Nontypeable *Haemophilus influenzae* isolates from the lower respiratory tract in Western Sichuan, China: MIC value of ofloxacin and Mutation characteristics of Quinolones Resistance determining region gene

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Abstract

Background: Fluoroquinolones are one of the most widely used antibiotics in the treatment of respiratory tract infections, and the mechanism of resistance to fluoroquinolones is considered to be related to the amino acid substitution of gyrA, gyrB, parC, and parE, the Quinolone Resistance-Determining Regions (QRDRs) of DNA cyclase type II topoisomerase and IV topoisomerase. The purpose of this study was to explore the molecular mechanism of quinolone resistance of Nontypeable *Haemophilus influenzae* (NTHi) isolates and analyze the mutation law of the QRDRs gene.

Results: MIC value of ofloxacin of 280 NTHi isolates from lower respiratory tract secretions in children group during 2003~2004 and in whole age group during 2013~2014 in Western Sichuan, China were monitored and the amino acid sequences of gyrA, gyrB, parC and parE gene in QRDRs were detected. The resistance rate of ofloxacin in adult group was 1.92% (n=52), while the NTHi strains with ofloxacin MIC value ≥ 0.5 showed an upward trend in all age groups. No ofloxacin-resistant strains were found in 57 NTHi strains isolated from the children patient. Four amino acid substitutions were found in GyrA genes, four in GyrB, three in parC and nine in parE genes. The results showed that different amino acid replacement patterns of the gyrA, gyrB, parC and parE gene had different effects on ofloxacin MIC values.

Conclusions: Ser-84-leu and asp-88-tyr/asn mutation of the gyrA, ser-84-lys/ile and ser-133-ala mutations of the parC and ala-400-val variation of the gyrB were the main factors leading to the increase of MIC value of ofloxacin of NTHi strains in Western Sichuan, China. It can be predicted that with the increase of quinolones exposure, the susceptibility of isolates from children to quinolones will be further reduced.

Background

Quinolones are commonly used antibiotics in adult patients with respiratory tract infection. Since the first discovery of quinolone-resistant strains in 1993[1], the quinolone resistant strains of *Haemophilus influenzae* (*H. influenzae*) have gradually increased in many countries and regions. The molecular mechanism of drug resistance has also been elucidated. Although quinolones are banned in pediatrics in China, our early studies found that *H. influenzae*, which is resistant to ciprofloxacin, were isolated from the respiratory tract of children and newborns[2]. In order to explore the molecular mechanism of fluoroquinolones resistance and the genetic regularity of drug resistance genes, we monitored the susceptibility to ofloxacin of NTHi isolates from lower respiratory tract secretions from children group during 2003 ~ 2004 and from whole age group during 2013 ~ 2014 in Western Sichuan, China; then we analyzed the nucleotide sequence of the QRDRs of gyrA, gyrB, parC and parE and the relationship between QRDRs mutation and ofloxacin MIC value. We also analyzed the trend of amino acid substitution of QRDRs gene of the above genes in this region during the past ten years. Part of the epidemiological study of this work was presented at the 20th Annual Congress of Chinese Pediatric Society in Xiamen China, 23 to 26 September 2015 and won the National Excellent Paper Award awarded by Chinese Pediatric Society.

Results

Strains serotype

All the 280 strains of *H. influenzae* showed p6 gene and fuck gene positive, while cap gene was negative. All strains were *H. influenzae* without capsule, NTHi isolates.

Susceptibility of ofloxacin

The results of antimicrobial susceptibility test by broth method showed that ofloxacin MIC of strain (n = 57) during 2003 ~ 2004 was 0.0156 ~ 1, and no ofloxacin resistant strain was found according to the breakpoints of Institute of Clinical Laboratory Standards (CLSI) 2016 guidelines; The frequency of ofloxacin resistant strains was 1.92% (1/52) and the ofloxacin MIC value was 0.0156 ~ 16 in adult group (n = 52) during 2013 ~ 2014; the ofloxacin MIC value was 0.0078 ~ 2 in 0 ~ 3yrs group (n = 142) and 0.0156 ~ 0.5 in 4 ~ 6yrs group during 2013 ~ 2014. Although the results of Rank sum test showed that the MIC value of ofloxacin during 2013 ~ 2014 was not statistically significant compared with that of 2003 ~ 2004 ($p = 0.139$), the strain of ofloxacin MIC ≥ 0.5 still showed an increasing trend in all age groups, as shown in S2 Table and in Table 2.

