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Original Research Article

Identification of multi-drug resistant genes in *P. aeruginosa* isolates from patients under mechanical ventilation and respiratory support in an intensive care unit

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Abstract

Purpose: *To determine multi-drug resistant (MDR) and metallo β-lactamase (MBL)-resistant genes from Pseudomonas aeruginosa isolated from intensive care unit (ICU) patients under mechanical ventilation and respiratory support.*
Methods: *P. aeruginosa was isolated from 387 purulent tracheobronchial secretions collected from ICU patients who were intubated and mechanically ventilated for at least 48 h. Antibiotic resistance was determined by minimum inhibitory concentration (MIC) assay while MDR genes, viz, blaTEM, blaOXA, blaVIM, blaCTX-M-15 were determined by polymerase chain reaction (PCR).*
Results: *A total of 144 (37.2 %) P. aeruginosa were isolated from the purulent tracheobronchial secretions. A majority of the isolates (51.4 %) were resistant to gentamicin. Meropenem-gentamicin was the predominant (35.4 %) resistant combination. Out of the 144 isolates, 102 (70.8 %) were positive for blaTEM gene, 51 (35.4 %) for were positive for blaOXA gene, 22 (15.3 %) were positive for blaVIM gene, while 19 (13.2 %) were positive for blaCTX-M gene.*
Conclusion: *The high prevalence of MDR P. aeruginosa indicates the need for continued monitoring of MDR P. aeruginosa especially in ICU patients who are under mechanical respiratory support.*

Keywords: *Multi-drug resistance genes, Mechanical ventilator, Respiratory support, Pseudomonas aeruginosa*

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INTRODUCTION

Mechanical ventilation is one of the fundamental elements of therapy for patients admitted in ICUs [1]. The ability of *Pseudomonas aeruginosa* to colonize in-patients is highly critical within ICUs. Mechanical ventilators used in the ICUs are associated with a higher risk of respiratory tract infections leading to ventilator-associated respiratory infections (VARI) and ventilator-associated pneumonia (VAP) [1]. *Pseudomonas aeruginosa* is one of the most common organisms associated with nosocomial pneumonia [2]. According to the United States National Healthcare Safety Network (NHSN)

data, *P. aeruginosa* was the second most common pathogen isolated from patients with VAP [2]. The National Nosocomial Infection Surveillance System (NNISS) in China reported that *P. aeruginosa* is the predominant pathogen isolated from the lower respiratory tract, accounting for 12.8 % during 1999 to 2001, 12.3 % during 2002 to 2004, and 13.4 % during 2005 to 2007 period [3,4].

A combination of β-lactams either with anti-pseudomonal quinolone or an aminoglycoside is the primary treatment choice for *P. aeruginosa* infections. However, increasing resistance towards various antibiotics has led to severe life-threatening conditions which pose a challenge in the treatment of *P. aeruginosa* infections. *Pseudomonas aeruginosa* exhibits resistance towards multiple antibiotics leading to the development of multidrug-resistant (MDR) strains. The increase in MDR strains is a global problem. Multi-drug resistant *P. aeruginosa* strains are associated with increased morbidity and mortality, prolonged hospital stay, and higher costs of treatment [5]. Infections caused by resistant *P. aeruginosa* are often associated with excessive use of antibiotics, and invasive procedures including hemodialysis, tracheostomy and mechanical ventilation catheter [6].

Carbapenems were considered effective antibiotics against *P. aeruginosa* infections. However, due to extensive use of these antibiotics, the resistance mechanism spread across hospitals. Multi-drug resistance especially the MBL-producing strains has been commonly reported in all regions of the globe. The most common MBL genes are imipenem (*IMP*), verona integron-encoded metallo-β-lactamase (*VIM*), Sao Paulo MBL (*SPM*), German imepenemase (*GIM*), and the recently reported Seoul imepenemase (*SIM*) families.

The true prevalence of MDR *P. aeruginosa* has not been well established mainly due to the ambiguity existing in the definition of MDR [5]. Different definitions of MDR have been used in the literature [7]. Majority of the published literature define MDR as strains which possess resistance to a minimum of three different antibiotic classes, mainly the anti-pseudomonal penicillins, aminoglycosides, fluoroquinolones, cephalosporins and carbapenems [5]. However, a group of experts from European Centre for Disease Prevention and Control and the Centers for Disease Control and Prevention, defined MDR as *acquired non-susceptibility to at least one agent in three or more antimicrobial categories* [7].

