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Prevalence, phenotype and genotype characteristics of antibiotic resistance in coastal beach practitioners of tropical China

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ABSTRACT

Objective: To investigate the genetic mechanism and prevalence of antibiotic resistance among the practitioners of two bathing beaches in tropical China.

Methods: Totally 259 strains were isolated from the rectal swabs of the practitioners and screened by MacConkey agar. A questionnaire survey was conducted. Kirby-Bauer test was used for phenotype, and double disc inhibition synergy test was used for extended-spectrum β -lactamase-producing *Enterobacteriaceae* (ESBL-E), which was further confirmed by VITEKII instrument. The genotype was detected by polymerase chain reaction, and the similarities of ESBL-E were analyzed using pulsed-field gel electrophoresis.

Results: The prevalence of ESBL-E was 13.1% (34/259), of which, *CTX-M* genes accounted for 44.1% (15/34), mainly *CTX-M-14* and *CTX-M-27*. Moreover, 19.1% (4/21) of the isolates were homologous. The resistance phenotypes of ESBL-E to chloramphenicol, ciprofloxacin, levofloxacin, ceftazidime, cefepime and gentamicin were different between two beaches(P<0.05). The prevalence of ESBL-E was caused by *CTX-M* genes at the island beach, while a high prevalence of ESBL-E was found at the city beach and clone transfer occurred.

Conclusions: ESBL-E-associated gentamicin antibiotic resistance risk may be greater for island beach practitioners. The city beach populations could face more potential risks owing to severe resistance and metastatic ability of ESBL-E. The environmental exposure mechanisms and transfer role of antibiotic resistance at public beaches with high density should be investigated.

KEYWORDS: Antibiotic resistance; Antibiotic resistance genes; Extended-spectrum β -lactamase-producing *Enterobacteriaceae*; Beach practitioners; Risk

1. Introduction

The spread of antibiotic resistance (AR) is a global environmental and health threat. It's estimated that 10 million people may die from infectious diseases caused by antibiotic resistant organisms in 2050 worldwide[1] (Cooke, 2016). China is a major producer and consumer of antibiotics. River and marine areas[2–4] (Dada *et al*., 2013; Drali *et al*., 2018; Liu *et al*., 2018), wastewater and soil[5–7] (Zhang *et al*., 2019; Liu SS *et al*., 2018; Liu *et al*., 2018) are reservoirs of AR. Many reports suggested that artificially

Significance

Antibiotic resistance has been found to be common in marine beaches, but there have been few studies on antibiotic resistance among coastal beach practitioners. The prevalence and resistance phenotype of ESBL-E were higher in city beach, the genotypes were mainly *CTX-M-14* and *CTX-M-27*, and 19.0% of the strains were homologous. This study may provide reference to different infection treatments for island and city beach practitioners in China, and the mechanism of clone transfer of ESBL-E deserves attention.

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manufactured poultry, livestock, and aquatic products are rich in AmpC β -lactamase (AmpC), extended-spectral β -lactamase-producing *Enterobacteriaceae* (ESBL-E) and even carbapenem-resistant *Enterobacteria* (CRE)[8–12] (Hong *et al*., 2019; Jorgensen *et al*., 2017; Duan *et al*., 2019; Wu *et al*., 2018; Wang *et al*., 2019). ESBL-E is resistant to third-generation cephalosporin and is categorized as a high-risk resistant strain and an "a critical priority" by the World Health Organization in 2017. Increasing prevalence of CRE is another cause for great concern. Presently, ESBL-E and CRE limit the therapeutic options for infection in clinic, but some of the severe AR and pathogen is sourced from community and healthy population. Moreover, the plasmid with a mobile colistin resistance gene (*mcr−1*) may break the last line of defense against antibiotic resistant *Enterobacteriaceae*[13] (Shen *et al*., 2018). The threat posed by AR is typically the horizontal transmission of antibiotic resistance genes (ARGs), including the transfer from sensitive strains to resistant ones, and from common strains to pathogenic ones. Previous studies showed that surfers were 4.09 times more resistant to *CTX−M* than non-surfers in England[14] (Leonard *et al*., 2018). However, the status of AR among coastal practitioners and the difference of AR between island and city beaches populations have not been fully studied.

