Analysis of antimicrobial resistance and class 1 integrons among strains from upper respiratory tract of healthy adults

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ABSTRACT

Objective: The distribution and characterization of integrons among opportunistic pathogens from nasopharynx of healthy adults.

Methods: A total of 1,019 nasopharyngeal samples from healthy adults were collected; bacteria were identified by API system; antibiotic susceptibility were tested by K-B method; class 1, 2 and 3 integrons were examined by degenerate primers of the genetic content of integrons were analyzed by PCR and DNA sequencing.

Results: Out of the 1,019 cases, 743 (72.9%) opportunistic pathogens were isolated. The top five commons organism identified were *Coagulase-negative staphylococcus* (n=404), *Staphylococcus aureus* (n=109), *Haemophilus influenzae* (n=74), *Streptococcus pneumoniae* (n=49) and *Klebsiella pneumoniae* (n=32). Eight (25.0%) isolates of *K. pneumoniae* produced the ESBLs. The isolated rates of *S. aureus* and *H. influenzae* were decreased with aging. 6.3% (2/32) *K. pneumoniae* isolates and 16.7% (1/8) *Proteus* isolates carried class 1 integrons. Among intI1-positive strains, sequencing analysis revealed that one of the integron positive *K. pneumoniae* isolates harbored gene cassette *aadA5-dfrA17*. *Proteus* isolates harbored gene cassette *aadA2-dhfrXII*.

Conclusions: The results stressed the need for continued surveillance of bacteria from the asymptomatic carriers.

KEY WORDS Antimicrobial resistance; upper respiratory tract; class 1 integron; gene cassettes; healthy adults

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Introduction

Upper respiratory tract infections (URTIs) and community-acquired pneumoniae (CAP) are highly prevalent diseases of respiratory system associated with significant morbidity and socioeconomic cost. Because the nasopharynx lies between the nose, sinuses, ears, larynx, and the lower respiratory tract, resident pathogens of the nasopharynx can be the source for both upper and lower respiratory tract infections (1,2). The nasopharynx is also a major source of secretions containing bacteria that can easily spread between individuals and these may subsequently become pathogenic in the new host. In this study, the issue of nasopharyngeal carriage is investigated, because it plays an important role in both the development of disease and the spread of pathogens. In most cases these nasopharyngeal flora are carried without causing clinical symptoms. However, when homeostatic conditions of the host are altered, microorganisms may invade adjacent sites causing disease. Considering studies isolates to antimicrobial susceptibility in China have largely focused on isolates recovered from clinical specimens in the context of clinical disease, in this study, the antimicrobial susceptibility of the major opportunistic pathogens from healthy adults was also performed.

Integrons are ancient structure that contains determinants of a site-specific recombination system to capture genes encoding antimicrobial resistance (3). They can locate within transposons

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or conjugative plasmids and contribute to the traffic leading to the acquisition of new genes in bacteria (4-8). The class 1 integron is prevalent on plasmids. It has been found in both gram-positive and especially in gram-negative bacteria (8-11). The study of integrons most focus on clinical strains, few on nasopharyngeal flora from healthy adult. In this study, we investigated the distribution and characterization of the integrons among opportunistic pathogens from healthy adults.

Materials and methods

Subjects

A total of 1,019 nasopharyngeal samples from healthy adults were collected during the period July 2009-July 2010. 1,019 volunteers (n=1,019; 661 male; 358 female; mean 50±17 years), randomly selected from registry of general practitioners in Nanjing without symptoms or signs of clinical illness were enrolled in the study over a six-month time period and all of them gave their consent for the participation in this study. The healthy volunteers included had not been following any history of respiratory tract infection, chronic basic lung disease, had not received antibiotic treatment for at least six months prior to inclusion and did not have any relation with the hospital environment.

