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Short Communication

Identification of high-level ceftriaxone-resistant *Neisseria gonorrhoeae* isolates with diverse *penA* alleles in Zhejiang, China

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ABSTRACT

Objectives: The prevalence of ceftriaxone-resistant *Neisseria gonorrhoeae* poses a significant threat to the effectiveness of gonorrhoea treatment. The aim of the present study was to analyse the characteristics of ceftriaxone-resistant *N. gonorrhoeae*, with a specific focus on high-level ceftriaxone-resistant strains.
Methods: A total of 207 strains of *N. gonorrhoeae* were collected from hospitals in Zhejiang, China, between 2019 and 2020. From this collection, we selected 8 strains of ceftriaxone-resistant *N. gonorrhoeae* for whole-genome sequencing, genotyping, and molecular profile analysis. For clonal strains (FC428-like), we conducted a phylogenetic analysis to understand their origin and evolutionary path.
Results: Among the selected strains, 5 demonstrated high-level ceftriaxone resistance (MIC 1–2 mg/L). The genotyping results showed that these isolates had a higher diversity of *penA* alleles than expected. Four isolates had mosaic *penA*-60.001 allele and the remaining four had different non-mosaic *penA* alleles. Phylogenetic analysis suggested that the emergence of FC428-like clones containing *penA*-60.001 may result from further dissemination of different FC428 subclones from different regions of China. The identification of high-level ceftriaxone resistance in non-mosaic *penA* gonococci, specifically in the ZJ20-3 isolate (*penA*-21.001) with an MIC of 2 mg/L, is a groundbreaking discovery.
Conclusions: We present a comprehensive analysis of ceftriaxone-resistant *N. gonorrhoeae* isolates in Zhejiang, highlighting a significant diversity of *penA* alleles. The identification of strains exhibiting resistance to ceftriaxone at high levels in our study underscores the potential threat to existing protocols for gonorrhoea treatment. Consequently, we strongly emphasize the urgent need to enhance surveillance initiatives focused on ceftriaxone-resistant *N. gonorrhoeae*.

1. Introduction

The emergence and spread of multidrug-resistant *N. gonorrhoeae* strains have become a significant global public health concern. The potential for untreatable gonorrhoea in the future is a reality; in early 2017, *N. gonorrhoeae* was listed among antibiotic-resistant pathogens that pose a global threat to human health and need priority efforts toward developing new antibiotics by

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the World Health Organization (WHO) [1]. Currently, ceftriaxone is the only remaining option for empirical monotherapy or dual antimicrobial treatment (ceftriaxone plus azithromycin) of gonococcal infections. However, research studies have demonstrated an escalating incidence of global ceftriaxone-resistant *N. gonorrhoeae*, signifying an increasing prevalence of this antimicrobial resistance (AMR) phenomenon on an international scale [2–4]. A retrospective observational study of AMR data from gonococcal isolates reported to WHO in 2017–18 revealed that 31% (21 out of 68 reporting countries) reported decreased susceptibility or resistance to ceftriaxone. The prevalence of decreased susceptibility or resistance to ceftriaxone ranged from 0% to 21% annually across countries, indicating an ongoing emergence of resistance in several nations [5]. Asia is the predominant source of documented ceftriaxone treatment failures in cases of gonococcal infections, with multiple ceftriaxone-resistant strains believed to have originated in this region and subsequently disseminated worldwide [6,7]. Notably, China has been specifically identified as a prominent hotspot for the emergence and dissemination of ceftriaxone-resistant strain [8–10]. In Zhejiang, there has been a consistent and notable rise in the occurrence of ceftriaxone resistance and decreased susceptibility among *N. gonorrhoeae* isolates in Hangzhou between 2015 and 2017. Specifically, the prevalence of ceftriaxone resistance increased from 1% to 5%, while the prevalence of decreased susceptibility rose from 9% to 27% [8]. This study presents an analysis of recently identified ceftriaxone-resistant *N. gonorrhoeae* isolates discovered in the Zhejiang region. These isolates display a diverse array of *penA* alleles, with a subset of them exhibiting high-level ceftriaxone resistance.