The beach is an open public place with a large number of domestic and international tourists. AR among beach practitioners can probably be transferred to the public. Sanya is the southernmost tourist city of China (Figure 1). Dadonghai (DDH) Beach is located in the city center, and Wuzhizhou (WZZ) Beach is approximately 3.3 km away from the nearest land. The beach practitioners from DDH and WZZ were selected as subjects and was for environment exposure and their rectal swabs were collected. The swabs were initially screened for Gram-negative bacteria and later for ESBL-E and drug-susceptibility. The ARGs and the similarity of ESBL-E were also studied. We aimed to understand the prevalence of multiple AR and find out the ARGs of ESBL-E among the populations. The results of this study will help in the prevention of risks of AR among occupational and recreational populations of beaches and provide a scientific basis for the prevention of AR.



Figure 1. Location of Sanya in China

2. Materials and methods

2.1. Sampling

Dadonghai(DDH)Beach is located at the longitude of 109. 529 and the latitude of 18. 227, while Wuzhizhou(WZZ)Beach is located at the longitude and latitude of 109. 772 and 18. 315, respectively. Both these beaches have a huge number of tourists. The cluster sampling of individuals was conducted at the two beaches in December of 2017. *Enterobacteriaceae* were screened for antibiotic susceptibility, followed by ARGs determination and on-site surveys.

2.2. Questionnaire survey

The questionnaire asked for details of basic conditions and environmental exposure. The basic conditions included age, gender and occupation. The environmental factors included frequency of seawater exposure (<1 times/week, 1~3times/week, 4~5times/week, 6~7times/week), frequency of sand exposure (<1 times/week, 1~3times/week, 4~5times/week, 6~7times/week), recent interval time of seawater exposure (⩽7days, > 7days) and recent interval time of sands exposure (⩽7days, > 7days). Seawater exposure was defined to exposure to seawater (above the ankle) for at least 20 minutes, sand exposure was defined to exposure to the sand without closed shoes or barefoot for at least 20 minutes. A analysis using the formula $n=t^2pq/d^2$ was performed to determine the sample size. Considering of the missing rate, we set the coefficient 0.05 to calculate more samples, and the sample size should be 235, and finally 247 persons and 259 qualified strains were explored.

2.3. Medical ethics and informed consent

The study was approved by the Ethics of Committee of Hainan Medical University. The forms were prepared following the prescribed guidelines. Informed consent forms were signed by all respondents and researchers.

2.4. Sample collection and screening of isolates

The samples were collected with sterilized rectal swabs, stored at 4 ℃ for 4 h and brought back to the laboratory. The samples were plated on MacConkey agar (Huankai, China), and individual colonies were purified by the partitioning method. The oxidase-negative strains were firstly chosen for *Enterobacteriaceae* identification.

2.5. Drug−susceptibility test and identification of bacteria

Kirby–Bauer disk diffusion test was performed using Mueller–Hinton agar and double disc inhibitory synergy test for ESBL-E (Oxoid, Hampshire, UK). The antibiotics used were ampicillin (AMP), cefazolin (CFZ), cefoxitin (FOX), ampicillin/sulbactam (AMS), ticarcillin/clavulanic acid (TIM), cefuroxime (CXM), compound sulfamethoxazole (SXT), chloramphenicol (CHL), imipenem (IPM), ceftazidime (CAZ), piperacillin (PIP), ceftriaxone (CRO), ciprofloxacin (CIP), levofloxacin (LVX),

minocycline (MI), gentamicin (GEN), tobramycin (TOB), aztreonam (ATM), piperacillin/tazobactam (TZP) and cefepime (FEP). All the results were interpretated according to the American Clinical Laboratory Specification of CLSI2020. A double-disc synergistic experiment confirmed the presence of ESBL-E, *Escherichia coli* ATCC 25922 (*E. coli*) and *Kelbsiella pneumoniae* ATCC700603 (*K. pneumoniae*) were used as quality controls, and identified by 16s RNA gene sequencing, with universal primers of 27F and 1492R(AGAGTTTGATCATGGCTCAG; TACGGYTACCTTGTTACGACTT)[15] (Kisand *et al*., 2002).