Nasopharyngeal samples

Samples were collected from both anterior nasopharynxes rotating a sterile polyester fiber-tipped swab moistened with sterile saline. Swabs were placed in 3 mL of Luria-Bertani broth and transported to the Department of Clinical Microbiology of the first Affiliated Hospital of Nanjing Medical University. All nasopharyngeal samples were inoculated on blood agar plates and chocolate agar plates (bioMerieux, France) and identified by the analytical profile index procedure (API-20NE system; bioMerieux, France).The reference strains used in this study were as follows: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853.

Antimicrobial sensitivity testing

Antimicrobial sensitivity testing was performed by the disc diffusion (Kirby-Bauer) method for Methicillin-resistant Coagulase Negative *Staphylococci* (MRCoNS), Methicillin-resistant *Staphylococcus aureus* (MRSA), *K. pneumoniae*, *Haemophilus* according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2010). The isolates were interpreted as susceptible or resistant according to the inhibition zone diameter using CLSI recommendations. Discs were obtained from Oxoid. The antimicrobials used for the susceptibility testing were as follows (μg per disc): fusidine (5 μg), penicillin (10 μg), oxacillin (1 μg), cefoxitin (30 μg), gentamicin (10 μg), rifampin (5 μg) ciprofloxacin (5 μg), levofloxacin (5 μg), moxifloxacin (5 μg), trimethoprim-sulfamethoxazole (1.25/23.75 μg), azithromycin (15 μg), erythromycin (15 μg), clindamycin (2 μg), chloramphenicol (30 μg), quinupristin/dalfopristin (15 μg), tetracycline (30 μg), amoxicillin/clavulanic acid (30 μg), cefoperazone/sulbacta (30 μg), piperacillin/tazobactam (100/10 μg), cefazolin (30 μg), cefuroxime (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), cefepime (30 μg), aztreonam (30 μg), imipenem (10 μg), meropenem (10 μg), amikacin (30 μg), ampicillin (10 μg), ampicillin/sulbacta (10/10 μg), Antimicrobial susceptibility test of MRCoNS, MRSA and *Streptococcus pneumoniae* was performed using the E-test (AB Biodisk, Solna, Sweden) on Mueller-Hinton agar (Oxoid, UK). The values of the minimal inhibitory concentration (MIC) of MRSNS/MRSA isolates to vancomycin and the values of the MIC of *Streptococcus pneumoniae* isolates to erythromycin, clindamycin, penicillin, vancomycin, levofloxacin, ampicillin, and cefotaxime were determined according to the manufacturer's instructions and to CLSI guidelines.

Detection of integrons

To study the distribution and characterization of the integron among opportunistic pathogens from healthy adults, all isolates were screened for class 1, 2 and 3 integrons by PCR using degenerate primers hep35 (5′ TGCGGGTYAARGATBTKGATTT 3′) and hep36 (5′ CARCACATGCGTRTARAT 3′) and *Hinf* I restriction analysis of the integrase gene product (12). Cassette regions of class 1integron were amplified using primers 5′CS and 3′CS as described previously (13). Cassette PCR products were sequenced. The resulting DNA sequences were analyzed by the BLAST program, available at the NCBI homepage (http://www.ncbi.nlm.nih.gov/BLAST/).

Statistical analysis

We analyze the difference of isolated rates between different age groups. The statistical analyses were performed using SPSS 17.0 software programs, employing the chi-aquare test. P values less than 0.05 were considered statistically significant.

Results

Bacteria group distribution

Out of the 1,019 samples, 743 (72.9%) opportunistic pathogens were isolated. The top five strains were as follows: *Coagulase-negative staphylococcus* (CoNS, n=404), *S. aureus* (n=109), *H. influenzae* (n=74), *S. pneumonia* (n=49) and *K. pneumonia* (n=32), other opportunistic pathogens such as *Escherichia coli*, *Enteroaerogen* were also detected (Table 1, Figure 1).

Table 1. Numbers of detection strains, detection ratio,composition ratio from upper respiratory tract of health adults.