Table 1
Primer sequences of PCR amplification of the gyrA, gyrB, parC and parE gene

Primers of Gene	Sequence(5'-3')	Product length (bp)	Procedure
gyrA-F	CCGCCGCGTACTGTTCT	375	(94 °C 5 min)*1 cycle; (94 °C 30 s, 58 °C 30 s, 72 °C 30 s)*36 cycles; (72 °C 7 min)*1 cycle; 4 °C∞.
gyrA-R	CCATTTGCTAAAAGTGC		
gyrB-F	GGAAAATCCTGCAGATGC	445	
gyrB-R	AAGCAACGTACGGATGTG		
parC-F	TGGTTTAAAACCCGTTCA	370	
parC-R	AGCAGGTAAATATTGTGG		
parE-F	GAACGCTTATCATCACGCCA	471	
parE-R	AGCATCCGCGAGAATACAGA		

Table 2
Distribution rate of ofloxacin MIC $\geq$ 0.5 strains in different Age groups (%)

Group	MIC value range	MIC = 0.5	MIC = 1	MIC = 2	MIC = 16
2003 ~ 2004					
0 ~ 3yrs group (n = 57)	0.0156 ~ 1	0	1.75%(1/57)	0	0
2013 ~ 2014					
0 ~ 3yrs group(n = 142)	0.0078 ~ 2	4.93% (7/142)	2.82% (4/142)	1.41% (2/142)	0
4 ~ 6yrs group(n = 29)	0.0156 ~ 0.5	8.31%(2/29)	0	0	0
$\geq$ 18yrs group(n = 52)	0.0156 ~ 16	9.62%(5/52)	3.85%(2/52)	1.92%(1/52)	1.92% (1/52)

Mutation of QRDRs gene and its effect on MIC value of ofloxacin

The amino acid substitutions in the gyrA sequence are ser-84-leu, asp-88-tyr/asn, ala-134-val and glu-142-lys. The amino acid substitution patterns of gyrB sequence are gly-399-glu, ala-400-val, glu-469-asp and thr-472-ile. ser-84-lys/ile and ser-133-ala as well as asn-138-ser mutations appear in the parC gene sequence. The amino acid substitution patterns in the parE sequence are as follows: asp-364-tyr, ala-369-thr, lys-378-cys, ala-383-thr, cys-405-ser, asp-420-asn, ala-426-val, val-466-met and ser-474-asn .

According to the mutation of the above allele, all the isolates were divided into a mutation group and a non mutation group, and two independent sample rank sum tests (Mann-Whitney U test) were carried out. The effect of amino acid substitution in QRDRs gene sequence on the MIC value of ofloxacin, the results of Mann-Whitney U test, is shown in Table 3. The results showed that ofloxacin MIC values of the strains with ser-84-leu, asp-88-tyr/asn and ala-134-val in gyrA sequence and with ala-400-val in gyrB sequence and with ser-84-lys/ile in parC sequence were significantly higher than those of no mutation group ($p \leq 0.05$); The changes of ofloxacin MIC values in the strains with glu-142-lys in gyrA sequence and with ser-133-ala and asn-138-ser in parC sequence were at the critical value compared with non-mutation group, $p = 0.053, 0.054$ and 0.07 ; However, there was no significant difference in the MIC value of ofloxacin between other amino acid substitution group and non-replacement group ($p > 0.05$).

