Ofloxacin MIC values and Mutation characteristics of Quinolones resistance determining region gene of *Haemophilus influenzae* isolates from the lower respiratory tract in Western Sichuan, China: Comparative study of children and adults

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Research

Keywords: Nontypeable Haemophilus influenzae, quinolone resistance determining region, gene mutation, amino acid substitution

Posted Date: May 6th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-482903/v1

Abstract

Background: In order to further investigate the trend of quinolone resistance and the gene variation characteristics of quinolone resistance determining region of non-typeable *Haemophilus influenzae* (*NTHi*) isolates from Chinese children's respiratory tract, We tracked the isolates of children in western Sichuan of China for ten years and compared them with adult groups in the same period.

Method: We monitored the MIC value of ofloxacin of *NTHi* isolates (n=280) from lower respiratory tract secretions in children group (n=57) during 2003~2004 and in whole age group (n=223) during 2013~2014 in Western Sichuan, China. The amino acid sequences of QRDRs, *gyrA*, *gyrB*, *parC* and *parE*, were detected; and the relationship between amino acid substitutions (*AAS*) of QRDRs and ofloxacin MIC value was analyzed. At the same time, the mutation trend of QRDRs gene in this region during the past ten years was compared analyzed.

Results: 1. All the strains (n=280) of *H. influenzae* included in the study were *NTHi*. No ofloxacin-resistant strains were found in 57 *NTHi* isolated from the children patient during 2003~2004. While strains with the minimum inhibitory concentration (MIC) value ≥ 0.5 showed an upward trend in all age groups during 2013~2014. **2.** The *AAS* are S84L, D88Y/N, A134V and E142K in *gyrA*, G399E, A400V, E469D and T472I in *gyrB*. S84R/I, S133A and N138S in *parC* and D364Y, A369T, R378C, A383T, G405S, D420N, A426V, V466M and S474N in *parE*. **3.** Compared to the non-mutation group, the change of ofloxacin MIC values of the strains with S84L, D88Y/N and A134V in *gyrA*, A400V in *gyrB* and S84R/I , S133A in *parC* showed statistical significance ($p \leq 0.05$ or approximate $p=0.05$); And the results of ordered multi-classification logistic analysis showed that the *AAS gyrA*-S84L (*OR*=139.824, 95% *CI*=55.730~350.811, p□0.001), *gyrA*-D88Y/N (*OR*=28.950, 95% *CI*=8.432~99.395, p□0.001), *parC*-S84R/I (*OR*=102.789, 95% *CI*=31.851~331.713, p□0.001), *parC*-S133A (*OR*=1.872, 95% *CI*=1.023~3.426, $p=0.042$) were risk factors for the increase of ofloxacin MIC value, respectively; the *AAS gyrB*-A400V (*OR*=0.517, 95% *CI*=0.322~0.831, $p=0.006$) was the factor affecting the decrease of ofloxacin MIC value. The *gyrA*'s S84L, *gyrA*'s D88Y/N, *gyrB*'s A400V, *parC*'s S84R/I and *parC*'s S133A mutations were the main factors affecting the MIC value of ofloxacin of *NTHi* isolates in Western Sichuan, China, respectively. **4.** The age group distribution of mutations S84L, D88Y/N and A134V in *gyrA* were significantly different ($p=0.013$, 0.034 and 0.010, respectively). The key mutation of multiple QRDRs of *NTHi* isolates in children in low age group is increasing rapidly. Along with increase of the mutation rate of S84L in *gyrA* and A400V in *gyrB* of isolates from 0~3 yrs old group ($X^2=6.089$, $p=0.014$; $X^2=25.181$, $p<0.001$), the ofloxacin MIC value increasing was statistically significant in the past ten years ($p=0.022$).