Strains	No. of detection strains	Detection ratio (%)	Composition ratio (%)
MSCoNS	404	39.6	54.3
MRSA	109	10.7	14.7
H. influenzae	74	7.3	10.0
Streptococcus pneumonia	49	4.8	6.6
Klebsiella pneumonia	32	3.1	4.3
Enteroaerogen	22	2.2	3.0
Escherichia coli	8	0.8	1.1
C.freumdii	8	0.8	1.1
Enterobactercloscae	7	0.7	0.9
Proteus	6	0.6	0.8
Acid yield clay "coli"	5	0.5	0.7
Acinetobacter	5	0.5	0.7
Stenotrophomonas-maltophilia	4	0.4	0.5
C.diversus	4	0.4	0.5
Pseudomonas aeruginosa coli	3	0.3	0.4
Serratialiquefaciens	2	0.2	0.3
Fungi	1	0.1	0.1
Total	743	72.9	100

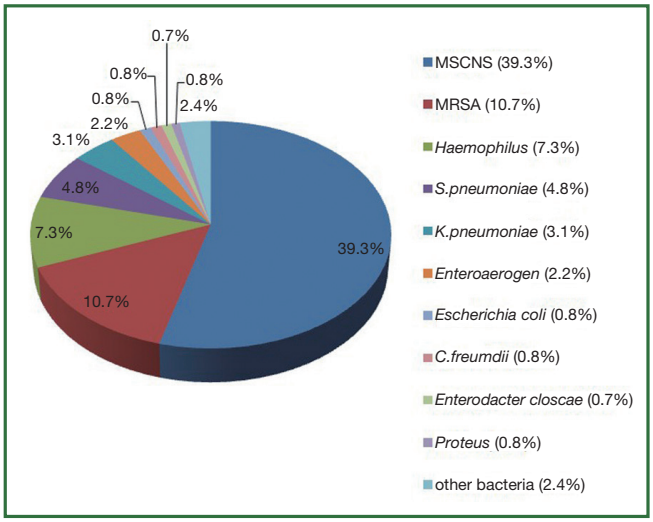

Figure 1. Distribution of isolates from upper respiratory tract.

Comparing different age groups, we found that the isolated rates of *S. aureus* and *H. influenzae* were decreased with aging (Table 2). The isolated rates of *S. aureus* and *H. influenzae* age group of 41-60 (*Staphylococcus aureus*, 10.0%; *H. influenzae* 5.9%) and age group of 61-90 (*S. aureus*, 7.3%; *H. influenzae* 4.2%) compared to age group of 19-40 (*S. aureus*, 14.8%; *H. influenza* 11.0%) were different (P<0.05).

Antimicrobial susceptibility profiles

Antimicrobial susceptibility of MRCoNS and MRSA isolates

A total of 162 MRCoNS isolates and 19 (MRSA) isolates of healthy adults origin obtained during the period 2009-2010 in Nanjing were tested for antibiotic susceptibility (Table 3). Of these, 100.0% was found to be fusidine and vancomycin susceptive, 96.9% was found to be rifampin susceptive. The susceptive rate for MRCoNS and MRSA of other antibiotic was lower. Of these, 100.0% was found to be penicillin resistant. Overall, the susceptive rate of MRSA was lower than the susceptive rate of MRCoNS.

Antimicrobial susceptibility of S. pneumoniae isolates

A total of 49 *S. pneumoniae* isolates were tested for antibiotic susceptibility by testing the values of MIC. Most of these isolates were high resistant rate to erythromycin (71.2%), clindamycin (76.9%), and penicillin (74.4%). None isolate was found to be vancomycin and amoxicillin resistant (Table 4).

Antimicrobial susceptibility of K. pneumoniae isolates

Out of 32 *K. pneumoniae* isolates, 8 (25.0%) isolates were ESBLs-producing *K. pneumoniae* isolates. Overall, antimicrobial resistant rate of *K. pneumoniae* isolates was at a low level (Table 5).