Table 3

The effect of amino acid substitution in QRDRs gene sequence on the MIC value of ofloxacin(Mann-Whitney U test, n = 280)

Variation Site	mutation strains	U	<i>p</i>
	n		
gyrA			
S84L	49	590.5	< 0.001
D88Y/N	10	709	0.007
A134V	5	314.5	0.029
E142K	39	3823	0.053
gyrB			
G399E	1	137.5	0.986
A400V	86	7179	0.05
E469D	5	642	0.79
T472I	1	73	0.529
parC			
S84R/I	19	404.5	< 0.001
S133A	32	3126	0.054
N138S	41	4006	0.07
parE			
D364Y	1	138	0.989
A369T	2	83	0.072
R378C	1	138	0.989
A383T	1	138	0.989
G405S	1	138	0.989
D420N	3	273	0.979
A426V	1	8	0.064
V466M	11	1361	0.621
S474N	7	741	0.28

Variation Site	mutation strains	U	<i>p</i>
	n		
notice: Amino acid abbreviation A-Ala, G-Gly, V-Val, L-Leu, I-Ile, M-Met, S-Ser, T-Thr, N-Asn, Q-Gln, D-Asp, E-Glu, K-Lys, R-Lys, C-Cys, Y-Tyr. <i>p</i> < 0.05 is statistically significant.			

The results of Mann-Whitney U test showed that $p < 0.05$ and p values were critical level (0.053 ~ 0.07) and 8 QRDRs mutation sites which met the statistical condition were used for logistic regression analysis of ordered multiple classifications, as shown in Table 4. With allelic variation (amino acid substitution) as independent variable and ofloxacin MIC value as a dependent variable, logistic regression analysis of ordered multiple classifications was carried out. The results showed that the amino acid substitution of ser-84-leu and asp-88-tyr/asn in gyrA gene was the main factor affecting the MIC value of ofloxacin ($p < 0.001$) and the effect of variation on the MIC value of ofloxacin was 139.824 times and 28.950 times of that of no variation, respectively; Amino acid substitution of gyrB gene for ala-400-val is the main factor affecting the MIC value of ofloxacin ($p = 0.006$) and the effect of variation on the MIC value of ofloxacin was 0.517 times of that of unmutated strains. The amino acid substitution of parC gene for ser-84-lys/ile and ser-133- ala were the main factors affecting the MIC value of ofloxacin ($p < 0.001$ and $p = 0.042$), and the effect of variation on MIC value of ofloxacin was 102.789 times and 1.872 times of that of no variation, respectively. The effect of ala-134-val and glu-142-lys in gyrA sequence, asn-138-ser mutation in parC sequence on the MIC value was not statistically significant, $p = 0.860$, 0.783 and 0.200, respectively.

Table 4

Effect of gene mutations of QRDRs on the variation of ofloxacin MIC value of NTHi isolates (by logistic regression analysis results)

Variation Site	Estimate	OR	Std. Error	Wald	<i>P</i> value	95% Confidence Interval	
						Lower Bound	Upper Bound
gyrA							
S84L	4.940	139.824	0.469	110.809	< 0.001	55.730	350.811
D88Y/D88N	3.366	28.950	0.629	28.597	< 0.001	8.432	99.395
A134V	0.135	1.144	0.762	0.031	0.860	0.257	5.096
E142K	-0.092	0.912	0.334	0.076	0.783	0.474	1.755
gyrB							
A400V	-0.660	0.517	0.242	7.418	0.006	0.322	0.831
parC							
S84R/I	4.633	102.789	0.598	60.063	< 0.001	31.851	331.713
N138S	0.627	1.872	0.308	4.133	0.042	1.023	3.426
S133A	-0.449	0.638	0.351	1.642	0.200	0.321	1.269
Notice: Amino acid abbreviation A-Ala, G-Gly, V-Val, L-Leu, I-Ile, M-Met, S-Ser, T-Thr, N-Asn, Q-Gln, D-Asp, E-Glu, K-Lys, R-Lys, C-Cys, Y-Tyr. <i>p</i> < 0.05 is statistically significant.							