2.6. Detection and comparison of antibiotic resistance genes of the ESBL–E

The bacterial strains were cultivated on LB broth media (Huankai, China) by incubating the plates overnight at 37 ℃. The cells were isolated and boiled at 100 ℃ for 5 minutes (min) for DNA extraction. After centrifugation at 12000 r/min for 7 min, the supernatant was separated as a template. The polymerase chain reaction (PCR) was performed using 2 Taq PCR MasterMix (Tiangen Biotech, China) and PCR instrument of Master cycler Pro (Eppendorf, USA). This reaction was conducted in a total volume of 25 μL containing 0.5~1.0 μl (10 μM) of each primer, 12.5 μl of Mix (optimized proportions of Taq DNA polymerase, dNTPs, $MgCl_2$ and buffer), 1.0 μl of template DNA and mensurable ddH_2O. Amplification condition was as follows: 5 min at 94 ℃, followed by 25-30 cycles (94 ℃, 30 s; 55~58 ℃, 90 s; 72 ℃, 60 s), and the extension step was performed at 72 ℃ for 10 min.

The primers of mobile colistin resistance gene (*mcr–1*)[16] (Liu Y *et al*., 2016), New Delhi metallo- β -lactamase 1, *CTX–M* extended-spectrum β -lactamase genes, bla_{KPC} carbapenemase genes (National Center for Biotechnology Information (NCBI) database 2017)[17] are shown in Table 1, the amplified ARGs were sent to Shanghai Sanggon Co, Ltd for sequencing. The sequencing results were submitted to NCBI to obtain the accession numbers.

Table 1. PCR primer targeting antibiotic resistance genes.

Targeted gene	Primer sequence (5'-3')	Amplicon size (bp)
mcr–1	CGGTCAGTCCGTTTGTTC CTTGGTCGGTCTGTA GGG	309
NDM–1	CAGCACACTTCCTATCTC CCGCAACCATCCCCTCTT	292
bla_{KPC}	GCTACACCTAGCTCCACCTTC TCAGTGCTCTACAAAAACC	1050
CTX–M	GGGCTGAGATGGTGACAAAGAG CGTGCGAGTTCGATTTATTCAAC	881

PCR: polymerase chain reaction

2.7. Homology detection of strains

According to antibiotic resistance phenotype, twenty-one ESBL *E. coli* samples were analyzed using pulsed-field gel electrophoresis (PFGE). The gel blocks were prepared by 40U XbaI endonuclease (Takara, Japan) enzymatic digestion, followed by electrophoresis. Initial switch time and final switch time was 6.76 s and 35.38 s respectively, run time was 19 h. The gel block was stained with GelRed nucleic acid dye for 20–30 min, and the image was generated using Gel Doc 2000 (Bio-Rad Laboratories, USA).

2.8. Statistical analysis

The characters of antibiotic resistance were analyzed by descriptive statistical methods. The rates of resistance to antibiotics and the differences between environmental exposure were compared using a *Chi*-square test or an exact Fisher test based on a four-grid table, the groups of environmental exposure would be combined if necessary ($\alpha = 0.05$).

3. Results

A total of 259 isolates were screened from the rectal swabs. If there is more than one strain derived from the same sample, the duplicate strain with the same drug-resistance spectrum would be deleted. The proportion of ESBL-E was 16.3% (24/147) at the DDH Beach and 8.9% (10/112) at the WZZ Beach. An isolate was resistant to the imipenem of carbapenems antibiotics, while the bla_{NDM}, bla_{KPC} genes were not been found; another one carrying *mcr–1* was found in the non-ESBL-E category (accession number MK550663). All isolates of ESBL-E were *E. coli* except one of *Klebsiella pneumonia*, and neither *NDM–1* nor *mcr–1* was found (Table2).

3.1. Antibiotic resistance patterns of the strains found in the practitioners of the two beaches

The high resistance rates to antibiotics were shown in the order: ampicillin 65.6% (n=170), sulfamethoxazole 29.7% (n=77), cefazolin 26.6% (n=69), ampicillin/sulbactam 26.3% (n=68), chloramphenicol 21.6% (n=56), and cefuroxime 18.9% (n=49). The low rates were attributable to amikacin and imipenem (< 10%). Further comparison showed that the resistant rates of ciprofloxacin, levofloxacin, sulfamethoxazole, chloramphenicol, ampicillin/sulbactam, ticarcillin/clavulanic acid, ceftazidime and cefepime were significantly higher at DDH Beach than that at WZZ Beach (P<0.05) (Table 3).