Antimicrobial susceptibility of H. influenzae isolates

A total of 74 of *H. influenzae* isolates were tested for antibiotic susceptibility (Table 6). None isolates was found to be ampicillin/sulbacta, piperacillin/tazobactam, cefazolin, cefuroxime resistant, 44.4% of isolates were trimethoprim-sulfamethoxazole resistant. Table 6 shows antimicrobial susceptibility of *H. influenzae* isolates.

Prevalence of class 1 integrons

We found a low rate of class 1 integrons (0.4%) among 743 opportunistic pathogens of healthy adults origin obtained during the period 2009-2010 in Nanjing. Out of the 32 *K. pneumoniae* isolates and 6 *Proteus* isolates, 2 (6.3%) *K. pneumoniae* isolates and 1 (16.7%) *Proteus* isolates carried class 1 integron genes (Figure 2A). One of the gene cassette positive *K. pneumoniae* is an ESBL-producer. Class 1 integrons were not detected in any gram-positive strains and any other gram-negative strains. No class 2 and 3 integrons were detected.

DNA sequence analysis

Among intI1-positivestrains, an amplicon of 1,584 bp was yieded

Table 2. The isolated rates of S.aureus, S.pneumoniae, Haemophilus, and Klebsiella pneumoniae in different age groups.

Age groups	No. of strains	S. aureus % [no.]	S. pneumonia % [no.]	H. influenzae % [no.]	K. pneumonia % [no.]
19-40	365	14.8 [54]	4.1 [15]	11.0 [40]	3.0 [11]
41-60	370	10.0* [37]	3.6 [13]	5.9* [22]	3.2 [12]
60-90	284	7.3* [18]	7.4 [21]	4.2* [12]	3.2 [9]

*Comparing to age group of 19-40, P<0.05.

Table 3. Antimicrobial susceptibility profiles of MRCoNS and MRSA nasopharyngeal isolates[a].

Antibiotic	MRCoNS (n=162)			MRSA (n=19)		
	R%	I%	S%	R%	I%	S%
Penicillin	100.0	0.0	0.0	100.0	0.0	0.0
Oxacillin	98.6	0.0	1.4	92.9	0.0	7.1
Rifampin	2.8	0.7	96.6	0.0	0.0	100.0
Ciprofloxaci	66.2	11.0	22.8	68.7	27.0	28.6
Levofloxacin	67.3	10.8	21.9	67.9	5.1	27.0
Moxifloxacin	16.6	13.1	70.3	42.9	7.1	50.0
TMP-sulfamethoxazole	49	4.1	46.9	57.1	0.0	42.9
Clindamycin	70.3	8.3	21.4	87.0	1.3	11.7
Azithromycin	81.2	0.7	18.1	85.7	0.0	14.3
Erythromycin	78.6	5.5	15.9	78.6	21.4	0.0
Gentamycin	86.2	4.8	9.0	78.6	7.1	14.3
Chloramphenicol	25.5	2.1	96.6	0.0	0.0	78.6
Quinupristin/dalfopristin	1.4	2.1	98.6	0.0	0.0	100.0
Tetracycline	0.0	1.4	98.6	0.0	0.0	100.0
Fusidine	0.0	0.0	100.0	0.0	0.0	100.0
Vancomycin	0.0	0.0	100.0	0.0	0.0	100.0

[a], R%, resistant rate; I%, intermediate rate; S%, susceptible rate.

Table 4. Antimicrobial susceptibility profiles of *Streptococcus pneumoniae* nasopharyngeal isolates.

Antibiotic	R%	I%	S%
Clindamycin	76.9	0.0	23.1
Erythromycin	71.2	0.0	28.8
Penicillin	74.4	16.3	9.3
Amoxicillin	0.0	0.0	100.0
Cefotaxime	5.9	1.1	93.0
Levofloxacin	5.4	11.6	83.0
Vancomycin	0.0	0.0	100.0

Table 5. Antimicrobial susceptibility profiles of *Klebsiella pneumoniae* nasopharyngeal isolates.

