cross-validation consistency; MBL, metallo-β-lactamase.

In the interaction model for the three factors (Fig. 2), the cells in the left of the figure, represent the MDRPA cases with the non-MDRPA cases on the right. This shows that only those patients infected with *P. aeruginosa* with OprD carriage, normal *gyrA*, and deficient in MBLs were associated with the lowest risk of MDRPA. Moreover, the interactions between the four factors (*gyrA* mutation, MBL presence,

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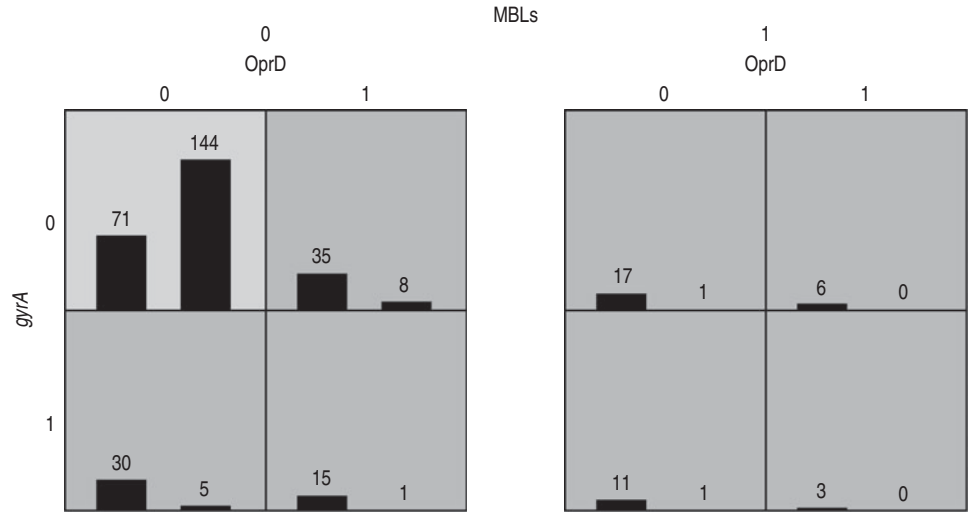

Fig. 2. A multifactor dimensionality reduction analysis of the three-factor [*gyrA*, OprD and metallo-*β*-lactamase (MBL)] interaction model.

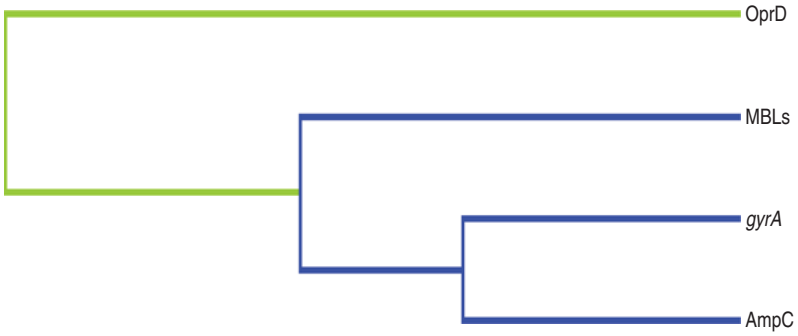

Fig. 3. A tree diagram of the interactions of *gyrA* mutation, production of metallo-*β*-lactamases (MBLs), AmpC positivity and loss of OprD, analysed by multifactor dimensionality reduction.

AmpC presence, and OprD loss) shown in the tree diagram (Fig. 3) all proved to be synergistic and the intensities of synergistic effect are linked to the distances between these factors which indicates that the interactions possibly promote the occurrence of MDRPA.

Association between positive number of screened factors and MDRPA rate

The relationships between the number of screened determinants (*gyrA* mutation, OprD loss and MBL presence) and MDRPA rate are displayed in Figure 4. According to results of the linear-by-linear association test, the rate of MDRPA increased significantly with the number of determinants ($P < 0{\cdot}001$). These results further support our premise that the interactions observed above in the determinants are indeed synergistic.

DISCUSSION

Over the past decades, several investigations have described the distribution of antimicrobial resistance profiles and molecular determinant carriage in *P. aeruginosa* isolates. However, epidemiological studies of interactions of molecular factors with antimicrobial resistance are rarely reported. In this work, we applied a multifactor dimensionality reduction approach to evaluate the roles of molecular-factor interactions in acquiring multidrug resistance in *P. aeruginosa* strains from infected patients.