Distribution trend of QRDRs gene mutation

The results of χ^2 test or Fisher exact probability analysis of the variation rate of QRDRs gene in past ten years are shown in S3 table. The trends of these eight QRDRs allele mutation over the past decade are shown in Fig. 1. Compared with 2003 ~ 2004, 17 allele mutation, which was found in the QRDRs gene sequence in this region during 2013 ~ 2014 (except lys-378-cys, val-466-met and ser-474-asn in parE gene sequence), increased by varying degrees. Compared with 2003 ~ 2004, the increase of mutation rate of ser-84-leu in gyrA gene sequence and ala-400-val in gyrB gene sequence during 2013 ~ 2014 was statistically significant, $\chi^2 = 9.904, 19.944$; $p = 0.002, p < 0.001$.

The distribution of the strains with 8 allelic mutations in the three age groups is shown in S4 table (results of χ^2 test or Fisher exact probability method). The distribution trend of the above QRDRs allele mutations in the three age groups is shown in Fig. 2. The age group distribution of ser-84-leu and asp-88-

tyr/asn as well as ala-134-val mutation in gyrA sequence was significantly different ($p = 0.013$, 0.034 and 0.010 , respectively), but the other five loci had no statistical significance, $p > 0.05$.

Discussion

Through an all age group epidemiological survey in 2013 ~ 2014, we found that *H. influenzae* isolated from sputum from patients with lower respiratory tract infection in Western Sichuan Province were all non capsular strains or called NTHi isolates(NTHi)[3, 4]. Ofloxacin resistance rate was 1.98% (2/101) in the group of ≥ 18 years old[4]. Although no resistant strain was found in the 0 ~ 17 years old group[3], the result of the more accurate broth drug sensitivity test showed that the strains of ofloxacin MIC ≥ 0.5 showed an increasing trend in all age groups. Indeed, *H. influenzae* isolated from all age groups is becoming less susceptible to ofloxacin. Our findings are very similar to the results of a simultaneous study by shoji S et al in Japan[10]. Unlike in China, tofloxacin (tosufloxacin) as one kind of fluoroquinolones has been approved for use in pediatric patients in Japan in 2010[11]. The reason why the strains of ofloxacin MIC ≥ 1 appeared in 0 ~ 3 years old patients and the susceptibility to ofloxacin was decreased in 0 ~ 17 years old patients in China should be paid more attention to. Since 1993, when quinolone-resistant strains were first reported [12], the strains with reduced susceptibility to quinolones have been found in elderly patients in many countries and regions[13, 14, 20–23]. Because a group of fluoroquinolones, such as tosufloxacin, levofloxacin, moxifloxacin, garenoxacin and sitafloxacin, etc., have an excellent transfer rate to the lungs and show a strong antibacterial activity against most community-acquired pneumonia-causing pathogens, including *S. pneumoniae*, *H. influenzae* and *Mycoplasma pneumoniae*[12], they are called as ‘respiratory quinolones’and are used as a first-line drug to treatment for adults with community-acquired pneumonia[15]. The widespread use of fluoroquinolones in adult patients may induce the emergence of more drug-resistant strains, but it does not explain why *H. influenzae* isolates with reduced ofloxacin sensitivity have been found more frequently in Chinese children. So the genetic pattern of quinolone-resistant strains isolated from Chinese children is worthy of further study.

The mechanism of action of the quinolones involves the disruption of DNA replication of type II topoisomerase[16]. Type II topoisomerases are currently recognized to include DNA gyrase, which is responsible for the formation and elimination of supercoiled structures in DNA strands, and topoisomerase IV, which cuts and re-ligates tangled DNA during DNA replication[16]. Each of these enzymes is composed of two dimers of subunit types A and B, which together form a tetramer. DNA gyrase is composed of gyrA and gyrB, topoisomerase IV comprises parC and parE [17]. The subunit A (gyrA and parC) possesses DNA cutting and ligating activity, the subunit B (gyrB and parE) possesses ATPase activity[18]. Quinolones bind to the exposed double-stranded DNA and form the DNA-DNA gyraseequinolone antibiotic complex, thereby preventing the re-ligation of DNA[19]. Substitutions of amino acids in each enzyme lead to the inhibition of these formations[19]. In particular, mutations in the QRDRs are closely related to resistance[19]. In particular, mutations in the QRDRs are closely related to resistance. Mutations in the QRDRs of *H. influenzae* have been demonstrated to occur in a stepwise manner with an increasing number of mutations yielding higher quinolone MIC[20–24]. A sequence of