2. Materials and methods

2.1. Clinical isolates and antimicrobial susceptibility testing

In a regional drug resistance surveillance study, a comprehensive collection of 207 *N. gonorrhoeae* isolates was obtained from 207 patients who were diagnosed with symptomatic urethritis (characterized by dysuria and/or urethral discharge) between the years 2019 and 2020. The collection took place in hospitals located in Deqing, Zhejiang Province. Urogenital specimens were obtained using sterile Dacron swabs and streaked onto Thayer-Martin (T-M) agar supplemented with 1% IsoVitaleX (Oxoid, USA). *N. gonorrhoeae* identification included routine clinical tests (oxidase, Gram staining, and glucose utilization) [11]. One isolate per patient was stored at −80°C following our previously described protocol [10]. Antimicrobial susceptibility testing was conducted using agar dilution, following our established methodology [10]. The results were interpreted according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST), using breakpoint tables for interpretation of MICs and zone diameters, version 12.0, 2022 (http://www.EUCAST.org). The main purpose of this study is to explore the resistance and molecular characteristics of ceftriaxone-resistant *N. gonorrhoeae* strains. Therefore, this study exclusively includes strains with ceftriaxone MIC values >0.125 mg/L.

2.2. Genotyping and phylogenetic analysis

In this study, nucleic acid extraction and purification were performed using the QIAamp DNA Mini Kit (QIAgen, Hilden, Germany) according to the manufacturer's protocol. The WGS of these isolates was performed using Illumina HiSeq X Ten sequencer (Illumina, San Diego, CA) with 150 bp paired-end reads. Raw sequence reads are available under BioProject PRJNA560592 in NCBI. And the data was used for *N. gonorrhoeae* sequence typing for antimicrobial resistance (NG-STAR) and multi-locus se-

Table 1
Clinical information and antimicrobial susceptibility of ceftriaxone-resistant *N. gonorrhoeae* in Zhejiang, China.

Isolate id	Patient gender	Date of clinic visit	Age	Sexual orientation	Culture result	Sampling site	Treatment	Residence of patients in the past six months	Gonorrhea history	Antibiotic history	MIC (mg/L)					
											CRO	CEF	AZM	CIP	PEN	SPT
ZJ19-F9	M	2/11/2019	19	hetero	POS	urethral	NA	NA	NA	NA	0.25/R	1/R	0.25/S	>8/R	0.25/S	16/S
ZJ20-23	M	31/7/2020	16	hetero	POS	urethral	SPT+CXT	local	NO	NO	0.5/R	1/R	0.5/S	8/R	2/R	16/S
ZJ20-215	M	16/4/2020	29	bisexual	POS	urethral	NA	local	NO	NO	1/R	>2/R	0.25/S	>8/R	4/R	16/S
ZJ20-230	M	14/5/2020	22	hetero	POS	urethral	NA	local	NO	NO	1/R	2/R	>4/R	>8/R	2/R	16/S
ZJ20-3	M	31/12/2019	31	hetero	POS	urethral	AZM	local	Yes	NO	2/R	>2/R	>4/R	>8/R	1/S	16/S
ZJ20-5	M	14/1/2020	22	hetero	POS	urethral	SPT+DOX	local	NO	NO	1/R	>2/R	≤0.03/S	>8/R	>16/R	32/S
ZJ20-9	F	10/3/2020	22	hetero	POS	cervix	SPT+DOX	local	NO	NO	0.5/R	>2/R	≤0.03/S	8/R	>16/R	32/S
ZJ20-12	M	26/3/2020	58	hetero	POS	urethral	SPT+DOX	local	NO	NO	1/R	2/R	>4/R	>8/R	4/R	16/S

AZM, azithromycin; CEF, cefixime; CIP, ciprofloxacin; CRO, ceftriaxone; CXT, cefoxitin; DOX, doxycycline; F, female; M, male; NA, not available; PEN, penicillin; POS, positive; R, resistant; S, susceptible; SPT, spectinomycin.

Table 2
Genotyping of ceftriaxone-resistant *N. gonorrhoeae* in Zhejiang, China.

Isolate	*penA*	*mtrR*	*porB*	*ponA*	*gyrA*	*parC*	23S rRNA	NG-STAR ST	*abcZ*	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*	MLST ST
ZJ19-F9	60.001	1	12	1	7	3	100	1143	126	39	67	157	148	153	65	1903
ZJ20-23	60.001	1	4	1	7	3	100	2239	126	39	67	157	148	153	65	1903
ZJ20-215	60.001	1	8	1	7	3	100	233	126	39	67	157	148	153	65	1903
ZJ20-230	60.001	1	8	1	7	53	2	2238	126	39	67	78	148	153	65	1600
ZJ20-3	21.001	19	12	1	7	3	100	1142	109	39	170	111	148	153	65	1901
ZJ20-5	12.001	22	8	1	34	93	100	2473	59	39	170	78	148	153	65	8123
ZJ20-9	13.001	1	8	1	1	177[a]	100	5117[b]	59	39	67	158	148	153	65	7827
ZJ20-12	2.001	1	8	1	5	100	100	5116[b]	109	39	67	78	148	153	65	7356

