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RESEARCH ARTICLE

Molecular characteristics of carbapenem-resistant Enterobacteriaceae isolates from a Chinese Tertiary hospital in Guangdong

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ABSTRACT

Objective: The aim of this study was to investigate the molecular characteristics of carbapenem-resistant Enterobacteriaceae (CRE) isolates from a teaching hospital in Guangdong, China, during the period between January 2008 and December 2011.

Methods: A total of 28 (0.9%) CRE isolates were collected among 3286 isolates. Antimicrobial susceptibility testing, Modified Hodge Test (MHT) and genotyping by pulsed-field gel electrophoresis were performed. Extended-spectrum β-lactamase (ESBL) and carbapenemase genes were detected using gene amplification and sequencing. Outer membrane porin (OMP) genes (*Omp*K35/*Omp*K36 for *K. pneumoniae*, *Omp*C/*Omp*F for *E. cloacae* were examined.

Results: Seven (25.0%) isolates of the 28 carbapenem resistant Enterobacteriaceae had a positive MHT. PCR and sequencing analysis revealed that 19 of the 28 isolates were molecularly confirmed to harbor ESBLs (15 isolates, 53.6%), AmpC (6 isolates, 21.4%), or IMP-4 (five isolates, 17.9%), and nine (32.1%) isolates of the 28 isolates possessed at least two different classes of β-lactamases. Specially, five (71.4%) of seven MHT-positive CRE isolates is due to the production of the carbapenemase-encoding gene IMP-4. Almost all of the CRE isolates in the present study loss gene expressions of *Omp*K35 and/or *Omp*K36 in *K. pneumoniae*, *Omp*C and/or *Omp*F in *E. cloacae*, except for one *K. pneumoniae*. PFGE analysis demonstrated genomic variability.

Conclusion: Carbapenem resistance in Enterobacteriaceae is due to the combined effect of β- lactamases with porin impermeability caused by loss of OMPs observed in these organisms. To prevent both CRE transmission and infections has become significantly urgent public health objectives. *J Microbiol Infect Dis 2014; 4(4): 145-151*

Key words: Molecular characteristics, Carbapenem-resistant, Enterobacteriaceae, IMP-4

Çin Guangdong Bölgesindeki bir üçüncü basamak hastanede izole karbapenem dirençli Enterobacteriaceae kökenlerinin moleküler özellikleri

öZET

Amaç: Bu çalışmada Çin'in Guangdong bölgesinde bulunan bir eğitim hastanesinde, Ocak 2008 ile Aralık 2011 tarihleri arasında dönemde izole edilen karbapenem dirençli Enterobacteriaceae (CRE), kökenlerinin moleküler özelliklerini incelemesi amaçlanmıştır.

Yöntemler: Çalışma döneminde 3286 suştan toplam 28 (% 0,9) CRE izolatı ayrıştırıldı ve çalışmaya alındı. Antimikrobiyal duyarlılık testleri, Modifiye Hodge (MHT) testi ve genotip incelenmesinde Pulsed-field jel elektroforez kullanıldı. Genişletilmiş spektrumlu β-laktamaz (GSBL) ve karbapenemaz genlerinin varlığı, gen amplifikasyonu ve dizi analizi kullanılarak tespit edildi. Bakterilerin Dış zar porini (OMP) genleri (*Omp*K35 / *Omp*K36 *K. pneumoniae*, *E. cloacae* için OMPC / *Omp*F) incelendi.

Bulgular: MHT ile 28 kökenin yedisi (% 25,0) dirençli Enterobacteriaceae olarak tanımlandı. PCR ve dizi analizi gibi moleküler yöntemlerle kökenlerden 15'inin (% 53,6) GSBL pozitif olduğu, altısının AmpC (% 21,4), beşinin IMP-4 (% 17,9) ve dokuzunun en az iki farklı sınıf dirence sahip olduğu görülmüştür. Özel olarak, yedi MHT pozitif kökenin beşinin (% 71,4) karbapenemaz kodlayan genlerinin IMP-4 üretimine bağlı iken PFGE analizinde bir *K. pneumoniae* dışında hemen hemen tüm CRE kökenlerinin, OMPC ve / veya *omp*F içinde *Omp*K35 ve / veya *Omp*K36 kaybı gen ifade kaybı gözlendi. PFGE analizi genomik değişkenlik gösterdi.