Background

Fluoroquinolones are one of the most widely used antibiotics in the treatment of respiratory tract infections. Since the first discovery of quinolone-resistant strains in 1993[1], the quinolone resistant strains of *H. influenzae* have gradually increased in many countries and regions. 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. Although quinolones have been banned in pediatrics after the listing of quinolones in China, our early research found that *H. influenzae* strains resistant to ciprofloxacin were still isolated from the respiratory tract of children and new borns [2]. To further explore the molecular mechanism of quinolones resistance and the variation characteristic of QRDRs genes of *H. influenzae* isolates from children's respiratory tract in China, we monitored the ofloxacin MIC values of the *H. influenzae* isolates from the lower respiratory tract secretions among all age groups during 2013 ~ 2014 in Western Sichuan, China and sequenced the QRDRs of *gyrA*, *gyrB*, *parC* and *parE*. On this basis, the relationship between different *AAS* patterns and ofloxacin MIC values was analyzed, and the *gyrA*, *gyrB*, *parC* and *parE* gene mutations of *H. influenzae* isolates in different age groups were compared. Partial 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.

Methods

Strain source

All strains were derived from two prospective epidemiological surveys in the Western Sichuan, China, 2003 ~ 2004 and 2013 ~ 2014[3, 4]. The patient's age ranged from 0 d to 93 yrs, with a male to female ratio of 167 : 113. Group of 0 ~ 28 d: n = 19, 6.79%; 29 d ~ 1 year group: n = 110, 39.29%; 2 ~ 3 yrs group: n = 68, 24.28%; 4 ~ 6 yrs group: n = 31, 11.07%; adult group (> 17 yrs) : n = 52, 18.57%. 57 strains were isolated from 2003 to 2004, and 223 were isolated from 2013 to 2014. 228 strains in 0 ~ 17 yrs 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.

Strain Identification and DNA extraction

Same as the literature [4].

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 was ≤ 2 μg/mL as sensitive and > 2 μg/mL as resistant. Quality controlling was performed in each experiment by testing the MIC of reference *H. influenzae* strain ATCC49247 which was purchased from the Clinical Laboratory Center of the Ministry of Health of the People's Republic of China.

QRDRs gene sequencing

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[5]. 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 Mg^{2+}) 2.5 μl, Taq polymerase (5 U/μl, Polymerase from Dalian Bao Bio Takara Technology Co., Ltd.) 0.5 μl, dNTP (10 mM) 1.0 μl, Primer (+ -)1 μl (10 uM), ddH_2O 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.

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 5min)*1cycle; (94°C 30s, 58°C 30s, 72°C 30s)*36 cycles; (72°C 7min)*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		

Data analysis

The enumeration data were expressed as percentage (%). There was a significant difference in χ2 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.

Results

Strains serotype

All the 280 strains of *H. influenzae* showed *p6* gene and *fuck* gene positive, while cap genes were negative. All *H. influenzae* strains were *NTHi*.

Susceptibility of ofloxacin

The results of antimicrobial susceptibility test by broth method showed that ofloxacin MICs of strains (n = 57) during 2003 ~ 2004 were 0.0156 ~ 1 μg/mL, and no ofloxacin resistant strain was found; The frequency of ofloxacin resistant strains was 1.92% (1/52) and the ofloxacin MIC value was 0.0156 ~ 16 μg/mL in adult group (n = 52) during 2013 ~ 2014; the ofloxacin MIC value was 0.0078 ~ 2 μg/mL in 0 ~ 3 yrs group (n = 142) and 0.0156 ~ 0.5 μg/mL in 4 ~ 6 yrs group during 2013 ~ 2014.

The results of Rank sum test showed that the ofloxacin MIC value increasing of *NTHi* isolates (n = 142) in 0 ~ 3 yrs group during 2013 ~ 2014 was statistically significant compared with that of 2003 ~ 2004 (n = 55, $p = 0.022$). And the *NTHi* isolates with ofloxacin MIC ≥ 0.5 μg/mL also showed an increasing trend in all age groups during 2013 ~ 2014, as shown in Table 2 and Table 3.

Table 2
The decade variation in MIC values of Ofloxacin of NTHi strains from 0-3yrs group (the results of Rank-Sum test)

Group	n	Median	Percentiles		*Wilcoxon*	*p*
			25th	75th		
2003–2004	55	0.0313	0.0313	0.0313	11690.0	0.022
2013–2014	142	0.0313	0.0156	0.0625		
Notes: $p < 0.05$ is statistically significant.						