Antibiotic	R%	I%	S%
Amoxicillin/clavulanic acid	39.2	9.6	51.2
Piperacillin/ tazobactam	5.9	0.0	94.1
Cefoperazone/sulbacta	14.3	14.7	71.0
Cefazolin	17.6	0.0	82.4
Cefuroxime	17.6	0.0	82.4
Ceftazidime	17.6	0.0	82.4
Cefotaxime	17.6	11.8	70.6
Cefepime	17.6	0.0	82.4
Aztreonam	19.4	11.8	58.8
Imipenem	5.9	5.9	88.2
Meropenem	17.6	0.0	82.4
Amikacin	17.6	5.9	76.5
Levofloxacin	44.1	5.6	50.3

in *K. pneumoniae* isolates, an amplicon of 1,974 bp was yieded in *Proteus* isolates (Figure 2B). Sequencing analysis revealed that amplicon 1,584 bp (Nanjing v3) harbored gene cassette *aadA5* and *dfrA17* (Figure 3A), amplicon 1,974 bp (Nanjing v1) harbored gene cassette *aadA2* and *dhfrXII* (Figure 3B), There

Table 6. Antimicrobial susceptibility profiles of *H. influenzae* nasopharyngeal isolates.

Antibiotic	R%	I%	S%
Ampicillin	5.6	1.6	92.8
Ampicillin/sulbacta	0.0	0.0	100.0
Piperacillin/tazobactam	0.0	0.0	100.0
Cefazolin	0.0	0.0	100.0
Cefuroxime	0.0	0.0	100.0
Cefotaxime	7.3	0.0	92.7
Levofloxacin	0.0	2.2	97.8
TMP-sulfamethoxazole	44.4	22.2	33.3

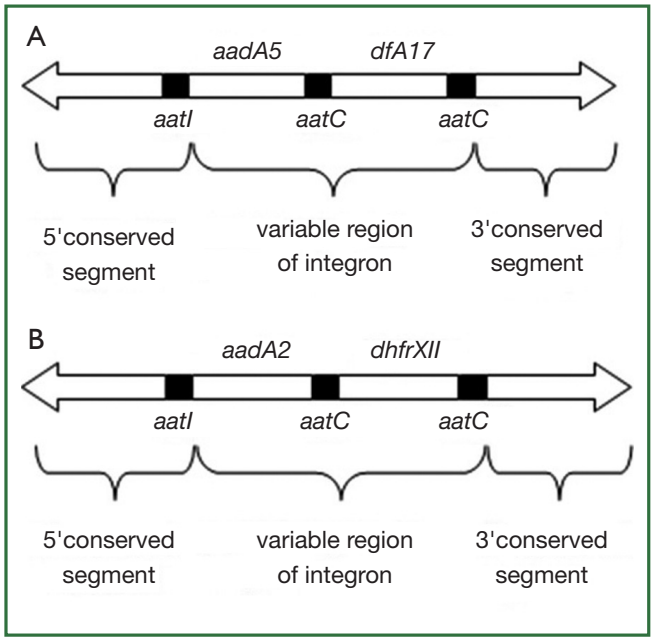

Figure 3. The cassette array of integron in *K. pneumonia* and *Proteus* from healthy adults. A. The cassette array of integron in K. pneumoniae from healthy adult. It contained four resistance genes,*aadA5* and *dfrA17*; B. The cassette array of integron in *Proteus* from healthy adult. It contained four resistance genes, *aadA2* and *dhfrXII*.

was no class 1 integron found in gram-positive strains and any other gram-negative strains (Table 1).