Sonuç: Enterobacteriaceae karbapenem direnci bu organizmalarda gözlenen OMP'lerin kayb sonucu oluşan porin'e değişimi ve β-laktamazların kombine etkisi nedeniyle oluşmaktadır. CRE yayılımı ve enfeksiyonlar hem anlamlı acil halk sağlığı hedefleri haline gelmiştir önlemek için.

Anahtar kelimeler: Moleküler özellikler, karbapenem dirençli, Enterobacteriaceae, IMP-4

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INTRODUCTION

Carbapenems are usually the only practicable therapeutic options for treatment of severe community-acquired or hospital infections caused by multi-drug resistant AmpC- or extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae.[1] However, it is reported that the universal susceptibility to carbapenems in Enterobacteriaceae is no longer guaranteed. In the Meropenem Yearly Susceptibility Test Information Collection Program, researchers suggested that meropenem resistance among clinical isolates of *Klebsiella pneumoniae* increased dramatically from 0.6% in 2004 to 5.6% in 2008.[2] According to the data reported to the National Healthcare Safety Network (NHSN) in 2006-2007, carbapenem resistance was reported in up to 10.8% of *K. pneumoniae* and isolates 4.0% of *Escherichia coli* which were associated with some device-related infections.[3]

Resistance to carbapenems may be the result of a number of mechanisms, including the production of *carbapenemases* such as class A KPCs, the class B metallo-β-lactamases VIM, IMP or *NDM*-1, or the class D *OXA*-type enzymes such as *OXA*-48.[4,5] In Enterobacteriaceae, resistance to carbapenems may also be due to the production of a class A ESBL or an AmpC-type enzyme accompanied by membrane impermeability.[4,6] Membrane impermeability may be due to mutations or alterations in porin channels resulting in porin non-functionality or may be due to complete loss of the *Omp*F and/or *Omp*C porin proteins. Active drug efflux may also play a role.[6-8]

In China, the first isolates (*Klebsiella pneumoniae*, *Serratia marcescens* and *Escherichia coli*) were identified in 2008, since Gram-negative bacteria with acquired carbapenemases are occasional.[9] KPC-2 and IMP-4 were believed to be the major causes of resistance to carbapenems among Enterobacteriaceae isolates in China.[9-11] However, given the regional distributions, studies on carbapenem-resistant Enterobacteriaceae in Guangdong Province the southern part of China, are scare, thus warranting further research into the molecular patterns and resistance profiles of carbapenem-resistant Enterobacteriaceae.

The aim of this study was to investigate molecular characteristics of carbapenem resistance in clinical isolates of Enterobacteriaceae isolates collected from a tertiary teaching hospital in Guangdong, China.

METHODS

Setting

This retrospective study was organized at a 2.548-bed tertiary teaching hospital in Guangdong which covers almost all specialties in southern China.

Isolation and identification of bacterial strains

A total of 28 (0.9%) carbapenem resistant Enterobacteriaceae (CRE) isolates with ertapenem MICs ≥2 μg/ml were collected from among 3286 Enterobacteriaceae isolates during the period from January 2008 to December 2011. Among the 28 CRE strains, 11 (39.3%) were isolated from urine,[10] (35.7%) from sputum, 5 (17.9%) from abscesses, 2 (7.14%) from wound. Identification of isolates was performed using VITEK-2 system microbiology analyzer (bioMérieux, Marcy l'Etoile, France). The 28 CRE isolates included [12] (42.9%) *Enterobacter cloacae*, 7(25.0%) *K. pneumoniae*, 7(25.0%) *E. coli*, 1 (3.57%) *K. oxytoca* and 1 (3.57%) *Citrobacter freundii*.

Antimicrobial susceptibility testing

Antimicrobial susceptibilities for isolates were detected initially by Gram-negative susceptibility (GNS) cards on the Vitek system (bioMérieux). Antimicrobials evaluated included aztreonam, piperacillin, ceftazidime, cefotaxime, imipenem, meropenem, cefepime, ampicillin /clavulanic acid cefoxitin, trimethoprim/sulfamethoxazole, ciprofloxacin, amikacin.