nucleotides associated with quinolones resistance in the *gyrA* and *parC* gene sequences is known as the QRDRs. Among them, the *parC* region of the *gyrA* gene was composed of the 202 ~ 531 base at the fifth terminal of the *gyrA* gene (that is, the base encoding the 68 ~ 177 amino acid residues of the *gyrA*) and the 152 ~ 456 base at the terminal of the *parC* gene (that is, the amino acid residue at the 51 ~ 152nd position of the *parC*). Previously, quinolone-resistant *H. influenzae* with substitutions at ser-84 and asp-88 in *gyrA*, as well as at gly-82, ser-84, glu-88 in *parC* have been reported[25, 26]. Kurt F et al [27] reported that in addition to the ser-84-leu and asp-88-asn in *gyrA* and the ser-84-ile in *parC*, the molecular characteristics of high levels of *H. influenzae* resistant to ciprofloxacin in southern Denmark also included K20R, asp-356-ala/thr-356-ala and met-481-ile mutation of *parC* and glu-151-lys, ile-159-ala, asp-420-asn and ser-599-ala mutation of *parE*. Hisashi S et al. [28] showed that five amino acid substitutions in *gyrA* (at ser-84 and asp-88) and *parC* (at gly-82, ser-84 and glu-88) were found to be closely related to the MIC by genetic transformation experiments. It was also observed that the degree of resistance is related to the number of the mutations[28].

In addition to ser-84-leu, asp-88-tyr/asn mutation, the *gyrA* sequence of ala-134-val and glu-142-lys mutation was also observed. In addition to ser-84-lys/ile mutation, ser-133-ala and asn-138-ser mutation were also observed in the QRDRs sequence of *parC*. We also observed the amino acid substitution of the *gyrB*, gly-399-glu, ala-400-val, glu-469-asp and thr-472-ile, which were rarely reported in Asia before. The amino acid substitution of 9 sites was also found in the *parE* sequence, including asp-364-tyr, ala-369-thr, lys-378-cys, ala-383-thr, cys-405-ser, asp-420-asn, ala-426-val, val-466-met and ser-474-asn. We tried to analyze the effect of QRDRs amino acid substitution on the MIC value of ofloxacin by statistical method (orderly multiple classifications logistic regression analysis), and the results showed that the ser-84-leu and asp-88-tyr/asn mutation in *gyrA* sequence were the main factors affecting the MIC value of ofloxacin ($p < 0.001$); and the effect of the variation on MIC value of ofloxacin was 139.824 times and 28.950 times of that of no variation, respectively. Ser-84-lys/ile and ser-133-ala, the amino acid substitution of *parE* sequence, were the main factors affecting the MIC value of ofloxacin ($p < 0.001$ and $p < 0.042$); and the effects of variation on MIC value of ofloxacin were 102.789 times and 1.872 times of that of no variation, respectively. Ala-400-val, the amino acid substitution of *gyrB* sequence, was the main factor affecting the MIC value of ofloxacin ($p = 0.006$); and the effect of variation on MIC value of ofloxacin was 0.517 times of that of unmutated strains. In addition, the results of Mann-Whitney U test showed that the ala-134-val and glu-142-lys variations of *gyrA* sequence and asn-138-ser variation of *parC* sequence were significantly different from those of unmutated group ofloxacin MIC, $p \leq 0.05$. However, the further ordered multiple classifications logistic regression analysis showed that the effect of the above three-locus variations on the MIC value of the strain was not statistically significant ($p = 0.860, 0.783$, and 0.200 , respectively). Our study also found that the amino acid substitution of *parE* sequence was complex, but the further statistical analysis indicated that the variation of *parE* sequence had no statistical significance on the MIC value of ofloxacin. Previously, Hisashi S et al.[28] verified the ser-84-leu, asp-88-tyr/asn, glu-142-lys mutation of *gyrA* and the ser-84-lys/ile and asn-138-ser mutation of *parC* by means of genetic transformation experiments, and the results were consistent with the results of this study. However, Hisashi S et al found that 8 strains, containing the met-121-leu and glu-142-lys mutations