Strains are categorized as MDR strains based on the definition published by Magiorakos *et al* [7]. In addition, due to geographical variations and participating centers, the SENTRY antimicrobial surveillance program was developed to track antimicrobial resistance trends nationally and internationally, fail to report the true prevalence of MDR *P. aeruginosa* [8]. With the lack of safe therapeutic antibiotics against MDR and pan-drug resistant strains, continued presence of such strains would pose serious challenges in infection control management [9]. Hence, it is extremely vital to understand the distribution of pathogens and antibiotic resistance patterns of pneumonia so as to achieve optimal antibiotic therapy. Although several studies have reported MDR *P. aeruginosa*, [5] limited data are available from China. The present study determined MDR resistant and MBL resistant genes from *P. aeruginosa* isolated from ICU patients who were under mechanical ventilation and respiratory support.

EXPERIMENTAL

Sample collection

A total of 387 purulent tracheobronchial secretions were collected from 387 non-repetitive patients who were admitted in ICU for various disabilities, and who were intubated and mechanically ventilated for at least 48 h between July 2015 and June 2017. Written informed consents were obtained from all the patients or their legal representatives after duly explaining the nature of the study. The study was approved by the institutional review board of The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China (approval no. TKG155661) and was carried out as per WHO guidelines [10]. Samples were collected in a sterile container and sent immediately for microbial analysis. After collection, the samples were liquefied by the addition of 1 % N-acetyl cysteine (equal volume v/v) and homogenized by vortexing (3000 rpm for one min). Then, 0.1 ml of the sample was diluted (1:100 with 9.9 ml of sterile physiological saline) and it was subjected to microbial culture [11].

Isolation and identification of *P. aeruginosa*

The samples were sub-cultured onto MacConkey agar at 37 ºC for 18 - 24 h. The non-fermenting, irregular, green-to-brown, catalase- and oxidase-positive colonies showing typical colony morphology of *P. aeruginosa* were subjected to identification using Vitek® 2 microbial identification system (Vitek® 2 software, version R02.03, Advanced Expert System software, version R02.00N). The isolates were stored in

nutrient broth (with 20 % glycerol) at -40 ºC until used for further analysis.

Minimum inhibitory concentration (MIC) assay

The MICs of various antibiotics i.e. gentamicin, amikacin, piperacillin-tazobactam, ciprofloxacin, cefepime, ceftazidime, polymyxin B, imipenem and meropenem (Sigma-Aldrich, USA) against *P. aeruginosa* were determined. The inoculum was prepared by direct suspension of colonies grown overnight on nutrient agar in 0.85 % saline, with turbidity adjusted to 0.5 McFarland's standard for inoculation. The MIC assay (micro broth dilution method) was performed at concentrations of each antibiotic ranging from 0.03 to 128 µg/mL; using Muller-Hinton broth (MHB) as described in Clinical Laboratory Standard Institute (CLSI) guidelines [12]. In essence, 10 µL of culture was inoculated into the various concentrations of MHB and incubated at 37 ºC for 24 h. After incubation, MIC was determined visually by the highest concentration showing the absence of growth.

Multiplex PCR

DNA was extracted from pure cultures by alkali lysis method and stored at -20 ºC until used for PCR. Multiplex PCR was used to determine extended-spectrum β-lactamases (ESBL)-MBL resistant genes such as bla_{TEM}, bla_{OXA}, $bla_{CTX\text{-}M\text{-}15}$, bla_{VIM} [9]. The PCR was performed using a 50-µL master mix containing 5 µL of template DNA, 5 pmoL of each primer (Table 1), dNTPs (2 mM), 3 units of Taq polymerase enzyme, 5 µL of 10x reaction buffer, and molecular grade PCR water in a total volume of 50 µL. PCR was performed using the thermocycler (Applied Biosystems, Verti Thermal Cycler, Thermo Fisher Scientific). The PCR cycling conditions were: initial denaturation at 94°C for 5 min followed by 35 cycles at 94 °C for 30 s; 56 °C for 30 s, extension at 72 °C for 1.5 min, and final extension at 72 °C for 7 min. After PCR, amplicons were resolved in 1.2 % agarose gel electrophoresis.