Susceptibility testing results were interpreted under the criteria recommended by the Clinical and Laboratory Standards Institute (CLSI, 2012). The quality control strain for susceptibility testing was *E. coli* ATCC 25922.

Modified Hodge test (MHT)

The MHT was conducted as recommended by the CLSI on Mueller-Hinton II agar plates using *E. coli* ATCC 25922 as the indicator isolate and a 10 μg ertapenem disk. A positive MHT was considered to indicate potential carbapenemase production. A confirmed KPC-producing isolate was used as a positive control.

PCR and sequence analysis of resistance genes

PCR was used to amplify the ESBL-encoding genes *bla*TEM-1, bla_{CTX-M}, bla_{SHV-1} and bla_{OXA-1}, the AMP-C gene bla_{DHA-1} and MIR, the carbapenemase-

encoding genes *bla*$_{GES}$, *bla*$_{IMI}$, *bla*$_{NMC}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{SME}$, *bla*$_{KPC}$, *bla*$_{OXA-48}$ and *bla*$_{NDM-1}$.[12-15] The coding sequences of (1) *Omp*K35 (forword:5'- TCCCTGCCCTGCTGGTAG-3';reverse: 5'-CTGGTGTCGCCATTGGTGG-3') and *Omp*K36 (forword:5'- GCGACCAGACCTACATGCGT -3';reverse: 5'- AGTCGAAAGAGCCCGCGTC') from *K. pneumoniae*, (2) *Omp*C (forword:5'- GCGACCAGACCTACATGCGT -3';reverse: 5'- TTCGTTCTCACCAGAGTTACCCT -3') and *Omp*F (forword:5'- TCCCTGCCCTGCTGGTAG -3';reverse: 5'-TAAGTGTTGTCGCCATCGTTG-3') from *E. cloacae* were amplified .Sequencing of all amplicons was carried out by an ABI PRISM BigDye 3.1 Terminator Cycle Sequencing Ready Reaction kit (P/N 402078).

Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) analysis was carried out as described by Wei Dai et al.[16] with the *XbaI* restriction endonuclease (TAKARA, Shiga, Japan) and the Fingerprinting II Informatix software package system (Bio-Rad Laboratories, Hercules, CA). The similarity of the PFGE banding patterns was interpreted by the Dice coefficient, and the data acquired were performed by the unweighted pair group method with arithmetic average (UPGMA) clustering by the Pearson correlation coefficient.

RESULTS

Bacterial identification, antimicrobial susceptibility and the modified Hodge test

In the present study, 28 isolates with an ertapenem MIC ≥2 g/ml were chosen for molecular testing. Among the 28 CRE isolates, 4 *Enterobacter cloacae* isolates (4/12) (Ecl5, Ecl18, Ecl22 and Ecl28), 3 *K. pneumoniae* isolates (3/7) (K31, K24 and K26), one *Escherichia coli* (1/7) isolate (E1) and one *Klebsiella oxytoca* isolate (1/1) (Kox19) were resistant to all three carbapenems tested (Tables 1). Seven (25.0%) isolates out of the 28 carbapenem resistant Enterobacteriaceae had a positive MHT.

PCR and sequencing analysis for β-lactamase expression

PCR on the total DNA of all carbapenem-resistant isolates showed differentβ-lactamase gene combinations (Tables 2). Among the 28 strains, twelve strains were positive for TEM, six strains were positive for CTX-M, and three strains were positive for SHV. Among the CTX-M-positive strains, DNA sequencing revealed that CTX-M-14 was most common (5/6), followed by CTX-M-15(1/6). *Bla*$_{DHA-1}$ and *bla*$_{MIR}$ were positive in 4 and 2 isolates, respectively. The two strains positive for MIR were ACT-like by sequencing. All IMP-4-positive strains were MHT positive, while MHT negative strains exhibited negative results for carbapenemase-encoding genes as we had detected, which suggested that other mechanisms may play a role. In all, these results suggested 19 of the 28 isolates were molecularly confirmed to harbor ESBLs (15 isolates, 53.6%), AmpC (six isolates, 21.4%), or IMP-4 (five isolates, 17.9%), and nine (32.1%) isolates of the 28 isolates possessed at least two different classes of β-lactamases.