of gyrA, the ala-400-val mutations of gyrB, the ser-133-ala and asn-138-ser mutations of parC, the val-466-met mutation of parE, showed a low range of moxifloxacin MIC (0.015–0.06 mg/ml) [28], which was not exactly consistent with our observation. According to the statistical results, we inferred that the QRDRs mutation sites affecting the MIC value of ofloxacin were gyrA's ser-84-leu (139.824 times), parC's ser-84-lys/ile (102.789 times), gyrA's ser-133-ala (28.950 times), parC's ser-133-ala (1.872 times) and gyrB's ala-400-val (0.517 times), respectively. While in the same period a further study on the mechanism of beta-lactam resistance of the NTHi strain, the results showed that different amino acid replacement patterns of the ftsI gene had different effects on the MIC values [29]. However, statistical analysis is not a substitute for further genetic transformation experiments and Ka/Ks evolutionary analysis, Ka/Ks is the ratio of the number of nonsynonymous substitutions per nonsynonymous site (Ka) to the number of synonymous substitutions per synonymous site (Ks), to clarify the real role of amino acid substitution in the mechanism of quinolone resistance emerging in the study and clarify the interrelationship between these amino acid substitutions.

In the past ten years, the strains with ser-84-leu mutation of gyrA sequence that led to the significant increase of the MIC value of ofloxacin, while the strains with ala-400-val mutation of gyrB sequence also increased significantly in western Sichuan increased significantly. It is suggested that not only a key mutation in the nucleic acid sequence in QRDRs of type II topoisomerase, but also the change of ATPase activity may be involved in the quinolone- resistance mechanism of *H. influenzae* strain.

Although the strains with the ser-84-leu, asp-88-tyr/asn and ala-134-val mutation, which were isolated from the 0 ~ 3yrs-old group and from the 4-6yrs-old group, were still at a low level, however, the strains with the glu-142-lys mutation of gyrA sequence, with ala-400-val mutation of gyrB sequence, and with the ser-84-lys/ile and ser-133-ala as well as asn-138-ser mutation, were similar to those of the adult group. It can be predicted that with the increase of quinolones exposure, the susceptibility of isolates from children to quinolones will be further reduced.

Conclusions

Ser-84-leu and asp-88-tyr/asn mutation of the gyrA, ser-84-lys/ile and ser – 133-ala mutations of the parC and ala-400-val variation of the gyrB were the main factors leading to the increase of MIC value of ofloxacin of NTHi strains in Western Sichuan, China. It can be predicted that with the increase of quinolones exposure, the susceptibility of isolates from children to quinolones will be further reduced.

Methods

Strain source

A prospective epidemiological survey was conducted on inpatients with lower respiratory tract infections (LRTI) in the western Sichuan China from January 2003 to December 2004 and November 2013 to April 2014 [3, 4]. At the same time, the bacteria in lower respiratory tract secretions were monitored. The first