Discussion

Bacterial resistance to antibiotics represents one of the most significant global health challenges of this century. Infections with multiple antibiotic-resistant bacteria have been increasing at an alarming rate. Many infections caused by isolates occur in persons with prior nasopharynx carriage such as *S. aureus* infections (14), and this carriage is an important risk factor for healthcare-associated infections. Antibiotic and vaccines modify

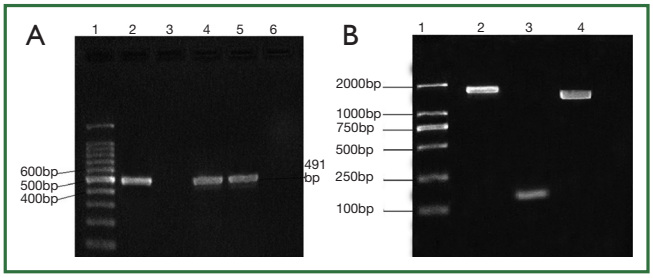

Figure 2. PCR products of integrase gene and variable regions of integrons in isolates from healthy adults. A. PCR products of integrase gene: 1. 100 bp marker; 2. stains of the positive control; 3-5. PCR positive products of integrase gene; 6.strains of the negative control; B. PCR products of variable regions of integrons in isolates from healthy adults. 1. DL 2,000 bp marker; 2. Nanjing v1 (1,974 bp); 3. Nanjing v2 (150 bp); 4.Nanjing v3 (1,584 bp).

the flora by removing organisms that are part of the commensal flora. Therefore, we investigated the nasopharynx carriage status of opportunistic pathogens and test the antimicrobial resistance of major opportunistic pathogens from healthy adults for guiding of empirical therapy and for focusing interventional control of antimicrobial resistance in different geographic areas.

In this study, the major isolates was Gram-positive bacteria, the five most common organism identified isolates were CoNS, Staphylococcus aureus, *H. influenzae*, *S. pneumoniae* and *K. pneumoniae*. However, it was different to the distribution of clinical flora. In clinical isolates (15), Gram-negative bacteria accounted for 70.0%, the major isolates were *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus* and *A. baumannii*. It presents those antibiotics empirical treatment should be according to different sources of patients. This study also showed that nasal carriage rate of *H. influenza* and *S. aureus* varied with age (Table 2). *H. influenzae* typically asymptomatically colonize the nasopharynx of young children. To our knowledge, nasal carriage rate of *H. influenzae* and *S. aureus* varied with age may be relevant to immunity.

ESBLs have been detected in a wide variety of Gram-negative bacteria. *K. pneumoniae* is still an important ESBLs producer, not only in the nosocomial setting but also in the community (16). In this study, 8 (25.0%) ESBLs-producing *K. pneumoniae* were found from 32 *K. pneumoniae* isolated from the nasal carriage in healthy adults in Nanjing. We should be alert to the result that ESBLs-producing *K. pneumoniae* were found from healthy adults although the detection rate was at a low level.

In recent studies, 59.4% CoNS is more prevalent than *S. aureus* in clinical gram-positive Staphylococcus isolates in China (17-22). This study revealed that 69.0% of nasopharynx carriage status of opportunistic pathogens isolates was *Staphylococcus* isolates, including 54.3% of CoNS and 14.7% of *S. aureus*. Our surveillance showed that resistant rate of MRSA

was higher than resistant rate of MRCoNS.

Nasopharyngeal carriage of *S. pneumoniae* may occur in up to 60.0% of healthy preschool children and up to 30.0% of older children and adults (23). In this study, it was up to 49 (4.8%) of *S. pneumoniae* isolates from healthy adults nasopharyngeal samples. Because the distribution of *S. pneumoniae* varies with age, geographic region and time (24), it was different nasopharynx carriage status of *Streptococcus pneumoniae*. Beta-lactams antibiotics and macrolides antibiotics were used for empirical therapy of CAP because the main strain of CAP was *S. pneumoniae*. However, resistant to penicillin and other antibiotics has increased dramatically worldwide over the past decades due to inappropriate antibiotic usage (25). Antibiotic resistance was commonly observed with beta-lactams and macrolides in Asia. The Asian Network for Surveillance of Resistant Pathogens (ANSORP) documented very high prevalence rates (>60.0%) of *Streptococcus pneumoniae* in Taiwan, Korea, Japan and Vietnam during 1996-1997 (26). In this study a trend towards increasing resistance to penicillin (74.4%), erythromycin (71.2%) and clidamycin (76.9%) was also seen.