Associations between carbapenemase and ESBL production

Among the 28 isolates, five of them carried one carbapenemase genes IMP-4,[15] possessed ESBL genes and six harbored AmpC genes. Of the 15 ESBL positive isolates, there were three (20.0%) carrying carbapenemase gene. Meanwhile, among the five IMP-4 positive isolates, three (60.0%) were found to be ESBL positive. Notably, three of the five IMP-4 positive strains (60.0%) possessed the *bla*-TEM gene. Specially, K26 and Ecl28 harbored both *bla*TEM and *bla*SHV genes.

Expression of outer membrane porin genes

The *Omp*$_{K35}$ gene was not amplified in one of the seven carbapenem-resistant *K. pneumoniae* isolates, which was K35. As compared with *omp*$_{K35}$, the *Omp*$_{K36}$ gene was found in only one of the carbapenem-resistant *K. pneumoniae* isolates, which was K32 (Table 2). Among 12 carbapenem-resistant *E. cloacae*.isolates, four isolates were amplified *Omp*C, and none was amplified *Omp*F.

Genetic diversity

The 28 ertapenem-resistant Enterobacteriaceaes were determined by PFGE of *XbaI*-digested DNA. The results revealed that the *K. pneumoniae*, *Enterobacter cloacae*, and *E. coli* isolates did not belong to the same PFGE cluster, suggesting that they were clonally uncorrelated.

Table 1. Antimicrobial susceptibility profiles of 28 carbapenem-resistant Enterobacteriaceae isolates as determined by disk diffusion and results of MHT

Isolate number	Disk diffusion													MHT
	ETP	MEM	IPM	PRL	ATM	AMC	FEP	CAZ	CTX	FOX	CIP	SXT	AMK	
K. pneumoniae														
K31	R	R	R	R	R	R	R	R	R	R	R	R	R	-
K35	R	I	R	R	R	R	S	R	R	R	R	R	I	-
K30	R	I	R	R	R	R	S	R	R	R	R	R	R	-
K32	R	S	S	R	R	R	R	R	R	R	R	S	S	-
K24	R	R	R	R	R	R	R	R	R	R	R	R	R	+
K26	R	R	R	R	R	R	R	R	R	R	R	R	R	+
K29	R	R	I	R	R	R	R	R	R	R	R	R	I	+
Enterobacter cloacae														
ECL5	R	R	R	R	R	R	S	R	R	R	R	R	R	-
ECL8	R	R	I	R	R	R	S	R	R	R	R	R	R	-
ECL4	R	R	I	R	R	R	S	R	R	R	R	R	R	-
ECL9	R	I	S	R	R	R	R	R	R	R	R	S	R	-
ECL7	R	S	I	R	R	R	S	R	R	R	R	S	R	-
ECL17	R	S	I	R	R	R	R	R	R	R	S	R	S	-
ECL6	R	S	S	R	R	R	R	R	R	R	S	R	S	-
ECL33	R	S	S	R	R	R	R	R	R	R	I	R	S	-
ECL14	R	S	S	R	R	R	S	R	R	R	I	R	R	-
ECL18	R	R	R	R	R	R	R	R	R	R	R	R	R	+
ECL22	R	R	R	R	R	R	R	R	R	R	R	R	R	+
ECL28	R	R	R	R	R	R	R	R	R	R	R	R	R	+
Escherichia coli														
E1	R	R	R	R	R	R	I	R	R	R	R	R	I	-
E11	R	I	I	R	R	R	R	R	R	R	R	R	I	-
E15	R	I	S	R	R	R	R	R	R	R	S	S	R	-
E2	R	S	S	R	R	R	R	R	R	R	S	S	S	-
E12	R	S	S	R	R	R	R	R	R	R	R	R	S	-
E3	R	S	S	R	R	R	S	R	R	R	R	R	R	-
E21	R	S	S	R	R	R	S	R	R	R	S	R	R	-
Citrobacter freundii														
CFR20	R	S	S	R	R	R	S	R	R	R	I	R	R	-
Klebsiella oxytoca														
KOX19	R	R	R	R	R	R	R	R	R	R	R	R	R	+

ETP = ertapenem; IMP = imipenem; MER = meropenem; PRL= piperacillin; AZT = aztreonam; AMC = ampicillin/ clavulanic; FEP =cefepime; CTX = cefotaxime; CAZ = ceftazidime; FOX=cefoxitin; CIP=ciprofloxacin; SXT=trimethoprim/ sulfamethoxazole; AMK=amikacin.