NOTE: Columns 2-8 and 10-16 denote the allele types of genes.
[a] New allele type;
[b] New NG-STAR ST;

quence typing (MLST) (NG-STAR, https://ngstar.canada.ca; MLST, https://pubmlst.org/organisms/neisseria-spp/). Novel NG-STAR STs in this study have been submitted to the database. Phylogenetic analysis was used to investigate the evolutionary of FC428-like isolates (have a close phylogenomic relationship to FC428 and share the extended-spectrum cephalosporin-resistant genetic determinant, mosaic *penA*-60.001 gene) and help infer their likely origin. To achieve this, a concatenate superset of SNPs relative to FA1090 was generated as previously described [12]. Based on the genome-wide SNP sites, a phylogenetic tree was constructed using Geneious Primer software with the PhyML 3.3.20180621 plugin using the HKY85 substitution model, 100 bootstrap replicates, and estimated proportion of invariable sites.

3. Results and discussion

3.1. Clinical isolates and antimicrobial susceptibility

Our study included a total of 8 isolates of ceftriaxone-resistant *N. gonorrhoeae*, as determined by the results of antimicrobial susceptibility testing. Clinical patient information and antimicrobial susceptibility data have been documented (Table 1). The observation that majority of patients belonged to the young age group (16–31 years old), with only one patient being 58 years old, is consistent with the fact that gonorrhoea is most commonly reported among sexually active young adults. Furthermore, our study reveals significant levels of resistance to third generation cephalosporins (ceftriaxone MIC 0.25 to 2 mg/L and cefixime MIC 1 to >2 mg/L). To our knowledge, there have been no reports of non-mosaic *penA N. gonorrhoeae* with ceftriaxone MIC values exceeding 0.5 mg/L [13]. Here, we identified three non-mosaic *penA* gonococcal, ZJ20-3 (*penA*-21.001), ZJ20-5 (*penA*-12.001), and ZJ20-12 (*penA*-2.001), with ceftriaxone MIC values ranging from 1 to 2 mg/L. All strains showed ciprofloxacin resistance, six showed penicillin resistance, and three exhibited moderate azithromycin resistance (MIC > 4 mg/L). However, all isolates were susceptible to spectinomycin, which could serve as an alternative treatment option for gonorrhoea in cases of cephalosporin resistance.

3.2. Genotyping and phylogenetic analysis of mosaic penA N. gonorrhoeae

Genotyping analysis revealed that four possessed FC428-associated mosaic *penA*-60.001 alleles (Table 2). Among these isolates, three shared the same MLST type (ST1903) as the original FC428 strain, whereas the remaining isolate shared a distinct MLST type (ST1600). NG-STAR analysis revealed the presence of four STs, with only one (ST 233) matching the original FC428 strain (Figure 1b). ZJ20-215 was found to have the same MLST and NG-STAR STs as SRRSH207 and SRRSH240, respectively, which were isolated from Hangzhou, Zhejiang. Similarly, ZJ20-230 had the same MLST ST and a closely related NG-STAR ST (ST2238 and ST2208 sharing six identical loci, except 23S rRNA) as the five other strains (SRRSH203, SRRSH204, SRRSH205, SRRSH214, and SRRSH229) isolated in Hangzhou [14]. All of these isolates demonstrated elevated ceftriaxone MIC (1 mg/L), which underscores the potential for these clones to disseminate more extensively in the region, warranting further attention and concern. The key resistance-mediating amino acid substitutions, A311V and T483S, as well as G545S, I312M, and V316T in the penicillin (PEN)-binding protein 2 (PBP2) encoded by the *penA*-60.001 gene, were highly associated with resistance to ceftriaxone, while the point mutation at position 2611 of 23S rRNA (ZJ20-230) confer moderate resistance to azithromycin [15] (Supplementary Table S1).