epidemiological investigation was conducted in Chengdu Children's Hospital; The subjects were 0 ~ 3 year old inpatients with LRTI. The four large public hospitals involved in the second epidemiological study included three general hospitals and one medical centre for women and children, with a total of more than 6000 beds. The service area covers 24.67 million people in western Sichuan (including 3.62 million people aged 0 ~ 17 years). The patient's age ranged from 0 to 93, with a male to female ratio of 167:113. Group of 0 ~ 28 days: n = 19, 6.79%; 29d ~ 1 year group: n = 110, 39.29%; 2 ~ 3yrs group: n = 68, 24.28%; 4 ~ 6yrs group: n = 31, 11.07%; adult group(> 17yrs): n = 52, 18.57%. 57 strains were isolated from 2003 to 2004, and 223 were isolated from 2013 to 2014. 228 strains in 0 ~ 17yrs group were respectively isolated from 180 cases (78.95%) of bronchopneumonia and 19 cases (8.33%) of neonatal pneumonia as well as 29 cases (12.72%) of bronchiolitis; and the main diagnosis of patients in over 17 yrs group (n = 52) included 31 cases (59.62%) of acute exacerbation of chronic obstructive pulmonary disease (AECOPD) and 18 cases (34.62%) of pulmonary infection as well as 3 cases (5.77%) of bronchitis.

The research program was approved by the Medical Ethics Review Committee of the institutional (the Approval No. CWSYLS-2013-R-1). Informed consent was obtained from patients or their parents or guardians according to the guidelines of the institutional review board on clinical samples. Sputum was sampled by a disposable nasal catheter or by endotracheal intubation with negative pressure sputum suction. Samples were obtained within the first 24 hours following admission. Each sample qualified as a sample from the lower respiratory tract when on gram stain was found to have neutrophil > 25, squamous cell < 10 or white blood cell/squamous epithelial cells $\geq$ 10, and the neutrophil phagocytosis of gram negative bacilli. The duplicate isolates of the same patient were excluded from the study to prevent repeated genetic background strains.

Strain Identification and DNA extraction

The bacterial isolation and preliminary identification of *H. influenzae* were carried out by the microbiology departments of the hospitals involved in the study. The identification was carried out in accordance with the 2nd edition of the National procedure for Clinical examination and the interlaboratory quality control. Strains were transported in magnetic bead tubes (provided by Beijing Youkang industry biotech co., Ltd.) and stored at -70°C refrigerator. The strain transport is carried out in accordance with the National Biosafety regulations. *H. influenzae* strains were initially screened by Gram staining and oxidase tests, factor V test and factor α + β (Media and factor sheets were available from OXOID, UK). Further identification was performed with a haemophilus identification card (Thermo scientific Inc., USA). Additional identification and serological typing of *H. influenzae* strains was carried out by PCR. This part of the study was conducted in the Respiratory Pathogens Laboratory of the National Center for Disease Prevention and Control Infectious Diseases Prevention and Control Institute (Beijing). Strain ATCC49247 was used as the positive laboratory standard. Extracted DNA of *H. influenzae* isolates was stored at 2–8°C. All PCR primers were synthesized by Beijing Tianyi Huiyuan Life Science & Technology Co. Ltd. The sequence and length of the primers used in PCR amplification are shown in S1 table. Molecular biological type by PCR is a reliable method for accurate identification of NTHi isolates[5, 6]. Outer membrane protein

P6 is a member of the class of outer membrane proteins known as peptidoglycan-associated lipoproteins, which are highly conserved among *H. influenzae* isolates[7]. The *fuck* gene has higher specificity for identification of *H. influenzae*[8]. The results of agar gel electrophoresis were as follows: the *p6* gene and the *fuck* gene were all positive for *H. influenzae*; both *p6* gene and *fuck* gene were positive, while the *cap* gene negative were NTHi strain[3–6].

Antimicrobial susceptibility test

The MIC value of ofloxacin was determined by broth dilution method, and the results were judged according to the breakpoint standard of Guidelines of CLSI 2016. The breakpoint of MIC to ofloxacin: S:≤2;R:>2. Quality control during the experiment with *H. influenzae* standard strain ATCC49247 was purchased from the Clinical Laboratory Center of the Ministry of Health of the People's Republic of China.