Statistical analysis

Continuous and categorical variables are presented as mean/ ranges and numbers/ percentages, respectively. ANOVA and chi-square tests were performed to determine the statistical significance using MINITAB statistical software (Minitab version 13.1; Minitab Inc, PA, USA). Values of $p < 0.05$ were considered statistically significant.

Table 1: Primer sequence of *ESBL-MBL* resistant genes

Gene	Primer sequence (5'-3")	Amplicon size (bp)
bla_{TEM}	Forward: TCAACATTTTCGTGTCGCCC Reverse: AACTACGATACGGGAGGGCT	766
bla_{OXA}	Forward: AGATCCTTGACCCGCAGTTG Reverse: CGCCGTCCCATCGAAAAATC	928
$bla_{CTX\text{-}M}$	Forward: AGACTGGGTGTGGCATTGAT Reverse: TTAGGTTGAGGCTGGGTGAAGT	676
bla_{VIM}	Forward: TGTCCGTGATGGTGATGAGT Reverse: GTGCTTCCGGGTAGTGTTGT	456

RESULTS

Patients and isolates

A total of 215 patients (55.6 %) were male, while 172 patients (44.4 %) were female (mean age = 49.3 ± 7.3 years). Prior antibiotic therapy was given to 154 (39.8 %) patients. Out of the 387 purulent tracheobronchial secretions, 144 (37.2 %) *P. aeruginosa* isolates were obtained, which was significantly higher than isolates of the other bacterial species ($p = 0.02$).

Antibiotic resistance

Amongst the 144 *P. aeruginosa* isolates tested for antibiotic susceptibility, 74 (51.4 %) were resistant to gentamicin, 63 (43.8 %) were resistant to ceftazidime, 63 (43.8 %) were resistant to imipenem, 62 (43.1 %) were resistant to meropenem, 61 (41.8 %) were resistant to ciprofloxacin, 58 (40.3 %) were resistant to cefepime, 54 (37.5 %) were resistant to piperacillin-tazobactam, 35 (24.3 %) were resistant to amikacin, while 31 (21.5 %) were resistant to polymixin B (Table 2). No significant difference was found in the presence of antibiotic resistance among the isolates (ANOVA, $F = 0.00$; $p = 1.00$).

A checkerboard analysis was performed to detect the cross-resistance of *P. aeruginosa* isolates to the related and unrelated antibiotics. The analysis showed that meropenem-gentamicin was the predominant resistant combination observed in 51 (35.4 %) isolates. Other common resistance combinations included meropenem-ciprofloxacin (49, 34.0 %), meropenem-ceftazidime (46, 31.9 %),

ciprofloxacin-imipenem (46, 31.9 %), ciprofloxacin-ceftazidime (43, 29.9 %), meropenem-piperacillin/tazobactam (43, 29.9 %) meropenem-cefepime (42, 29.2 %) and imipenem-cefepime (42, 29.2 %) (Table 3). In addition, 58 (40.3 %) of the tested isolates were MDR strains.

Resistance genes

Out of the 144 isolates, 102 (70.8 %) were positive for bla_{TEM} gene, 51 (35.4 %) were positive for bla_{OXA} gene, 22 (15.3 %) were positive for bla_{VIM} gene, while 19 (13.2 %) were positive for bla_{CTX-M} gene. The presence of *blaTEM* gene was significantly high among all the isolates tested by PCR (χ^2 = 138.07; DF = 3; p = 0.00). However, no significant difference was found in the presence of resistance genes among the isolates which were resistant to various antibiotics (ANOVA, F = 0.89; p = 0.537). Among these, bla_{TEM}-bla_{OXA} was the most common gene combination in 17 (11.8 %) isolates, while bla_{OXA}-bla_{VIM}, bla_{OXA}-bla_{CTX-M}, bla_{TEM}-bla_{OXA}-bla_{CTX-M} were the second most common gene combinations detected in 4 (2.8 %) isolates.