3.2. Comparison of the resistance phenotype of ESBL–E at the two beaches

The resistance rate of ESBL-E to multiple antibiotics was also significantly higher at DDH than that at WZZ (P<0.05). The resistance rate of the 6 antibiotics in two groups were chloramphenicol (50%, 20%), ciprofloxacin (70.8%, 10%), levofloxacin (66.7%, 10%), ceftazidime (70.8%, 10%), cefepime (58.3%, 0%) and gentamicin (40%, 4.2%), The differences were statistically significant (P<0.05, Table 4). It can be deduced that the differences in antibiotic resistance between the two beach populations may lead to varying clinical anti-infective treatment methods.

Table 2. Genotypes and phenotypes of the screened isolates.

Source of isolates	Phenotype of ESBL-E*	Genotypes of ESBL-E n #(%), N&=34			Genotypes of non-ESBL-E n (%), $N$$=225
		CTX–M	*mcr*	*KPC /NDM–1*	*mcr*
Dadonghai beach	24	5 (20.8)	0	0	1 (0.44)
Wuzhizhou beach	10	10 (100)	0	0	

*: extended-spectrum β -lactamase-producing *Enterobacteriaceae;* #: the number of some genetypes; &: the number of ESBL-E; $: the number of non-ESBL-E.

Table 3. Comparison of the antibiotic resistance prevalence among practitioners between two beaches.

Antibiotics	DDH		WZZ		$\chi2$	P
	n=147	%	n=112	%		
Ciprofloxacin	24	16.3	4	3.6	10.726	0.001
Levofloxacin	23	15.6	3	2.7	11.837	0.001
Sulfamethoxazole	57	38.8	20	17.9	13.315	<0.001
Chloramphenicol	43	29.3	13	11.6	11.678	0.001
Ampicilin-sulbactam	48	32.7	20	17.9	7.187	0.007
Ticarcilline	30	20.4	9	8.0	7.608	0.004
Ceftazidime	18	12.2	2	1.8	9.759	0.002
Cefepime	14	9.5	0	0	11.276	<0.001

DDH: Dadonghai beach; WZZ: Wuzhizhou beach

Table 4. Comparison of antibiotic resistance of ESBL-E among practitioners between two beaches.

Antibiotics	WZZ (n=10)		DDH (n=24)		P
	Resistance	Sensitivity	Resistance	Sensitivity	
Ciprofloxacin	1	9	17	7	0.002
Levofloxacin	1	9	16	8	0.007
Chloramphenicol	2	8	12	12	0.073
Gentamicin	4	6	1	23	0.019
Ceftazidime	1	9	17	7	0.002
Cefepime	0	10	14	10	0.002

3.3. Type of the extended–spectrum β –lactamase resistantce genes of ESBL–E

The 34 isolates of ESBL-E accounted for 13.1% (34/259) of the samples. These isolates carried 3 types of *CTX–M* genes, namely, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-27}}$, and $bla_{\text{CTX-M-65}}$. The type with the highest frequency was *CTX–M–14* (8/15). All ESBL-E at WZZ Beach carried *CTX–M*, whereas only 20.8% (5/24) at DDH Beach. The above difference was statistically significant (P<0.01), and the details are shown in Table 5 (accession numbers: MN367300, MN367302-MN367310, and MK652430-MK652434). The genotype of *CTX–M* seems to be related to the different occupation of the practitioners (Table 5), however the statistical difference was not analyzed because of small sample size of positive strains among occupational groups.

Table 5. Distribution of genotypes and phenotypes of β -lactamase *CTX–M* in ESBL-E.