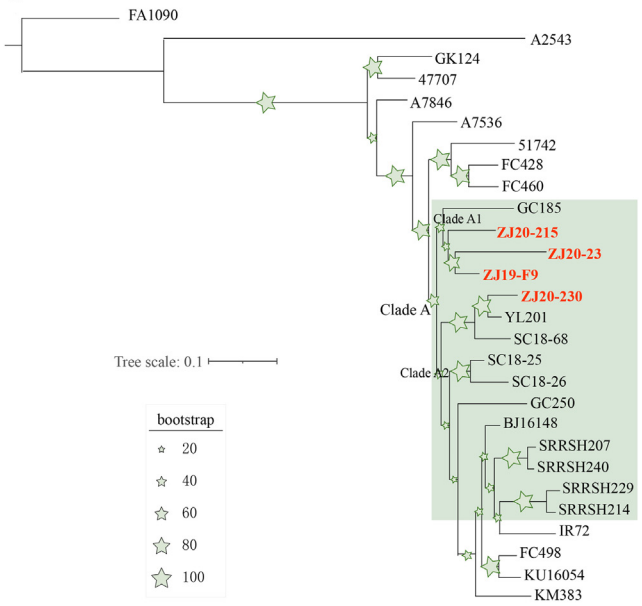
Phylogenetic analysis suggested that FC428-like clones from China were highly concentrated in clade A, which was further separated into two subclades, A1 and A2 (Figure 1a). Clade A1 consisted of ZJ20-215, ZJ20-23, and ZJ19-F9, as well as GC185 from Changsha [16]. Another newly isolated ZJ20-230 strain clustered with clade A2, showing a close relationship with YL201 from Shenzhen [9]. Interestingly, although SRRSH207, SRRSH214, SRRSH229 and SRRSH240 [14] are located in clade A2, they do not cluster near ZJ20-3, indicating that sustained transmission and multiple introductions of different FC428 subclones have occurred in Zhejiang.

3.3. Genotyping and AMR determinants of non-mosaic penA N. gonorrhoeae

The newly identified non-mosaic *penA* strains belong to different NG-STAR and MLST STs (Table 2). Genogroup MLST ST7827 (ZJ20-9), which originated in Asia, was the predominant MLST in China during 2012 to 2013 but was not particularly associated with reduced susceptibility to cephalosporins at that time [12]. However, it subsequently emerged and dominated in Norway and Netherlands [17], with the occurrence of decreased susceptibility. Considering the emergence of ceftriaxone resistance observed in this study and the rapid spread of the genogroup, close monitoring of the prevalence and drug resistance profile in China is needed. Additionally, ZJ20-9 was isolated from heterosexual females, whereas previous observations showed that MLST ST7827 isolates were almost exclusively isolated from men and transmitted among men who have sex with men [17]. However, more information about the patient's sexual partner is not available.

Drug-resistant non-clonal *N. gonorrhoeae* is attributed to diverse molecular mechanisms. The primary mechanism associated with ceftriaxone resistance is the presence of mutations in the *penA* gene. Specifically, substitutions in the PBP2 A501 residue have been shown to contribute to ceftriaxone resistance in non-clonal *N. gonorrhoeae* strains [15]. In our study, we identified four iso-

a



b

Sample	Country	Year	MLST	NG-STAR	MIC(mg/L)		
					CRO	CFM	AZM
A2543	Australia	2018	12039	996	0.5	NA	>256
GK124	Denmark	2017	1903	233	0.5	1	0.5
47707	Canada	2017	1903	233	1	2	0.5
A7846	Australia	2017	1903	233	0.5	NA	0.25
A7536	Australia	2017	1903	233	0.5	NA	0.25
51742	Canada	2018	1903	233	0.5	2	0.25
FC428	Japan	2015	1903	233	0.5	1	0.25
FC460	Japan	2015	1903	233	0.5	1	0.25
GC185	China	2017	1903	1143	1	NA	0.5
ZJ20-215	**China**	**2020**	**1903**	**233**	**1**	**>2**	**0.25**
ZJ20-23	**China**	**2020**	**1903**	**2239**	**0.5**	**1**	**0.5**
ZJ19-F9	**China**	**2019**	**1903**	**1143**	**0.25**	**1**	**0.25**
ZJ20-230	**China**	**2020**	**1600**	**2238**	**1**	**2**	**>4**
YL201	China	2020	1600	2238	0.75	NA	12
SC18-68	China	2018	7363	233	⩾0.5	NA	0.5
SC18-25	China	2018	1903	233	⩾0.5	NA	0.5
SC18-26	China	2018	1903	233	⩾0.5	NA	1
GC250	China	2018	7365	1143	0.5	NA	2
BJ16148	China	2016	1903	233	0.5	1	0.25
SRRSH207	China	2019	1903	233	1	2	0.1
SRRSH240	China	2019	1903	233	1	2	0.1
SRRSH229	China	2019	1600	2208	1	2	0.3
SRRSH214	China	2019	1600	2208	1	2	0.1
IR72	Ireland	2018	1903	1133	0.5	1-2	0.38-0.5
FC498	Japan	2015	1903	233	0.75	1.5	0.5
KU16054	Japan	2016	1903	233	0.5	1.5	0.19
KM383	Japan	2017	1903	233	0.5	1.5	0.125