Only one (0.7 %) isolate was positive for all four genes tested (Table 4). A comparison of antibiotic resistance and the presence of genes are shown in Table 5. Out of the 58 MDR isolates, 39 (67.2 %) were positive for bla_{TEM}, 32 (55.2 %) were positive for bla_{OXA}, 16 (27.6 %) were positive for bla_{VIM}, while 11 (19 %) were positive for bla_{CTX-M} genes. All the resistant isolates tested positive for any of the four tested genes. A total of 28 isolates which showed intermediate resistance towards the tested antibiotics were positive for bla_{TEM} (12), bla_{OXA} (8), bla_{CTX-M} (5) and bla_{VIM} (3) genes.

Table 4: MDR resistance genes among *P. aeruginosa* isolates

Gene	No of isolates (%)
blaTEM	76 (52.8)
blaOXA	17 (11.8)
blaVIM	10 (6.9)
blaCTX-M	7 (4.9)
blaTEM, blaOXA	17 (11.8)
blaTEM, *blaVIM*	1 (0.7)
blaOXA, blaVIM	4 (2.8)
blaOXA, blaCTX-M	4 (2.8)
blaTEM, blaOXA, blaVIM	1 (0.7)
blaOXA, blaVIM, blaCTX-M	3 (2.1)
blaTEM, blaVIM, blaCTX-M	2 (1.4)
blaTEM, blaOXA, blaCTX-M	4 (2.8)
blaTEM, blaOXA, blaVIM, blaCTX-M	1 (0.7)

Table 2: Antibiotic resistance and MIC of *P. aeruginosa* isolates (n =144)

Antibiotic	No of isolates			MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	Range (µg/mL)
	S	I	R			
GN	56	14	74	8	32	0.5-64
CAZ	62	19	63	16	128	2 - 128
IPM	73	8	63	2	64	0.25 – 64
MEM	72	10	62	1	32	0.5 - 64
CIP	69	16	61	2	8	0.06 – 8
CPM	72	14	58	8	64	≤ 0.03 - ≥128
PTZ	76	14	54	4	≥ 128	8- ≥128
AM	91	18	35	4	64	0.06 - ≥128
POL	95	18	31	1	32	0.06 – 64

Gentamicin (GN), amikacin (AM), piperacillin-tazobactam (PTZ), ciprofloxacin (CIP), cefepime (CPM), ceftazidime (CAZ), polymyxin B (POL), imipenem (IPM) and meropenem (MEM)

Table 3: Antibiotic cross resistance among *P. aeruginosa* isolates

Antibiotic	No. of isolates								
	GN	CAZ	IPM	MEM	CIP	CPM	PTZ	AM	POL
GN	**74**	32	24	51	35	35	27	30	22
CAZ	32	**63**	29	46	43	28	39	29	19
IPM	24	29	**63**	25	46	42	35	32	26
MEM	51	46	25	**62**	49	42	43	26	17
CIP	35	43	46	49	**61**	18	19	14	22
CPM	35	28	42	42	18	**58**	26	24	25
PTZ	27	39	35	43	19	26	**54**	23	16
AM	30	29	32	26	14	24	23	**35**	18
POL	22	19	26	17	22	25	16	18	**31**

Gentamicin (GN), amikacin (AM), piperacillin-tazobactam (PTZ), ciprofloxacin (CIP), cefepime (CPM), ceftazidime (CAZ), polymyxin B (POL), imipenem (IPM) and meropenem (MEM)

Table 5: Antibiotic resistance and MDR genes in *P. aeruginosa* isolates

Antibiotic	*blaTEM* (n = 102)	*blaOXA* (n = 51)	*blaVIM* (n = 22)	*blaCTX-M* (n = 19)
GN (n =74)	58	41	17	16
CAZ (n = 63)	51	35	8	12
IPM (n = 63)	48	25	12	6
MEM (n = 62)	50	31	15	11
CIP (n = 61)	32	23	8	5
CPM (n = 58)	45	29	17	3
PTZ (n = 54)	36	21	6	8
AM (n = 35)	16	18	16	13
POL (n = 31)	14	11	3	5

DISCUSSION

Rapid and accurate identification of infectious agents is crucial for the initiation of appropriate therapy that has important consequences in patient's clinical outcome. It has been reported that rapid identification of infectious agents leads to a substantial reduction in the time taken to initiate effective antimicrobial therapy, and also decreases hospital cost and mortality [13]. Several studies have reported that Vitek® 2 microbial identification system correctly identifies bacterial strains, with accuracy ranging from 85.3 - 100 % [14 - 16]. In this study, rapid Vitek® 2 microbial identification system was used successfully to identify the isolates up to the species level.