Occupation of host	Strain Number	Genotypes	Phenotypes	Source beach
Seawater sports coach or lifeguard	171	$bla_{\text{CTX-M-14}}$	SXT+AMP+GEN+CXM+CFZ	WZZ
	237	$bla_{\text{CTX-M-14}}$	SXT+AMP+GEN+AMS+CRO+CXM+CFZ	WZZ
	259b	$bla_{\text{CTX-M-14}}$	AMP+CRO+CXM+CFZ	WZZ
	260	$bla_{\text{CTX-M-14}}$	SXT+AMP+CXM+CFZ	WZZ
	275	$bla_{\text{CTX-M-14}}$	SXT+AMP+GEN+CRO+CXM+CFZ	WZZ
	283	$bla_{\text{CTX-M-14}}$	CIP+LEV+AMP+CRO+GEN+CXM+CFZ	WZZ
	216a	$bla_{\text{CTX-M-27}}$	SXT+AMP+CRO+CXM+CFZ	WZZ
	98	$bla_{\text{CTX-M-14}}$	CIP+LEV+AMP+CRO+CXM+CFZ	DDH
	110a	$bla_{\text{CTX-M-14}}$	SXT+AMP+CHL+CRO+CXM+CFZ	DDH
Sanitation worker	35	$bla_{\text{CTX-M-27}}$	CIP+LEV+SXT+AMP+CRO+CXM+CFZ	DDH
	20b	$bla_{\text{CTX-M-27}}$	CIP+LEV+SXT+AMP+CRO+CXM+CFZ	DDH
	41	$bla_{\text{CTX-M-27}}$	SXT+AMP+CRO+CXM+CFZ	DDH
Others	185b	$bla_{\text{CTX-M-27}}$	CIP+LEV+SXT+AMP+CRO+FEP+CXM+CFZ	WZZ
	194	$bla_{\text{CTX-M-27}}$	SXT+AMP+CRO+CXM+CFZ	WZZ
	163b	$bla_{\text{CTX-M-65}}$	CIP+LVX+AMP+CXM+CFZ	WZZ

SXT: sulfamethoxazole; AMP: ampicillin; GEN: gentamicin; CXM: cefuroxime; CFZ: cefazolin; AMS: ampicilin-sulbactam; CRO: ceftriaxone; CIP: ciprofloxacin; LVX: levofloxacin; CHL: chloramphenicol; FEP: cefepime.

3.4. Comparison on antibiotics resistance prevalence between different environmental exposure factors

Only gentamicin resistance was statistically significant. Except for the frequency of seawater exposure, age, gender, occupation, frequency of sands exposure, recent time interval of exposure to sand and seawater were significantly negatively correlated with the incidence of gentamicin resistance (P<0.05). Seawater exposure is a major factor (>3 times/week) in higher gentamicin resistance rates, which is respectively 1.4% (1/69) and 9.5% (18/190) in two different exposure groups (χ^2=4.795, P=0.029).

3.5. Homology analysis of ESBL–E

Totally 25 strains of ESBL-producing *E. coli* were selected for homology analysis by PFGE, four of them were failed. The ESBL-producing *E. coli* belonged to 18 genetic types, in which four strains (19.0%), DH73B, DH39A, DH53B, DH65 had 100% homology, suggesting that they were derived from the same clone. The affected individuals had a similar drug-resistance spectrum, suggesting the same bacterial resistance phenotype caused an ARGs transfer at DDH Beach. The other 17 types were sporadic and entailed different types (Figure 2).

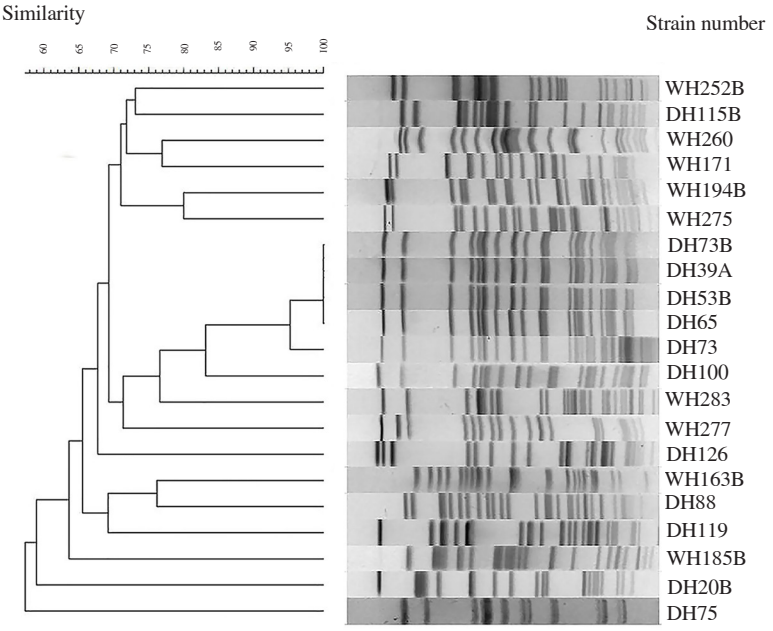

Figure 2. Homology analysis of extended-spectrum β -lactamase-producing *Enterobacteriaceae*.