Table 3
Proportion of strains with ofloxacin MIC ≥ 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 = 55)	0.0156 ~ 1	0	1.81%(1/55)	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
≥ 18yrs group(n = 52)	0.0156 ~ 16	9.62%(5/52)	3.85%(2/52)	1.92%(1/52)	1.92% (1/52)
Note: 55 of 57 cases during 2003 to 2004 belong to 0 ~ 3 years group ,and 2 cases are of 4 ~ 6 years old.					

Mutation of QRDRs gene and its effect on ofloxacin MIC value

The *AAS* in the *gyrA* gene were S84L, D88Y/N, A134V and E142K (gyrA-S84L, gyrA-D88Y/N, gyrA-A134V and gyrA-E142K). The *AAS* of *gyrB* gene were G399E, A400V, E469D and T472I (*gyrB*-G399E, *gyrB*-A400V, *gyrB*-E469D, *gyrB*-T472I). Mutations S84R/I and S133A and N138S appeared in the *parC* gene (*parC*-S84R/I, *parC*-S133A and *parC*-N138S). The *AAS* in the *parE* gene were as follows: D364Y, A369T, R378C, A383T, G405S, D420N, A426V, V466M and S474N (*parE*-D364Y, *parE*-A369T, *parE*-R378C, *parE*-A383T, *parE*-G405S, *parE*-D420N, *parE*-A426V, *parE*-V466M and *parE*-S474N).

According to whether the above alleles were mutated, all strains were respectively divided into mutant group and non-mutant group, compared between the two groups using the Mann-Whitney U test. The effect of *AAS* in QRDRs genes on the ofloxacin MIC value and the results of Mann-Whitney U test were shown in Table 4. Compared to the non-mutation group, the change of ofloxacin MIC values of the strains with S84L, D88Y/N in *gyrA*, A400V in *gyrB* and S84R/I, S133A in *parC* showed statistical significance ($p \leq 0.05$); The changes of ofloxacin MIC values in the strains with *gyrA*-A134V, *gyrA*-E142K, *parC*-S133A, *parC*-N138S, *parE*-A369T, *parE*-A426V, respectively were at the critical value compared with non-mutation group ($p = 0.053, 0.054, 0.07, 0.072, 0.064$). There was no significant difference in the MIC value between the other *AAS* groups and non-mutation group ($p > 0.05$).

Table 4
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	*p*
	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

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. $p < 0.05$ is statistically significant.

An ordered multi-classification logical regression analysis was performed on the 8 mutation sites with Mann-Whitney U test *p* values less than 0.05 or close to 0.05. The results of ordered multi-classification logistic analysis, with the 8 *AAS*, respectively, as independent variable and ofloxacin MIC value as a dependent variable, showed that the *AAS gyrA*-S84L (*OR* = 139.824, 95% *CI* = 55.730 ~ 350.811, *p*＜0.001), *gyrA*-D88Y/N (*OR* = 28.950, 95% *CI* = 8.432 ~ 99.395, *p*＜0.001) and *parC*-S84R/I (*OR* = 102.789, 95% *CI* = 31.851 ~ 331.713, *p*＜0.001) as well as *parC*-S133A (*OR* = 1.872, 95% *CI* = 1.023 ~ 3.426, *p* = 0.042) were risk factors for the increase of ofloxacin MIC value, respectively; the *AAS gyrB*-A400V (*OR* = 0.517, 95% *CI* = 0.322 ~ 0.831, *p* = 0.006) was the factor affecting the decrease of ofloxacin MIC value. The above results are shown in Table 5.

Table 5
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	*P* value	95% Confidence Interval	
						Lower Bound	Upper Bound
gyrA-S84L	4.940	139.824	0.469	110.809	< 0.001	55.730	350.811
gyrA-D88Y/N	3.366	28.950	0.629	28.597	< 0.001	8.432	99.395
gyrA-A134V	0.135	1.144	0.762	0.031	0.860	0.257	5.096
gyrA-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
parC-S133A	0.627	1.872	0.308	4.133	0.042	1.023	3.426
parC-N138S	-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. $p < 0.05$ is statistically significant.

Distribution trend of QRDRs gene mutation

The results of χ2 test or Fisher exact probability analysis of the mutation rate of QRDRs genes of the isolates from 0 ~ 3 yrs group in the past decade are shown in S1 table. The *AAS* patterns of the QRDRs genes isolated from 0 ~ 3 yrs old (n = 142) were more diverse, with seven new mutation sites, including the *gyrB*-G399E and *gyrB*-E469D, and *parE*-D364Y, *parE*-A369T, *parE*-A383T, *parE*-G405S and *parE*-D420N,

compared with 2003 ~ 2004 (n = 55), as shown in S1 table; the results of Rank-Sum test show that the increase of *gyrA*-S84L, *gyrB*-A400V in 0 ~ 3 yrs old group isolates from 2013 to 2014 was statistically significant(X^2 = 6.089, 25.181; p = 0.014, p < 0.001). Meanwhile, the increase of ofloxacin MIC value in these isolates also shows statistical significance (p = 0.022), as shown in Table 2. The trends of these eight QRDRs allele mutation, with the p < 0.05 and p close to 0.05 in 0 ~ 3 yrs old group isolates throught by the Mann-Whitney U test, are shown in Fig. 1.

The distribution of the strains with the 8 allelic mutations in the different age groups is shown in S2 table. The results of χ2 test or fisher exact probability method show that the distribution of D88Y/N and A134V mutation rate in *gyrA* sequence as well as S84R/I mutation rate in *parC* sequence in different age group was significantly different (p = 0.027, 0.029 and 0.036), respectively; The p value of A400V variation in *gyrB* distribution difference in different age groups is also close to 0.05 (p = 0.052), but age group distribution of the other four loci had no statistical significance (p > 0.05). The distrubtion trende of QRDRs variation in neonatal group and 29 d ~ 3 yrs group during the past decade is shown in Fig. 1. Compared with the isolates from 2003 ~ 2004, the *gyrA*-S84L, *gyrA*-E142K, *gyrB*-A400V, *parC*-S84R/I, *parC*-S133A variation of 29 d ~ 3 yrs group isolates (n = 123) showed different increasing trend during 2013 ~ 2014, among which *gyrB*-A400V variation increased the most. The *gyrA*-S84L, *parC*-S84R/I and *parC*-S133A variation of 0 ~ 28 d group isolates (n = 9) also increased by varying degrees.

The distribution trend of the above QRDRs mutations in different age group during 2013 ~ 2014 is shown in Fig. 2. The *gyrA*-D88Y/N, *gyrB*-A400V, *parC*-S84R/I and *parC*-S133A variation rates of 0 ~ 28 d group isolates (n = 9) from 2013 ~ 2014 were higher respectively than those in adults (n = 52). The *gyrA*-E142K, *gyrB*-A400V, *parC*-S84R/I and *parC*-S133A variation rates of isolates from 29 d ~ 1 year group (n = 64) in 2013 ~ 2014 were higher at different degrees than those in adult groups.

The *gyrA*-E142K, *gyr*B-A400V, *parC*-S133A and *parC*-N138S variation rate of 2 ~ 3 yrs isolates (n = 59) in 2013 ~ 2014 were higher than those in adults at different degrees, among them, the rate of *gyrB*-A400V variation increased fastest.

The *gyrA*-S84L, *gyrA*-D88Y/N, *gyrA*-A134V, *parC*-S84R/I and *parC*-N138S variation rates of isolates from 4 ~ 6 yrs groups (n = 31) in 2013 ~ 2014 were lower than those in adult group, and the variation rate of *gyrA*-E142K, *gyrA*-E142K and *parC*-S133A was slightly higher than that in adult group.

The *gyrB*-A400V variation rates of isolates from groups 29 d ~ 1 year (n = 64) and 2 ~ 3 yrs (n = 59) during 2013 ~ 2014 were higher than those in adult groups (n = 52) in the same period, respectively. The *gyrB*-A400V variation rate of 2 ~ 3 yrs groups increased faster. The *parC*-S84R/I variation rates of 0 ~ 28 d group (n = 9) and 29 d ~ 1 year group (n = 64) were also significantly higher than those of adult group (n = 52). The *parC*-S133A variation rates of isolates from groups 0 ~ 28 d (n = 9), 29 d ~ 1 year, 2 ~ 3 yrs and 4 ~ 6 yrs were also higher than those in adult groups, but no statistical difference was shown.

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 *NTHi* [3, 4]. Ofloxacin resistance rate was 1.98% (2/101) in the group of ≥ 18 yrs old [6]. Although no resistant strain was found in the 0 ~ 17 yrs old group [3], the result of the more accurate broth drug susceptibility 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 susceptibility to ofloxacin. Our findings are very similar to the results of a simultaneous study by shoji S et al in Japan [7]. Unlike in China, tofloxacin (tosufloxacin) as one kind of fluoroquinolones has been approved for use in pediatric patients in Japan in 2010 [8]. As a result, it is not difficult to explain the possible reasons for the MIC value of ofloxacin isolated from respiratory secretions of Japanese children. Since 1993, when quinolone-resistant strains were first reported [9], the strains with reduced susceptibility to quinolones have been found in elderly patients in many countries and regions [10, 11, 17–20]. 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* [9], they are called as 'respiratory quinolones' and are used as a first-line drug for adults with community-acquired pneumonia [12]. The widespread use of fluoroquinolones may induce the emergence of more drug-resistant strains in adult patients, but it does not explain why *H. influenzae* isolates with reduced ofloxacin sensitivity have been found frequently in Chinese children. The causes of high ofloxacin MIC value of *NTHi* strains in respiratory tract secretions of children in China should be highly concerned.

The mechanism of the quinolones killing bacteria involves the disruption of DNA replication of type II topoisomerase [13]. 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 [13]. 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 [14]. The subunit A (gyrA and parC) possesses DNA cutting and ligating activity, the subunit B (gyrB and parE) possesses ATPase activity [15]. Quinolones bind to the exposed double-stranded DNA and form the DNA-DNA gyrase-equinolone antibiotic complex, thereby preventing the re-ligation of DNA[16]. The substitutions of amino acids in each enzyme lead to the inhibition of the formation of antibiotic complex. In particular, mutations in the QRDRs are closely related to resistance [16]. 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 value [17–21]. A sequence of nucleotides associated with quinolones resistance in the *gyrA* and *parC* gene sequences is known as the QRDRs. Among them, the QRDR region of the *gyrA* gene was composed of the 202 ~ 531st base at the fifth terminal of the *gyrA* gene (that is, the base encoding the 68 ~ 177th amino acid residues of the gyrA) and the 152 ~ 456th 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 those at gly-82, ser-84,

glu-88 in *parC* have been reported [22, 23]. Kurt F et al [24] 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, D420N and ser-599-ala mutation of *parE*. Hisashi S et al. [25] 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 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 [25].

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 *gyrA*'s S84L, *gyrA*'s D88Y/N, *gyrB*'s A400V, *parC*'s S84R/I and *parC*'s S133A mutations were the main factors affecting the MIC value of ofloxacin ($p < 0.001$, $p < 0.001$, $p = 0.006$, $p < 0.001$ and $p < 0.042$), respectively; and the effect of the variation on MIC value of ofloxacin was 139.824 times, 28.950 times, 0.517 times, 102.789 times and 1.872 times of that of no mutation group, respectively. In addition, the results of Mann-Whitney U test showed that the *gyrA*'s A134V and *gyrA*'s E142K variations were significantly different from those of non-mutated group ofloxacin MIC ($p = 0.029$, 0.053). However, the further ordered multiple classifications logistic regression analysis showed that the effect of the above two-locus variations on ofloxacin MIC value of the strain was not statistically significant ($p = 0.860$, 0.783). We also found that the *AAS* 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. Hisashi S et al.[25] verified previously the S84L, D88Y/N, E142K mutation of *gyrA* and the S84R/I and N138S 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 eight strains, containing *gyrA*-M121L and *gyrA*-E142K mutation, *gyrB*-A400V mutation, *parC*-S133A and *parC*-N138S mutations, the *parE*-V466M mutation, et al, showed a low range of moxifloxacin MIC (0.015–0.06 μg/ml) [25], 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 S84L (139.824 times), *parC*'s S84R/I (102.789 times), *gyrA*'s D88Y/N (28.950 times), *parC*'s N138S (1.872 times) and *gyrB*'s A400V (0.517 times), respectively. However, statistical analysis is not a substitute for further genetic transformation experiments and Ka/Ks evolutionary analysis. The latter can help 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, accompanied by a significant rising in the MIC value ofloxacin ,the *gyrA*'s S84L and *gyrB*'s A400V mutation rates of *NTHi* isolates in the 0 ~ 3 yrs old group in western Sichuan were significantly increased. 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. Through a detailed age grouping analysis, we found that the *gyrA*'s D88Y/N, *parC*'s S84R/I and *parC*'s S133A mutation rates of *NTHi* isolates from group 0 ~ 28d were much higher than those in adult group during 2013 ~ 2014; The *gyrA*'s E142K, *gyrB*'s A400V, *parC*'s S84R/I and *parC*'s S133A mutation rate in 29 d ~ 1 year old group was higher than that of adult group at

the same time; and the *gyrA*'s E142K, *gyrB*'s A400V and *parC*'s S133A in 2 ~ 3 yrs old group isolates continued to exceed adult levels; The *gyrB*'s A400V and *parC*'s S84R/I mutation rate of 4 ~ 6 yrs old group strains was close to or below the adult group level, but the *gyrA*'s E142K and *parC*'s S133A mutation rates still exceeded the adult group level. While there were fewer cases in our two epidemiological surveys during 2003 ~ 2004 and 2013 ~ 2014 in the 0 ~ 28 d groups and no large sample levels in the 29 d ~ 1 year old groups, the abnormal increase in the key mutation rate of QRDRs genes in these low age groups' isolates is very worrying in the context of the continuous ban of quinolones in children under 12 yrs of age in China. Besides the transmission factors of adult quinolone-resistant *NTHi* strains to children, the effect of quinolones residues in the environment and food chain on the resistance of *NTHi* isolates in children must also attract our attention. The genetic variation mechanism of quinolone resistance gene of *NTHi* strains in children is still worthy of our further exploration.

Conclusions

The *gyrA*'s S84L, *gyrA*'s D88Y/N, *gyrB*'s A400V, *parC*'s S84R/I and *parC*'s S133A mutations were the main factors affecting the MIC value of ofloxacin of *NTHi* isolates in Western Sichuan, China, respectively. The key mutation of multiple QRDRs of *NTHi* isolates in children in low age group is increasing rapidly.

Abbreviations

NTHi, Nontypeable Haemophilus influenzae; QRDRs, the Quinolone Resistance-Determining Regions; MIC, minimum inhibitory concentration; yrs, years; LRTI, lower respiratory tract infections; CLSI, Institute of Clinical Laboratory Standards; PCR, Polymerase chain reaction; *AAS*, amino acid substitutions;

Declarations

Acknowledgments

Thanks to all the co-workers involved in the clinical cases of the Third People's Hospital of Chengdu (TPHC), the Women & Children's Medical Center of Chengdu (WCMCD)and the People's Hospital of Deyang (PHD) as well as the Medical Center of Dujiangyan (MCD).

Authors' Contributions

Wang XL designed the experiment and wrote the first draft and together with Xie J, Guo YB and Shao ZJ further finalized the design. Zhu BQ, Hu J and Wang ZH planned the laboratory procedures. Guo HM and Huang LF conceptualized the statistical analyses. Yang LL and Liu HW performed sample collection. All authors had helped in revision and approved the final manuscript.

Funding

This study was funded by Science□Technology Department of Sichuan Province (2013JY0121) and Science□Technology Department of Chengdu (2014-HM01-00272-SF). The funding bodies had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

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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Tables

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 5min)*1cycle; (94°C 30s, 58°C 30s, 72°C 30s)*36 cycles; (72°C 7min)*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 The decade variation in MIC values of Ofloxacin of NTHi strains from 0-3yrs group (the results of Rank-Sum test)

Group	n	Median	Percentiles		*Wilcoxon*	*p*
			25th	75th		
2003-2004	55	0.0313	0.0313	0.0313	11690.0	0.022
2013-2014	142	0.0313	0.0156	0.0625		

Notes: $p<0.05$ is statistically significant.