The prevalence of *P. aeruginosa* was 37.2 %. A meta-analysis by Ding *et al*. [4] on 28 studies in China reported an overall 19.4 % prevalence of *P. aeruginosa* in VAP, which is much lower than that obtained in the present study (37.2 %). Similarly, another systematic review from Mainland China reported that 20.6 % of isolates from ICU patients with VAP were *P. aeruginosa* [17]. In other Asian countries, the prevalence of *P. aeruginosa* was much lower than that reported in this study, as indicated by these results: Thailand (18 %), Malaysia (18 %) and the Philippines (19 %) [18].

However, a study in Brazil reported a higher percentage (51.9 %) of *P. aeruginosa* isolates from ICU patients, when compared with the prevalence of 37.2 % obtained in the present study. Studies have also reported that mechanical ventilator is a risk factor for *P. aeruginosa* infections [19]. A declining trend in the prevalence of *P. aeruginosa* isolates associated with VAP has been reported [4]. However, the present study obtained much higher percentage of *P. aeruginosa* than those reported in other Asian countries. This could possibly be due to the differences in periods of the studies. The Asian studies were conducted between 1999 and 2012. Thus, there could be fluctuations in the prevalence of *P. aeruginosa* over time, which may account for the differences in the prevalence figures. This variation in the prevalence implies that it is risky to adopt an attitude of complacence by continuing to refer to declining trend in the prevalence of *P. aeruginosa* in the Asian region based on previous findings. There should be a continuous monitoring system focused on *P. aeruginosa* infections in the clinical departments of hospitals, especially in the ICUs.

The inherent resistance of *P. aeruginosa* to a broad range of antibiotics, and its ability to develop MDR and acquired resistance through chromosomal mutations pose serious challenges during treatment [8, 16]. Given the increasing resistance towards various antibiotics, MDR is expected to become more prevalent in several hospitals. In this study, 51.4 % (MIC range: 0.5 - 64 µg/mL) of the isolates were found to be resistant to gentamicin, while only 24.3 % (MIC range: 0.06 ≥ 128 µg/mL) of isolates were resistant to amikacin. Similarly, a meta-analysis in China reported gentamicin resistance in 51.1 % and amikacin resistance in 22.5% of the isolates in VAP cases [4].

A study in India reported comparable resistance (18 %) to amikacin, but much lower resistance values to gentamicin (38 %), ceftazidime (26 %), cefepime (5.5 %), meropenem (18.5 %), imipenem (20.5 %) and ciprofloxacin (25.5 %) [9], when compared to the 37.2 % obtained in the present study. In contrast, a study in Romania reported higher resistance to amikacin (46.9 %), imipenem (49.8 %), piperacillin/tazobactam (49.7 %), meropenem (50.5 %), ceftazidime (53.1 %), ciprofloxacin (60.3 %) and gentamicin (64.2 %) [20]. Similarly, a study in Brazil reported higher antibiotic resistance for cefepime (48.1 %), imipenem (64.8 %), meropenem (64.8 %), piperacillin/tazobactam (46.3 %) and ceftazidime (63 %) [19]. In the present study, the highest resistance was to gentamicin (51.1 %), while the lowest resistance was to polymixin B (21.5 %).

Extended spectrum β-lactamases are the major factor for acquired β-lactam resistance in *P. aeruginosa* -. In China, ESBL-producing *P. aeruginosa* isolates ranged from 35.3 – 64.7 % which was higher than that reported from India (22.2 %) [–21-23]. Among β-lactams, carbapenems (meropenem and imipenem) due to their stability against ESBLs are considered as the most potent agents for MDR *P. aeruginosa* [24]. In the present study, the activity of imipenem and meropenem were comparable, wherein 43.1 % (MIC range 0.25 - 64 µg/mL) of the isolates were resistant to imipenem and 41.8% (MIC range 0.5-64 µg/mL) of isolates were resistant to meropenem.