4. Discussion

In this study, we analyzed the environmental influencing factors and micro-mechanism of ESBL-E among the special beach practitioners. The prevalence of ESBL-E among the studied population (13.9%) was similar to that of healthy people in Okinawa, Japan (12.2%)[18] (Higa *et al*., 2019). The positive rate of *CTX–M* (5.8%,15/259) in ESBL-E patients was close to the antibiotic resistance reported recently among surfers in coastal water (6.3%)[14](Leonard *et al*., 2018). Various antibiotic-resistant phenotypes significantly differed between the city beach and the island beach. The AR of population was more severe at the city beach of DDH as compared at the island beach of WWZ. It is not clear whether the differential characteristic is popular in other island and city regions. More regional samples should be taken to explore the preliminary observation.

Among various antibiotics, sulfamethoxazole, Cefazolin and ampicillin showed the maximum drug resistance to the total isolates, and certain similarity on resistance to some antibiotics was also reported in other areas[7,19,20] (D Cortes Vélez *et al*., 2012; Liu *et al*., 2014; Liu *et al*., 2017). These observations may be due to the large-scale and long-term use of certain antibiotics [4,21,22,23] (Liu *et al*., 2018; Huang *et al*., 2019; Gustafson *et al*., 1997; Maravic *et al*., 2015). The resistance to chloramphenicol, ciprofloxacin, levofloxacin, ceftazidime, cefepime and gentamicin in the ESBL-E differed between the two bathing beaches. Since the high risk of ESBL-E, the prevention and control of these six types of antibiotics should be treated as an emergency.

CTX–M β -lactamases were involved in the primary resistance mechanism in ESBL-E among the island beach population, the variant *CTX–M–14* was found to be predominant, and this variant was similarly reported[24,25] (KW Seo, 2020; Gundran *et al*., 2019). In contrast, the detection rates of *CTX–M* were lower at city beach DDH while the rates of ESBL-E were high, indicating that other

mechanisms may have contributed to the antibiotic resistance in the tested populations. The city beach populations could face greater potential risks owing to severe resistance and metastatic ability of ESBL-E. The significant ESBL genes in Philippines were reported to be *bla* (*TEM–1*), *bla* (*SHV–11/12*), and *CTX–M–15*, which was not the same with our findings[25](Gundran *et al*., 2019). Some subtypes of *CTX–M* were reported probably from different sources of the world, and some types such as *CTX–M–15* and *CTX–M–27* had different virulence[26](Ivana, J *et al*., 2018), therefore, further researches about ARGs, virulence and source of different ESBL-E are needed. It is worth noting that the beach practitioners were possible to face higher risk of gentamicin resistance due to their more seawater exposure. Gentamicin was widely used in Livestock and poultry industry in China, it has thermal stability and good water solubility. Further studies are probably required for control of these kind of antibiotics or related ARGs in coastal environment.

The PFGE analysis of ESBL-E in the population tested in this study suggested that clonal transfer occurred at the city beach. However, whether the transmission was due to physical contact of individuals or indirect movement of water or sand was unknown. The environmental exposure of practitioners is much more than the beach travelers, thus it is necessary to investigate their environment exposure and the AR status of water and sand. Our findings are expected to provide insights into the prevention and control of AR risk for the beachgoers.

5. Conclusions

There are probably various opportunities to prevent the infection of ESBL-E in different occupational population. Further studies should be conducted to control the risk and determine the transfer role of AR on beach environment and beachgoers.

Conflicts of interest statement

All authors declare no conflict of interest

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Authors' Contributions

Liang F, Hu GY, Wu JZ was responsible for samples collection, detection of genotypes, questionnaire and writing the draft of this articles; Li TJ, Yi GH designed the methods for similarity and exploring phenotypes detection; Huang HR reviewed the development of each process; Li MX checked the results and English; Long WF wrote and revised the manuscript and controlled the content of whole review.

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