QRDRs gene sequencing

The strain DNA was extracted and stored in 2–8°C refrigerator. The sequencing of the *gyrA*, *gyrB*, *parC* and *parE* gene were completed by Beijing Tianyi Huiyuan Life Science and Technology Co., Ltd. The primer sequence and base length of *gyrA*, *gyrB*, *parC* and *parE* genes are shown in Table 1[9]. PCR amplification reaction system: add the following ingredients to the 0.2 ml centrifuge tube: genomic DNA 1.0 µl, 10 × Buffer(contain 2.5 mM Mg²⁺) 2.5 µl, Taq polymerase(5U/µl, Polymerase from Dalian Bao Bio Takara Technology Co., Ltd.) 0.5 µl, dNTP (10 mM) 1.0 µl, Primer (+ -)1 µl (10uM), ddH₂O 0.5 µl, bulk volume 25 µl. Light and elastic mixing, instantaneous centrifugation collection of droplets from the tube wall to the tube bottom, PCR reaction on the PCR amplification instrument, the reaction parameters are as follows: (94 °C 5 min) *1 cycle; (94 °C 30 s, 58 °C 30 s, 72 °C 30 s)*36 cycles; (72 °C 7 min)*1 cycle. After the reaction, the 2 µl PCR product was detected by 1% agarose gel electrophoresis. The amplified PCR fragment was confirmed. PCR products were recovered by AxyPrepDNA gel recovery kit and the specific operation was carried out according to the kit specification. The purified PCR products were sequenced by sequenator(Applied Biosystems 3730-XL). Experimental instruments also included PCR instrument (Applied Biosystems 2720 Thermal cycler), Centrifuge(Eppendorf 5804R), GEL imaging system(UVP BioImaging System). Nucleotide sequences are translated into amino acids using sequencer 4.1.0 sequencing analysis software. The 4.1.0 sequencing analysis software was used, and all obtained data are entered into Genbank, and the sequence was compared with that of *H. influenzae* Rd kw20; The accession numbers of *gyrA* gene, *gyrB* gene, *parC* gene and *parE* gene were HM113386.1, AJ508044.1, HG983321.1 and AJ508046.1, respectively.

Data analysis

The enumeration data were expressed as percentage (%). There was a significant difference in χ^2 or Fisher exact probability test between the two groups by using Statistical Software R (v3.3.1), and the difference was statistically significant with $p < 0.05$. The MIC values of the two groups were compared by Rank Sum test. The median and quartile intervals were used to describe the center and the discrete trend, and $p < 0.05$ is statistically significant for the difference. Two independent sample rank sum test (Mann-Whitney u test) and Multivariate Ordered Logistic Regression Analysis with spss20.0 Statistical Software

for the data satisfying the condition. The independent variable is the mutation point of the *gyrA* gene, *gyrB* gene, *parC* gene and *parE* gene, respectively; and the dependent variable is the value of ofloxacin MIC value, in Multivariate Ordered Logistic Regression Analysis. The criteria is 0.05, exclusion criteria is 0.10; $p < 0.05$ is statistically significant for the difference.

Abbreviations

NTHi :Nontypeable *Haemophilus influenzae*; QRDRs: the Quinolone Resistance-Determining Regions; MIC: minimum inhibitory concentration; *H. influenzae*: *Haemophilus influenzae*; yrs: years; Fig: Figure; LRTI: lower respiratory tract infections; CLSI: Institute of Clinical Laboratory Standards; PCR: Polymerase chain reaction;

Declarations

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Authors' Contributions

XLW designed the experiment and wrote the first draft and together with JX, YBG and ZJS further finalized the design. BQZ, JH and ZHW planned the laboratory procedures. HMG and LFH conceptualized the statistical analyses. LLY and HWL performed sample collection. All authors had helped in revision and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The research program was approved by the Medical Ethics Review Committee of the institutional (the Approval No. CWSYLS-2013-R-1).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Trend of partial QRDRs variation in the past decade.

Fig2.Comparison of partial QDRDs variation in different age groups

Figure 2

Comparison of partial QDRDs variation in different age groups.

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