A comparable resistance to imipenem (41.1 %) and meropenem (38.9 %) was reported in China [4]. The study also reported comparable resistance values for ceftazidime (40.3 %), cefepime (38.5%) and piperacillin/tazobactam (38.9 %), while a study in India reported much lower resistance (25 %) for meropenem and comparable resistance (25 %) for piperacillin/tazobactam [9]. In contrast, a study in Brazil reported much higher degrees of resistance (64.8 %) to meropenem and imipenem in *P. aeruginosa* isolated from ICU patients [19].Although the majority of the isolates were resistant to gentamicin, antibiotic resistance did not differ significantly among the isolates (p = 1.00). In the present study, 40.3 % of isolates were MDR strains, which is higher than that reported in Brazil (37 %) [19]. All the identified MDR isolates were resistant to gentamicin.

The ESBL enzymes encoded by *TEM, CTX-M, GES, PER, SHV, VEB* and *IBC* family of genes were prevalent among *P. aeruginosa*. In addition, OXA-type ESBL enzymes have been reported. The bla_{TEM} gene was predominantly carried by *P. aeruginosa*, being narrow spectrum β-lactamases derived from mutation of single nucleotide from the TEM-1 and TEM-2, leading to TEM-3 and other variants which confer resistance to penicillin group of antibiotics. In this study significantly higher number of isolates (70.8 %) was positive for bla_{TEM} gene. This is similar to current findings in a study in India which reported that bla_{TEM} was the predominant gene present among the *P. aeruginosa* isolates (72.5 %) [9]. In that study [9], the bla_{OXA} (33.5 %) and bla_{VIM} (11.5%) genes were comparable. However, the $bla_{CTX\text{-}M}$ gene (5 %) was lower than that the value obtained in the present study. In another study, 93 % of the isolates were positive for bla_{OXA} gene which was much higher than that reported in the present study [16]. In *P. aeruginosa*, the bla_{OXA} gene is considered a naturally occurring gene.

The high prevalence of this gene raises an alarm because this may lead to potential horizontal gene transfer where other co-inhabiting bacteria species may possess Class D β-lactamases. In one study [25], 19.6 % of *P. aeruginosa* isolates were positive for $bla_{CTX\text{-}M}$ gene, which is higher than that seen in the present this study. Although the presence of bla_{TEM} gene was significantly higher, the presence of resistance genes did not differ significantly among the isolates which were resistant to various antibiotics.

It has been reported that if an isolate was positive for $bla_{CTX\text{-}M}$ gene but did not possess any of the other beta-lactamase genes, then the patient from whom the isolate was obtained can be treated using aminoglycosides, quinolones, carbapenems, and fourth and fifth generation cephalosporins, to the exclusion of cefotaxime and other third-generation cephalosporins [9]. In this study, a total of 7 (4.9 %) isolates which were positive for $bla_{CTX\text{-}M}$ gene, did not amplify any of the other tested genes. The study [9] also reported that if an isolate showed presence of $bla_{VIM/\mathrm{DIM}}$, bla_{TEM} and $bla_{\mathrm{CTX\text{-}M}}$ genes, the patient should be treated with colistin, polymyxin, aztreonam, or a combination of drugs [9]. In the present study, 2 (1.4 %) isolates were found to possess this combination of genes, which suggests that these patients should be treated with polymyxin, aztreonam, or a combination of drugs for better patient management.

CONCLUSION

P. aeruginosa is the predominant isolate from ICU patients under mechanical ventilation and respiratory support in this study. A majority of the isolates are resistant to gentamicin, and the predominant gene in the isolates is bla_{TEM}. Compared to other studies, especially studies from Asia, the high prevalence of MDR *P. aeruginosa* indicates the need for continued monitoring of MDR *P. aeruginosa* especially in ICU patients under mechanical respiratory support.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. This study was

designed by Xiaoli Yang, Hongping Yao, Congying He, Jinfang Sheng. The data was acquired by Chun Zhang, Cui Li, Congying He, Jinfang Sheng. This study was supervised by Xiaoli Yang, Hongping Yao, Congying He, Jinfang Sheng.

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