Figure 1. (A) Maximum-likelihood tree based on 15,100 SNPs extracted from globally disseminated *N. gonorrhoeae* strains of *penA*-60.001 clones. The phylogenetic tree root was placed on FA1090. The four isolates from the current study are highlighted in red, while isolates within the light green boxes represent FC428-related clones found in regions of China, and the remaining strains are foreign *penA*-60.001 strains. The green five-pointed star on the branches represents the bootstrap value. The tree scale bar indicates the average number of SNPs per site. (B) Phenotypic and molecular characteristics of isolates related to the FC428 clone. NA indicates not available.

lates that did not possess the mosaic *penA* allele but contained PBP2 A517G substitutions. Among these four isolates, two exhibited double substitutions of PBP2 A501V and A517G (Supplementary Figure S1). Furthermore, prior research has documented that substitutions occurring at amino acid positions 120 and 121 within the putative loop 3 of PorB are correlated with resistance to ceftriaxone [18]. Our study aligns with these findings (Supplementary Figure S1), further supporting the association between these PorB substitutions and ceftriaxone resistance. It should be noted that despite the identification of these resistance determinants, they do not fully account for the observed drug resistance. Therefore, it is likely that additional factors play a role in conferring resistance, warranting further investigation. Our findings also revealed previously unreported mutations, such as H541N, P552V, and K555Q in PBP2, S539G and H553N in RpoB, and I229V in RpoD (Supplementary Figure S1). However, in order to assess the significance of these allelic variants, it is crucial to gather and extensively analyse data on the presence of the same alleles in both ceftriaxone-sensitive and ceftriaxone-resistant strains.

4. Conclusions

In summary, our study identified 8 ceftriaxone-resistant *N. gonorrhoeae* isolates from Zhejiang, China, 5 of which showed high-level resistance to ceftriaxone. Molecular genetic analysis revealed that these isolates have complex genetic backgrounds and harbour diverse *penA* alleles. Ceftriaxone-resistant clonal isolates, characterized by the presence of the mosaic *penA*-60.001 allele, are exhibiting a notable expansion throughout China. Additionally, our analysis reveals the presence of four distinct non-mosaic *penA* alleles within non-clonal ceftriaxone-resistant isolates. The ceftriaxone-resistant strain ZJ20-3, with an MIC value of 2 mg/L, has the potential to render current treatment regimens ineffective.

Zhejiang, an eastern coastal province of China, has a high incidence of gonorrhoea and significant population mobility, making it a potential hotspot for the dissemination of resistant strains. Strengthening the surveillance of AMR is of paramount importance and serves as a fundamental prerequisite. In this regard, rapid and cost-effective molecular assays, such as high-resolution melting assay, have demonstrated significant potential in the diagnosis of *N. gonorrhoeae* infection and the prediction of AMR [10]. However, their ability to detect both mosaic and non-mosaic *penA* alleles is currently limited. Furthermore, it is essential to acknowledge that ceftriaxone resistance is linked to multiple genetic markers beyond the *penA* gene. The accessibility of WGS, based on next-generation sequencing platforms or other sequencing platforms, is progressively improving, holding the potential to provide more precise genomic insights. To enhance future surveillance of gonorrhoea resistance, it is crucial to prioritize the advancement of detection technologies, conduct comprehensive research on drug resistance mechanisms, and establish effective links between AMR and molecular detection methods. Additionally, there is an urgent need to strengthen the development of novel antimicrobial agents and implement effective antimicrobial stewardship strategies to tackle the current global crisis of *N. gonorrhoeae* AMR.

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Competing interests: None declared.

Ethical approval statement: This study was conducted in compliance with the medical ethical committee of the Shanghai Skin Disease Hospital (approval number 2021-20KY) and registered in the Chinese Clinical Trial Registry (TRN: ChiCTR2100048771, registration date: 20210716). The patient provided written informed consent for the publication of their case details.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2023.08.007.

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