H. influenzae is the second main pathogens in CAP. Resistance to ampicillin was first reported in the 1970s, and during the subsequent decades it has steadily increased. The resistance to ampicillin and other beta-lactam antibiotics is usually due to the production of a plasmid-encoded beta-lactamase, TEM-1 or ROB-1 (27). In Romania, 26.0% of *H. influenzae* stains isolated from patients with community acquired respiratory tract infections were resistant to amoxicillin (28). In France, 17.0% and 0.5% of *H. influenzae* stains isolated from the nasal carriage in children 3 months to 3 years of age were resistant to amoxicillin/clavulanic acid and cefotaxime (29). In this study, 5.6% and 7.3% *H. influenzae* stains isolated from the nasal carriage in healthy adults were resistant toampicillin and cefotaxime. In total, the resistant rate of *H. influenzae* was at a low level.

Of 32 *K. pneumoniae* isolate, 44.1% and 39.2% were resistant to Levofloxacin and amoxicillin/clavulanic acid, but 94.1% and 88.2% were susceptive to piperacillin/tazobactam and imipenem. It showed that we can choose piperacillin/tazobactam and imipenem as the first choice for empirical treating community-acquired *K. pneumoniae* infection.

Integrons are natural highly efficient recombination and expression systems able to capture genes as part of genetic elements known as gene cassettes (30). In recent studies, class 1 integrons were investigated from animals, water, human stools (31-34), however, to the best of our knowledge, this is the first report focusing on prevalence of class 1 integrons of strains from upper respiratory of healthy adults. In this study we investigated the occurrence, distribution, and cassette content of class 1 integrons among all 743 opportunistic pathogens of healthy adults origin obtained during the period 2009-2010 in Nanjing, China. No integron was found in gram-positive bacterium. Of Gram-negative bacterium, out of the 32 *K. pneumoniae* isolates and 6 *Proteus* isolates, 2 (6.3%) *K. pneumoniae* isolates and 1 (16.7%) *Proteus* isolates carried class 1 integron genes. In clinical Gram-negative isolates, Martinez's study showed that integrons positive rate was 43.0% (5). Resistant gene cassette *aadA5-dfrA17* (1,584 bp) was found in class 1 integron positive *K. pneumoniae* isolate, and *aadA5-dfrA17* encoded aminoglycoside resistance gene and trimethoprim resistance gene. Resistant gene cassette *aadA2-dhfrXII* in class 1 integron positive *Proteus* isolate and *aadA2-dhfrXII* encoded aminoglycoside resistance gene and sulfonamides resistance gene. Although it was lower class 1 integron positive rate of strains from healthy adults than isolates associated with the clinical setting, class 1 integron harboring multidrug-resistance genes has been identified among strains isolated from upper respiratory of healthy adults. The results stressed the need for continued surveillance of bacteria from the asymptomatic carriers.

In conclusion, with a new generation of antimicrobial appearance and extensive use, respiratory infections become one of the most common infections. Abuse of antimicrobial agents causes respiratory tract infection pathogens change and increase. Therefore, it was essential to monitor resistance of common pathogen from community-acquired infections and dynamically analyze pathogen distribution and resistance of the respiratory infection. Moreover, in this study we found class I integron harboring multidrug-resistance genes among strains isolated from upper respiratory of healthy adults. The results stressed the need for continued surveillance of bacteria from the asymptomatic carriers.

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