Identification and expression analysis of ceftriaxone resistance-related genes in *Neisseria gonorrhoeae* integrating RNA-Seq data and qRT-PCR validation

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ABSTRACT

Objectives: The aim of the study is to identify ceftriaxone resistance-related genes in *Neisseria gonorrhoeae*.
Methods: Differences in gene expression were compared between ceftriaxone-susceptible *N. gonorrhoeae* isolates [minimum inhibitory concentration (MIC) = 0.002–0.004 mg/L] and isolates with decreased ceftriaxone susceptibility (MIC = 0.125–0.5 mg/L) using RNA-Seq (RNA sequencing).
Results: Total RNA of 10 clinical isolates was used to make libraries and generated an average of 24.07 Mb reads per sample; these were assembled into 1871 mRNA genes. Moreover, 21 differentially expressed genes (DEGs) were found between the *N. gonorrhoeae* isolates with susceptibility and decreased susceptibility to ceftriaxone with a fold change of ≥ 2 ($P < 0.05$), among which 11 were upregulated and 10 were downregulated. Furthermore, all DEGs were verified by quantitative reverse transcription PCR (qRT-PCR), which detected 25 clinical isolates with decreased ceftriaxone susceptibility and 21 ceftriaxone-susceptible isolates. In addition, seven DEGs revealed relative expression levels by $2^{-\Delta\Delta Ct}$ and showed a statistical significance ($P \leq 0.05$). Analysis of Gene Ontology (GO) terms and KEGG pathway for functional enrichment showed that six DEGs were related to the cellular component and one DEG was related to the biosynthesis of antibiotics, and these results might be related to ceftriaxone resistance.
Conclusions: Examining ceftriaxone resistance-related genes in *N. gonorrhoeae* is necessary owing to the high morbidity and antimicrobial resistance of *N. gonorrhoeae*, especially its eventual resistance to third-generation extended-spectrum cephalosporins (cefixime and ceftriaxone). Moreover, this report provides a new direction for the study and control of ceftriaxone-resistant *N. gonorrhoeae*.

1. Introduction

Gonorrhoea is a sexually transmitted infection (STI) caused by the Gram-negative bacterium *Neisseria gonorrhoeae*. Gonorrhoea has become a global public-health issue owing to its high morbidity and antimicrobial resistance, especially the emergence of resistance to third-generation extended-spectrum cephalosporins (ESCs), namely cefixime and ceftriaxone [1–3]. The World Health Organization (WHO) reported 78 million new cases of gonorrhoea worldwide in 2012, with a global incidence rate of 19 per 1000 females and 24 per 1000 males; moreover, high prevalence values were reported in the Western Pacific and Africa [4]. Rapidly increasing rates of gonorrhoea have recently emerged. The US Centers for Disease Control and Prevention (CDC) reported 468 514 cases in 2016, which was an 18.5% increase from 2015 [5]. China reported 138 855 new cases in 2017, which was 20.7% higher than in 2016 [6]. The prevalence of gonorrhoea is becoming serious. In 2016, the WHO Global Strategy for STIs for 2016–2021 was approved by the UN World Health Assembly and one important goal of this strategy is to reduce the morbidity of gonorrhoea by resolving its broad-spectrum antimicrobial resistance [1,7].

Most countries use ESCs as the first choice for treating gonorrhoea [2,8]. However, resistance to cefixime, which was first identified in Japan in the early 2000s [9], has spread in Asia, France, Australia, Canada, Norway, South Africa and the UK [10].

The first strain with high-level resistance to ceftriaxone [minimum inhibitory concentration (MIC) = 2 mg/L], called a 'superbug', was reported in Japan in 2011 [11], followed by verified treatment failures in France, