| | |
|---|---|
| Title | Molecular characterization of fluoroquinolone-resistant Mycobacterium tuberculosis clinical isolates from Shanghai, China |
| Author(s) | Zhu, C; Zhang, Y; Shen, Y; Siu, GKH; Wu, W; Qian, X; Deng, G; Xu, Y; Lau, R; Fan, X; Zhang, W; Lu, H; Yam, WC |
| Citation | Diagnostic Microbiology and Infectious Disease, 2012, v. 73 n. 3, p. 260-263 |
| Issued Date | 2012 |
| URL | http://hdl.handle.net/10722/157707 |
| Rights | NOTICE: this is the author's version of a work that was accepted for publication in Diagnostic Microbiology and Infectious Disease. Changes resulting from the publishing process, such as peer review, editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes may have been made to this work since it was submitted for publication. A definitive version was subsequently published in Diagnostic Microbiology and Infectious Disease, 2012, v. 73 n. 3, p. 260-263. DOI: 10.1016/j.diagmicrobio.2012.03.025 |

# Molecular Characterization of Fluoroquinolone-Resistant Mycobacterium tuberculosis Clinical Isolates from Shanghai ,China

Cuiyun Zhu[1], Yongxin Zhang[2], Yinzhong Shen[1], Gilman Kit Hang Siu[3], Wenjuan Wu[1], Xueqin Qian[1], Guilin Deng[1], Yan Xu[1], Ricky Lau[3], Xiaoyong Fan[1], Wenhong Zhang[2], Hongzhou Lu[1,2]* Wing-Cheong Yam[3]*

*Co-Corresponding author

Correspondence:

Hongzhou Lu, luhongzhou@fudan.edu.cn.

Phone:(8621) 3799-0333. Fax: (8621) 5724-8782.

Wing- Cheong Yam, wcyam@hkucc.hku.hk.

Phone : 852-22554821 Fax: 852-28551241

[1.]Shanghai Public Health Clinical Center Affiliated to Fudan University, Shanghai, China

[2.]Shanghai Huashan Hospital Affiliated to Fudan University, Shanghai, China

[3.] Centre of Infection and Department of Microbiology, Queen Mary Hospital, The University of Hong Kong, Hong Kong Special Administrative Region, China

This work was supported by two grants from the Major Programs of the National Science & Technology during the Eleventh Five-Year Plan Period （No. 2008ZX10001-008；2009ZX10003-017）.

## Abstract

China is one of countries with the highest prevalence of fluoroquinolones resistant ($FQ^r$) *Mycobacterium tuberculosis*. Nevertheless, the knowledge on molecular characterization of $FQ^r$ *M. tuberculosis* strains of this region remains very limited.

This study was to investigate the frequencies and types of mutations present in $FQ^r$ *M. tuberculosis* clinical isolates collected in Shanghai, China.

A total of 206 $FQ^r$ *M. tuberculosis* strains and 21 ofloxacin sensitive ($FQ^s$) *M. tuberculosis* strains were isolated from patients with pulmonary tuberculosis in Shanghai. The phenotypic drug susceptibilities were determined by the proportion method and the mutations inside quinolone-resistance-determining region (QRDR) of *gyrA* and *gyrB* genes were identified by DNA sequence analyses.

Among 206 $FQ^r$ *M. tuberculosis* strains, 44% (90/206) were multi-drug resistant (MDR) isolates and 39% (81/206) were extensive drug resistant (XDR) isolates. Only 9% (19/206) were mono-resistant to Ofloxacin. In total, 79.1% (163/206) of $FQ^r$ isolates harboured mutations in either *gyrA* or *gyrB* QRDR. Mutations in *gyrA* QRDR were found in 75.7% (156/206) $FQ^r$ clinical isolates. Among those *gyrA* mutants, a majority (75.6%) harboured mutations at amino acid position 94, with D94G being the most frequent amino acid substitution. Mutations in *gyrA* QRDR showed

100% positive predictive value for $FQ^r$ *M. tuberculosis* in China. Mutations in *gyrB* were observed in 15.5% (32/206) of $FQ^r$ clinical isolates. Ten novel mutations were identified in *gyrB*. However, most of them also harboured mutations in *gyrA*, limiting their contribution to $FQ^r$ resistance in *M. tuberculosis*.

Our findings indicated that, similar to other geographic regions, mutations in *gyrA* showed to be the major mechanism of $FQ^r$ resistance in *M. tuberculosis* isolates. The mutations in *gyrA* QRDR can be a good molecular surrogate marker for detecting $FQ^r$ *M. tuberculosis* in China.

## 1 Introduction

In the last decades, tuberculosis (TB) remains one of the major life-threatening diseases worldwide due to the emergence of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB(XDR-TB) . MDR-TB is defined as *M. tuberculosis* strains that are resistant to both isoniazid and rifampin (World Health Organization, 2011) .Globally, about 440,000 MDR-TB cases is estimated to emerge, and 150,000 persons died with MDR-TB each year (World Health Organization, 2011). A recent report showed an alarming increase in the number of tuberculosis patients in the South Asian subcontinent, with China being singled out as having one of the greatest burdens of MDR-TB, with a poor prognosis and high mortality among HIV-infected

individuals (World Health Organization, 2010). XDR-TB is defined as MDR-TB strains which are further resistant to any fluoroquinolone (FQ) and any of the second-line anti-TB injectable drugs (amikacin, kanamycin or capreomycin) (World Health Organization, 2011). By March 2011, 69 countries, including China, had reported to WHO with at least one case of XDR-TB. There is an estimated 25,000 cases of XDR-TB emerging every year (World Health Organization, 2010).

Fluoroquinolones, which are the backbone drugs for MDR-TB treatment has been introduced into clinical practices in China since late 1980s (Xu et al., 2009). It has been widely used in treatment for undiagnosed respiratory bacterial infections for more than two decades. Since TB patients are not treated normatively, FQ-resistant ($FQ^r$) TB has become more prevalent in China (Hu et al., 1992, Jiang, 1992, Vien le et al., 2011, Wise, 2003, Xu et al., 2009, Zhou et al.,2011). Although the molecular characterization of fluoroquinolone resistance ($FQ^r$) in *Mycobacterium tuberculosis* has been well studied in our neighbouring regions such as Hong Kong, Taiwan and Russia (Chan et al., 2007, Huang et al., 2005, Lau et al., 2011, Mokrousov et al., 2008, Umubyeyi et al.,2007)， only a few studies based on limited strains of $FQ^r$ *Mycobacterium tuberculosis* were reported for China (Cui et al., 2011, Sun et al., 2007, Xu et al.,2009).

This study recruited a large cohort of $FQ^r$ *M. tuberculosis* clinical isolates

from Shanghai and its neighbouring cities in China to provide a conclusive and representative figures for molecular characterization of $FQ^r$ *M. tuberculosis* using sequence analyses of the drug target genes for fluoroquinolones, *gyrA* and *gyrB*.

## 2 Materials and methods:

**2.1 Selection of *M. tuberculosis* clinical isolates and drug susceptibility test.** All *M. tuberculosis* isolates were originally isolated from patients with pulmonary tuberculosis in the period of 32 months (September 2007 – April 2010). These patients were descriptive epidemiologically unlinked and originated from third-grade hospitals in Shanghai and its neighboring cities in China. All strains were cultured on Löwenstein-Jensen medium and identified by niacin accumulation test and nitrate reduction test (Clinical and Laboratory Standards Institute, 2008). The phenotypic susceptibilities of these isolates to major first-line drugs: isoniazid (INH) (0.2μg/mL), rifampin (RIF) (40μg/mL) and ethambutol (EMB) (2μg/mL) as well as the seondary drug streptomycin (STR) (4μg/mL) were examined by using the Löwenstein-Jensen medium proportion method (World Health Organization, 2001). Three second-line drugs were chosen for MDR-TB treatment in Shanghai and drug susceptibility tests were performed by Bactec MGIT 960,using the following concentrations: ofloxacin (OFX) (2.0 μg/ml), capreomycin

(CAP) (2.5 μg/ml), amikacin (AMK) (1.0μg/ml) (World Health Organization, 2008) .A total of 206 ofloxacin-resistant strains were obtained and selected for this project. Of these 206 ofloxacin-resistant strains, 56 were isolated from new cases whereas 150 were isolated from re-treated case. A additional 21 ofloxacin-susceptible strains were also randomly selected as the denominators for molecular characterization of FQr *M. tuberculosis* strains in this project.

**2.2 DNA extraction.** A loopful of *M. tuberculosis* colonies was collected from Löwenstein-Jensen slant and suspended in sterilized water to provide bacterial suspension of McFarland standard 1. The suspension was centrifuged at 10,000× g for 5 min. The supernatant was discarded and the sediment was resuspended in a 40 μl DNA extraction solution (QIAGEN,Hilden,Germany) by vortex. Subsequently, the tube was incubated at 100℃ for 15 min, followed by centrifugation at 13,000 × g for 10 min. The supernatant was ready for PCR and was preserved at -20℃ until use.

**2.3 *gyrA* and *gyrB* PCR-sequencing.** PCR-sequencing protocols were performed to detect mutations in fluoroquinolones-resistance determining regions (QRDR) in *gyrA* and *gyrB* according to Lau et al (Lau et al, 2011). The DNA sequences were assembled and edited by using BioEdit

software version 7.0.5.3. The genetic polymorphisms of *gyrA* and *gyrB* were compared with those sequences of *M. tuberculosis* strain H37Rv in GenBank accession number: NC_000962.2 (Takiff et al, 2004).

## 3. Results:

**3.1 Drug susceptibility profiles.** The 206 FQ-resistant isolates were tested for susceptibility to INH, RIF, STR, and ETH. A total of 43.9% (n = 90/206) of isolates were MDR and 39.3% (n=81/206) belonged to XDR. The drug susceptibility profiles of the 206 FQ-resistant isolates with different *gyrA/B* mutations patterns were shown in table 1.

Among 21 ofloxacin-susceptible strains, 76.2% (n=16/21) of them were pan-susceptible, 19.0% (n=4/21) were mono-resistant (one was resistant to STR and three were resistant to INH) while 4.8% (n=1/21) of isolates were resistant to both STR and INH.

**3.2 Distribution of *gyrA* mutations among *M. tuberculosis* clinical isolates.** The *gyrA* QRDR PCR were amplified successfully for all 227 isolates. Upon sequence analyses, no deletion and insertion were found. All the strains possessed a natural polymorphism at amino acid position 95 with serine substituted by thronine (AGC → ACC), which had shown to be unrelated to fluoroquinolone- resistance in *M. tuberculosis* (Ginsburg et al, 2003). None of 21 $FQ^S$ isolates harboured

resistance-associated mutation in *gyrA* QRDR, whereas 156 of 206 $FQ^r$ clinical isolates harboured resistance-associated mutations in this region, given the specificity and sensitivity to be 100% and 75.7% respectively.

Among the 156 *gyrA* mutants, 151 harbored single mutation at amino acid positions 88, 90, 91 or 94 whereas 5 showed double mutations in both 90 and 91or both 90 and 94.

Position 94 was the most frequent resistance-associated mutation site found in $FQ^r$ clinical isolates, resulting in seven different amino acid substitutions: D94G (n = 57), D94A (n = 23), D94C(n=2) D94Y (n = 1), D94N(n = 20), D94V(n = 1) and D94H (n = 3), and accounted for 57.3% of fluoroquinolone- resistance in *M. tuberculosis* isolates. Position 90 is the second most prevalent mutation site, which accounted for 16% of $FQ^r$ clinical isolates. The mutation patterns of *gyrA* QRDR among 206 $FQ^r$ clinical isolates were shown in table 2.

Among 156 *gyrA* mutants, 61 (39.1%) were XDR and 74 (47.3%) were MDR, accounting for 75.3% (61/81）XDR and 82.2% (74/90)MDR $FQ^r$ *M .tuberculosis* in our collection Table 1.

**3.3 Distribution of *gyrB* mutations among *M. tuberculosis* clinical isolates.** The *gyrB* QRDR PCR were also amplified successfully for isolates. Among 206 ofloxacin-resistant *M. tuberculosis* clinical isolates, 32 of them harboured mutations in *gyrB* gene. Of them, 78.1%(n=25/32)

also harboured mutations in *gyrA* QRDR. No *gyrB* mutations were found in those 21 $FQ^s$ isolates. A total of 18 amino acid substitutions were found in *gyrB* gene, with position 424 being the most frequent mutation site (Table 2). Among the 18 amino acid substitutions, 10 were novel mutations (Table 2) that were first reported in this study.

Among 32 *gyrB* mutants, 13 (40.6%) were XDR and 17 (53.1%) were MDR, accounting for 16% (13/81）XDR and 18.9% (17/90)MDR $FQ^r$ *M. tuberculosis* in our collection (Table 1).

## 4. Discussion

Despite continued efforts directed to improve tuberculosis control programs at national level, China remains as a major region with the greatest burden of MDR-TB. In addition to resistance towards INH and RIF, 27.4% of MDR-TB also showed resistance to FQ, which is the most potent drug against MDR-TB. This may reflect the extensive usage of FQ in treatment for undiagnosed bacterial infections in China (Jiang, 1992, World Health Organization, 2010).

In present study, a total of 75.7% of $FQ^r$ isolates were showed to harbour mutations in *gyrA* QRDR, with amino acid position 94 being the most predominant mutation site. The reported frequency is similar to that in Hong Kong (75%) and that in Rwanda (75%) although it is lower than that in Russia (83%) and higher than that in Taiwan (50%) (Chan et al.,

2007, Huang et al., 2005, Mokrousov et al., 2008, Umubyeyi et al.,2007), showing that mutations in *gyrA* QRDR was the key factor leading to quinolone-resistance in *M. tuberculosis* in China.

As reported in other studies (Huang et al, 2005, Lau et al, 2011, Mokrousov et al, 2008), *gyrB* QRDR mutations only accounted for a minority of FQ$^r$ *M. tuberculosis*. Ten novel mutations were found in this study. However, transformation studies are needed to confirm their contribution in FQ$^r$ *M. tuberculosis*.

In this study, we recruited a large cohort of 206 FQ$^r$ *M. tuberculosis* clinical isolates to investigate the types and frequencies of *gyrA* and *gyrB* mutations in FQ$^r$ *M. tuberculosis* circulating in China. Our findings are highly representative for molecular patterns of FQ$^r$ *M. tuberculosis* in this region. A previous study with fewer FQ$^r$ *M. tuberculosis* samples reported that only 8% of their strains had neither *gyrA* nor *gyrB* mutations (Cui et al., 2011). However, this study revealed more than 20.9% of our FQ$^r$ isolates harbored no known FQ resistance mechanisms, indicating that more comprehensive information would be available when more strains are included for investigation.

For those isolates with no known mutations, it has been suggested that the mechanism for resistance in such isolates may be mediated by active efflux pumps, as *in vitro* studies have shown that the use of efflux pump inhibitors resulted in the reduction of MIC levels of FQ (Escribano et al,

2007, Louw et al, 2011, Singh et al, 2011).

Although development of $FQ^r$ is a critical step for a MDR strain converting to be XDR, either *gyrA* or *gyrB* alone, or in combination, did not represent as a reliable marker for predicting XDR-TB, with more than 50% *gyrA* or *gyrB* mutants are non-XDR strains. Amikacin resistance and capreomycin resistance are also the important criteria in defining XDR-TB. Mutations in the 1400 region of *rrs* have been detected in isolates that are resistant to these drugs. Detection of both *gyrA* and *rrs* mutations may be more reliable for prediction of XDR phenotype.

In conclusion, our study indicated that the molecular characterization in $FQ^r$ *M. tuberculosis* collected in China is similar to those reported elsewhere, with mutations in *gyrA* QRDR being the most predominant resistance determinant. PCR-sequencing of *gyrA* QRDR is a reliable molecular detection marker for $FQ^r$ *M. tuberculosis* in China

**Acknowledgements**

This study was supported by two grants from the the Major Programs of the National Science & Technology during the Eleventh Five-Year Plan Period（No. 2008ZX10001-008；2009ZX10003-017）

## References

Chan RC, Hui M, Chan EW, Au TK, Chin ML, Yip CK, AuYeang CK, Yeung CY,

243 Kam KM, Yip PC, Cheng AF.(2007) Genetic and phenotypic characterization
244 of drug-resistant Mycobacterium tuberculosis isolates in Hong Kong. *J*
245 *Antimicrob Chemother* 59:866-73.
246 Clinical and Laboratory Standards Institute. (2008) Laboratory detection and
247 identification of Mycobacteria; Approved guideline. M48-A
248 Cui Z, Wang J, Lu J, Huang X, Hu Z. (2011) Association of mutation patterns in
249 gyrA/B genes and ofloxacin resistance levels in Mycobacterium tuberculosis
250 isolates from East China in 2009. *BMC Infectious Disease* 11: 78-82.
251 Escribano I, Rodriguez JC, Llorca B, Garcia-Pachon E, Ruiz M, Royo G. (2007)
252 Importance of the efflux pump systems in the resistance of Mycobacterium
253 tuberculosis to fluoroquinolones and linezolid. *Chemotherapy* 53: 397-401.
254 Ginsburg AS., Grosset JH, and Bishai WR.(2003) Fluoroquinolones,tuberculosis, and
255 resistance. *Lancet Infect. Dis* 3:432–442.
256 Hu WZ, Li LJ, Guan L.(1992) Fluroquinolones in the treatment of lower respiratory
257 tract infections. *Chinese Joural of Antibiotics* 17:122-125.
258 Huang TS., Kunin CM., Lee SSJ., Chen YS., Tu HZ., Liu YC.(2005) Trends in
259 fluoroquinolone resistance of Mycobacterium tuberculosis complex in a
260 Taiwanese medical centre: 1995-2003. *J Antimicrob Chemother* 56:
261 1058-1062.
262 Jiang SC (1992) Fluroquinolones in the treatment of bacterial digestive tract
263 infections. *Chinese Joural of Antibiotics* 17:118-121.
264 Lau RW, Ho PL, Kao RY, Yew WW, Lau TC, Cheng VC, Yuen KY, Tsui SK, Chen X,

Yam WC.(2011) Molecular characterization of fluoroquinolone resistance in Mycobacterium tuberculosis: functional analysis of gyrA mutation at position 74. *Antimicrob Agents Chemother* 55:608-614.

Louw GE, Warren RM, Gey van Pittus NC, Leon R, Jimenez A, Hernandez-Pando R, McEvoy CR, Grobbelaar M, Murray M, van Helden PD, Victor TC. (2011) Rifampicin Reduces Susceptibility to Ofloxacin in Rifampicin-resistant Mycobacterium tuberculosis through Efflux. *American Journal of Respiratory and Critical Care Medicine* 184: 269-276.

Mokrousov I, Otten T, Manicheva O, Potapova Y, Vishnevsky B, Narvskaya O, Rastogi N. (2008) Molecular characterization of ofloxacin-resistant Mycobacterium tuberculosis strains from Russia. *Antimicrob Agents Chemother* 52:2937-9.

Singh M, Jadaun GPS, Ramdas, Srivastava K, Chauhan V, Mishra R, Gupta K, Nair S, Chauhan DS, Sharma VD, Venkatesan K, Katoch VM. (2011) Effect of efflux pump inhibitors on drug susceptibility of ofloxacin resistant mycobacterium tuberculosis isolates. *Indian J Med Res* 133: 535-540.

Sun Z, Zhang J, Zhang X, Wang S, Zhang Y, Li C. (2007) Comparison of gyrA gene mutations between laboratory-selected ofloxacin-resistant *Mycobacterium tuberculosis* strains and clinical isolates. *Int J Antimicrob Agents* 31:115-21

Takiff HE, Salazar L, Guerrero C, Philipp W, Huang WM, Kreiswirth B, Cole ST, Jacobs WR Jr, Telenti A. (1994) Cloning and nucleotide sequence of Mycobacterium tuberculosis gyrA and gyrB genes and detection of quinolone

resistance mutations. *Antimicrob Agents Chemother* 38:773-780.

Vien le TM, Abuoun M, Morrison V, Thomson N, Campbell JI, Woodward MJ, Van Vinh Chau N, Farrar J, Schultsz C, Baker S.(2011) Differential phenotypic and genotypic characteristics of qnrS1-harboring plaSTRids carried by hospital and community commensal enterobacteria. *Antimicrob Agents Chemother* 55:1798-802.

Umubyeyi AN, Rigouts L, Shamputa IC, Fissette K, Elkrim Y, de Rijk PW, Struelens MJ, Portaels F. (2007) Limited fluoroquinolone resistance among Mycobacterium tuberculosis isolates from Rwanda: results of a national survey. J *Antimicrob Chemother* 59:1031-3.

Wise R.(2003) Maximizing efficacy and reducing the emergence of resistance..*J Antimicrob Chemother* 51 Suppl 1:37-42.

World Health Organization (2001) Guidelines for drug susceptibility testing for second line anti-tuberculosis drugs for DOTS plus.(WHO/CDS/TB/2001.288).

World Health Organization (2008) Policy guidance on drug-susceptibility testing (DST) of second-line antituberculosis drugs. WHO/HTM/TB/2008.392.

World Health Organization.(2010) Multidrug and extensively drug-resistant TB (M/XDR-TB): global report on surveillance and response.:WHO/HTM/TB/2010.3

World Health Organization.(2011) Towards universal access to diagnosis and treatment of multidrug-resistant and extensively drug-resistant tuberculosis

by 2015 :WHO progress report : WHO/HTM/TB/2011.3

Xu P, Li X, Zhao M, Gui X, DeRiemer K, Gagneux S, Mei J, Gao Q. (2009) Prevalence of Fluoroquinolone Resistance among Tuberculosis Patients in Shanghai, China. *Antimicrob Agents Chemother* 53:3170-72

Zhou TL, Chen XJ, Zhou MM, Zhao YJ, Luo XH and Bao QY (2011) Prevalence of plaSTRid-mediated quinolone resistance in Escherichia coli isolates in Wenzhou, Southern China, 2002-2008. *Jpn J Infect Dis* 64:55-7.

Table 1: The association between drug resistance patterns and mutations in gyrA and gyrB in 206 FQ-resistant isolates.

| Resistance Pattern[a] | gyrA only | gyrB only | gyrA + gyrB | Non-gyrA nor gyrB | Total |
|---|---|---|---|---|---|
| FQ only | 6 | 0 | 0 | 10 | 16 |
| FQ, INH | 3 | 0 | 0 | 1 | 4 |
| FQ, STR | 1 | 0 | 0 | 0 | 1 |
| FQ, EMB | 1 | 0 | 0 | 0 | 1 |
| FQ, CAP | 1 | 0 | 1 | 0 | 2 |
| FQ, INH, STR | 3 | 0 | 0 | 0 | 3 |
| FQ, INH, EMB,AMK | 0 | 0 | 0 | 1 | 1 |
| FQ, INH, STR, EMB | 1 | 0 | 0 | 0 | 1 |
| FQ, INH, STR,AMK | 1 | 0 | 0 | 0 | 1 |
| FQ, INH,STR,AMK,CAP | 0 | 0 | 0 | 1 | 1 |
| FQ, INH,CAP | 0 | 0 | 1 | 0 | 1 |
| FQ, RIF, STR, EMB | 1 | 0 | 0 | 0 | 1 |
| FQ,AMK,CAP | 1 | 0 | 0 | 0 | 1 |
| FQ, EMB,AMK,CAP | 0 | 0 | 0 | 1 | 1 |
| FQ, MDR | 59 | 2 | 15 | 14 | 90 |
| XDR | 53 | 5 | 8 | 15 | 81 |
| Total | 131 | 7 | 25 | 43 | 206 |

[a] FQ: Fluoroquinolones; INH: Isoniazid; STR: Streptomycin; EMB: Ethambutol; CAP: Capreomycin; AMK: Amikacin; RIF: Rifampcin; MDR: Multidrug resistance which is defined as *M. tuberculosis* strains that are resistant to both isoniazid and rifampin; XDR: Extensively drug-resistance which is defined as MDR-TB strains which are further resistant to any fluoroquinolone and any of the second-line anti-TB injectable drugs (amikacin, kanamycin or capreomycin)

Table 2: Mutation patterns in *gyrA* and *gyrB* identified in 206 FQ-resistant isolates by sequencing

| gyrA mutation | gyrB mutation | No. of isolates(%) |
|---|---|---|
| A90V | Wild type | 28(13.59) |
| A90V S91P | Wild type | 3(1.47) |
| A90V D94H | Wild type | 1(0.49) |
| A90V D94N | Wild type | 1(0.49) |
| S91P | Wild type | 5(2.4) |
| S91P | N464S | 1(0.49) |
| D94A | Wild type | 14(6.8) |
| D94A | T465P* | 2(0.97) |
| D94A | N464T* | 2(0.97) |
| D94A | N464K | 1(0.49) |
| D94A | I458M | 1(0.49) |
| D94A | E481Q D483H | 1(0.49) |
| D94A | E424K* | 1(0.49) |
| D94C | Wild type | 2(0.97) |
| D94G | Wild type | 46(22.3) |
| D94G | E424K* | 4(1.9) |
| D94G | A469V* | 1(0.49) |
| D94G | E522Q* | 3(1.47) |
| D94G | D414P* | 1(0.49) |
| D94G | D414K* | 1(0.49) |
| D94G | R457G | 1(0.49) |
| D94H | Wild type | 3(1.47) |
| D94N | Wild type | 17(8.25) |
| D94N | N464S* | 1(0.49) |
| D94N | V461A | 1(0.49) |
| D94N | E419K* E424K* R460K | 1(0.49) |
| D94V | N464T* | 1(0.49) |
| D94Y | Wild type | 9(4.37) |
| D94Y | E419K* | 1(0.49) |
| G88A | Wild type | 1(0.49) |
| Wild type | S434A | 1(0.49) |
| Wild type | E424K* | 4(1.9) |
| Wild type | G425E | 1(0.49) |
| Wild type | E419K* T465P* | 1(0.49) |
| Wild type | Wild type | 43(20.9) |
| | | 206(100) |

*: Novel mutations