Table 3 Proportion of strains with ofloxacin MIC≥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=55)	0.0156~1	0	1.81% (1/55)	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
≥18yrs group(n=52)	0.0156~16	9.62% (5/52)	3.85% (2/52)	1.92% (1/52)	1.92% (1/52)

Note: 55 of 57 cases during 2003 to 2004 belong to 0~3 years group ,and 2 cases are of 4~6 years old.

Table 4 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	*p*
	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

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. $p<0.05$ is statistically significant.

Table 5 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	*P* value	95% Confidence Interval	
						Lower Bound	Upper Bound
gyrA-S84L	4.940	139.824	0.469	110.809	<0.001	55.730	350.811
gyrA-D88Y/N	3.366	28.950	0.629	28.597	<0.001	8.432	99.395
gyrA-A134V	0.135	1.144	0.762	0.031	0.860	0.257	5.096
gyrA-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
parC-S133A	0.627	1.872	0.308	4.133	0.042	1.023	3.426
parC-N138S	-0.449	0.638	0.351	1.642	0.200	0.321	1.269

Figures

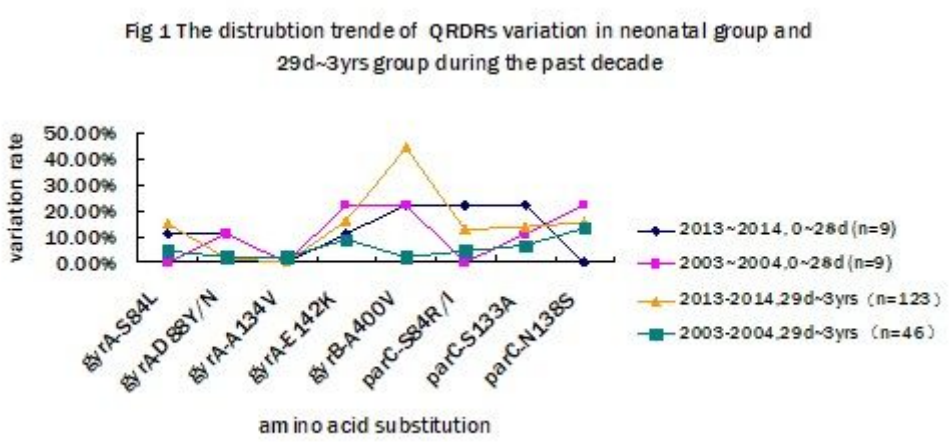

Figure 1

(caption in figure)

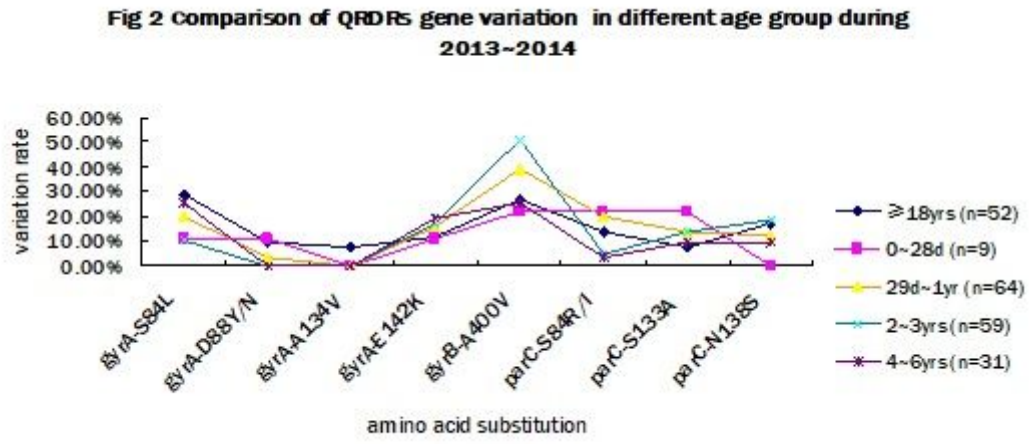

Figure 2

(caption in figure)

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