R = resistant; I = intermediate; S = susceptible.

Table 2. β-lactamase gene distribution and outer membrane porin-encoding genes as determined by PCR and sequencing

Isolate number	β-lactamase gene expression						Porin encoding gene expression	
	TEM	SHV	CTX-M	MIR	DHA-1	IMP-4	*Omp*K35/ *Omp*C	*Omp*K36/ *Omp*F
				K. pneumoniae				
K31	-	-	CTX-M-14	-	-	-	+	-
K35	-	-	CTX-M-14	-	+	-	-	-
K30	+	-	-	-	-	-	+	-
K32	-	-	-	-	-	-	+	+
K24	-	-	-	-	-	-	+	-
K26	+	+	-	-	-	+	+	-
K29	-	-	-	-	-	+	+	-
				Enterobacter cloacae				
ECL5	-	-	-	-	-	-	-	-
ECL8	+	-	-	-	-	-	-	-
ECL4	-	-	-	-	-	-	-	-
ECL9	+	-	-	+	-	-	-	-
ECL7	+	+	-	-	+	-	-	-
ECL17	-	-	CTX-M-14	+	-	-	-	-
ECL6	-	-	-	-	+	-	-	-
ECL33	+	-	-	-	-	-	-	-
ECL14	-	-	-	-	-	-	+	-
ECL18	+	-	-	-	+	+	+	-
ECL22	-	-	-	-	-	+	+	-
ECL28	+	+	-	-	-	+	+	-
				Escherichia coli				
E1	-	-	-	-	-	-		
E11	-	-	-	-	-	-		
E15	+	-	CTX-M-14	-	-	-		
E2	+	-	CTX-M-14	-	-	-		
E12	-	-	CTX-M-15	-	-	-		
E3	+	-	-	-	-	-		
E21	-	-	-	-	-	-		
				Citrobacter freundii				
CFR20	+	-	-	-	-	-		
				Klebsiella oxytoca				
KOX19	-	-	-	-	-	-		

DISCUSSION

In consideration of the frequency with which Enterobacteriaceae cause infections,[17] the high mortality relevant to infections caused by CRE,[18] and the potential for widespread carbapenem resistance transmission via mobile genetic elements, CRE are typically problematic currently. In the present study, our results represented that during the period from January 2008 to December 2011 in Guangdong area, the number of detection of CRE (0.9%) was below the national average.[19] We assumed that, the small number of CRE detection was reliable, since during that period, the prevalence of CRE in our region was uncommon, before that the appearance of novel β-lactamases with direct carbapenem-hydrolyzing activity was responsible for an increased prevalence of CRE.