Mutation of the *gyrA* gene proved to be the strongest single factor in our multifactor dimensionality reduction model, and was found in 31·4% of MDRPA patients compared to 4·4% of non-MDRPA patients. This mutation has been considered to be one of the most dominant risk factors associated with resistance to fluoroquinolones [25]. Moreover, *gyrA* mutations

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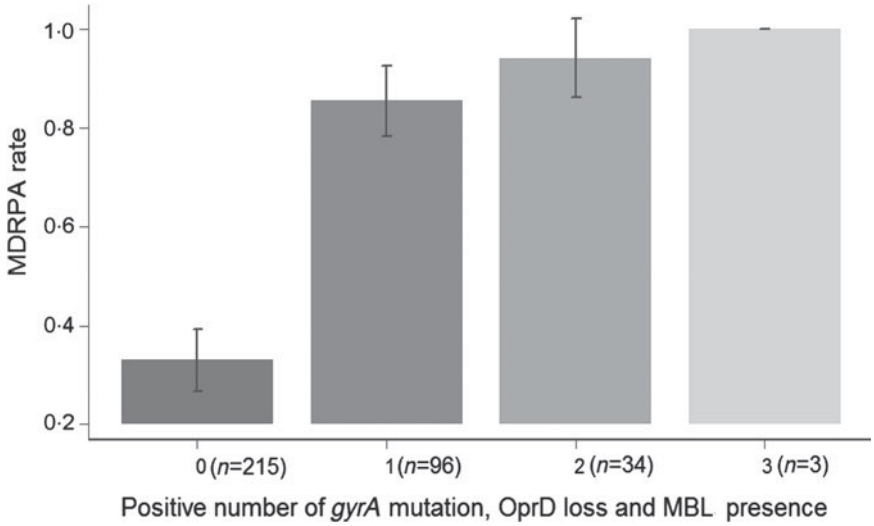

Fig. 4. Association of *gyrA* mutation, OprD loss and production of metallo-β-lactamases (MBLs) with rate of multidrug-resistant *Pseudomonas aeruginosa* (MDRPA).

were found to be the most stable genetic markers in *P. aeruginosa* high-risk XDR/MDR ST175 clones in several outbreaks in Spain [26–29]. Another key molecular determinant in our model, OprD loss, was observed in 31·4% of MDRPA strains and 5·6% of non-MDRPA strains. Loss of OprD in the outer membrane contributes mainly to resistance to carbapenems, especially imipenem. A study by Hocquet *et al*. [30] of 18 *P. aeruginosa* isolates found that only four *P. aeruginosa* isolates, with significantly higher minimum inhibitory concentrations for values of imipenem, were correlated with OprD deficiency, and resistance was little affected by other tested mechanisms. Moreover, changes in amino-acid sequences of OprD in several *P. aeruginosa* isolates from Sweden and Norway was the predominant mechanism of high-level carbapenem resistance reported by Giske *et al.* [31].

The production of MBLs also proved to be an important determinant in our optimal multifactor dimensionality reduction model as almost 20% of MDRPA were MBL producers compared to 1·2% of non-MDRPA strains. These findings are partly consistent with an earlier study from Japan which reported a 75% MBL carriage rate in *P. aeruginosa* isolates which were all were classified as MDRPA [32]. Similarly, production of MBLs was identified as a major mechanism for MDRPA acquisition in Indian isolates [33]. By contrast, production of MBLs appears to have been rare in isolates from Spain [20, 34], Bulgaria [35], Germany [36] and the USA [37]. These marked geographical variations may be due to multiple factors such as differences in antimicrobial prescribing practice, human genetics, and infection control policies.

Besides the impacts of the three dominant factors identified here, MDRPA acquisition was also influenced by interactions of multiple resistance genes and enzymes. It is clear that each determinant group markedly elevated the resistant rates to all antibiotic classes tested (Fig. 1). Nevertheless, all other factors, except bla_{PER}, were statistically correlated with MDRPA in the univariate logistic regression analysis (Table 1). Such interplay of multiple factors is supported by previous studies which noted the combined effects of diminished OprD production, presence of AmpC and efflux systems on carbapenem resistance [38]. By contrast with another study which reported an antagonistic effect between production of MBLs and loss of OprD in carbapenem-resistant *P. aeruginosa* [39], we found that interactions of tested factors were all synergistic (Figs 3 and 4).

The influence of several molecular factors on the spread of infection and its control should not be ignored although the latter was not included as an effective MDRPA determinant in our study. For example, the presence of $bla_{\mathrm{OXA\text{-}10}}$, bla_{PER}, and integron carriage was associated with antimicrobial resistance and some factors proved to be significant predictors of MDRPA by logistic regression analysis. Thus, their relationships with MDRPA infections should be examined in further large-scale studies.

In summary, we have shown the interaction of multiple genes/enzymes and MDRPA infections in

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hospital patients using multifactor dimensionality reduction analysis. Our findings indicate that a combination of OprD loss, *gyrA* mutation and production of MBLs may facilitate the prediction of multidrug resistance in *P. aeruginosa,* and this may be widely applied to the early screening of clinical MDRPA strains in local diagnostic laboratories. Additional studies with a larger sample size and more molecular determinants from various regions and countries are warranted to establish the validity of such an approach to improve infection control in hospitals.

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DECLARATION OF INTEREST

None.

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