Out of the 28 carbapenem resistant Enterobacteriaceae, seven (25.0%) isolates had a positive MHT. To our knowledge, the main factors of carbapenems resistance are carbapenemases, typically KPC and MBLs, such as IMP and VIM.[20] The genes encoding carbapenemases are frequently taken along by plasmids which usually carry other resistance genes simultaneously, causing multidrug-resistant bacteria to a great extent. In the present study, among the three *K. pneumoniae* isolates (K24, K26 and K29) with positive results of MHT, K26 and K29 both harboured IMP-4 MBL, but no resistance-encoding genes mentioned above was observed in K24, which suggested the presence of additional resistance mechanisms. As widely documented in the literature, KPC β-lactamases are now the most prevalent carbapenemases among *K. pneumoniae* in Asia.[21] It is reported by Hu et al. that the *bla*KPC gene was found in 75.3 % isolates and the gene encoded KPC-2-type carbapenemase in all cases, in a teaching hospital in Shanghai, China.[22] However, our results showed that class B metallo-β-lactamases (MBLs) of IMP type was predominant in carbapenem resistant Enterobacteriaceae in our region. The same was observed for the three *Enterobacter cloacae* isolates Ecl 18, Ecl 22 and Ecl 28 harbouring IMP-4. In worldwide, the transmission of acquired MBLs in Enterobacteriaceae is regarded as an emerging clinical threat.[23] Currently, at least nine different types of acquired MBLs have been reported, and the most important types for epidemiological transmission and clinical relevance are the VIM-type, IMP-type, SPM-type, and NDM-type enzymes. Notably, the SPM-type is mostly confined to Brazil and to Pseudomonas aeruginosa among these types.[24] In *E. cloacae*, carbapenemase production is largely attributed to MBLs such as VIM-1and IMP-8.[25] It is believed that the pattern of the epidemiology of acquired MBLs are country specific and due to several factors, including hospital practices and local antibiotic use, which could partly responsible for these differences. Although widespread dissemination of NDM-type Enterobacteriaceae has been reported in the UK, Pakistan, India and other countries,[26,27] and in the present study, 28 isolates of CRE were screened for the *bla*NDM-1 gene; however, we found no NDM-1-producing isolate. On the other hand, among the 21(21/28) MHT-negative strains, we found that 46.4% (13/28) of isolates (K32, Ecl9, Ecl7, Ecl17, Ecl6, Ecl33, Ecl14, E15, E2, E12, E3 E21 and CFR20) were found to be susceptible to imipenem and meropenem, respectively, and all of them showed negative results of MHT, without carbapenemase-encoding genes as we had detected in the present study. Therefore, these results indicated that the gradual accumulation of multiple resistance determinants rather than carbapenemase production accounted for imipenem and meropenem resistance probably.

Many studies have shown the emergence of carbapenem resistance in Enterobacteriaceae producing ESBLs and/or AmpC-type enzymes following prolonged carbapenem treatment owing to loss of, or variations in, expressed porins.[28] These present study indicated 19 of the 28 isolates were molecularly confirmed to harbor ESBLs (15 isolates, 53.6%), AmpC (six isolates, 21.4%), or IMP-4 (5 isolates, 17.9%), and 9 (32.1%) isolates of the 28 isolates possessed at least two different classes of β-lactamases.

Among the seven *K. pneumoniae* isolates, one carried IMP-4 encoding TEM-type and SHV-type ESBLs. Out of the 11 *E. cloacae*. isolates, Ecl 18 carried IMP-4 produced both TEM-type ESBL and DHA-1-type AmpC as well. Ecl 28 habouring IMP-4 produced TEM-type and SHV-type ESBLs.

As well as the production of ESBLs and/or plasmid borne AmpC β-lactamases can result in carbapenem resistance, the absence or reduced expression of the two major porins (*Omp*K35 and *Omp*K36 in *K. pneumoniae*, *Omp*C and *Omp*F in *E. cloacae* has been involved in carbapenem resistance by previous authors, and the present data pointed to these conclusions.[6] Almost all of the CRE isolates in the present study loss gene expressions of *Omp*K35 and/or *Omp*K36 in *K. pneumoniae*, *Omp*C and/or *Omp*F in *E. cloacae*, except for one *K. pneumoniae* (K32). K32 harbouring *Omp*K35 and *Omp*K36 encoding genes, but it was not detected any β-lactamase encoding gene. The potentially probable mechanism of K32 is still to be determined pending western blotting or sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of porin proteins to determine whether translation to a protein is actually occurring.

The present PFGE results revealed that the *K. pneumoniae*, *Enterobacter cloacae*, and *E. coli* isolates did not belong to the same PFGE cluster, suggesting that they were clonally uncorrelated. It was possible because most of the patients came from other hospital carrying resistant bacteria before they were admitted to this hospital. As well, the present study has a limitation in that strain collection was performed during a wide time span, which probably resulted in no clonal relation.

In conclusion, the exceeding emergence of acquired carbapenemase resistance in Enterobacteriaceae strains in China is disturbing, because it exerts pressures on the already difficult task of treating antimicrobial-resistant infections. Therefore, it is significant to take prompt detections for the containment of carbapenemase-producing strains and for the very prevention of nosocomial transmission. However, there were some limitations in this study still, including a retrospective study, the small number of cases, and all cases from a single institution. In future investigations, more studies about alterations in membrane permeability and differences in levels of expression of harbored genes will be determined.

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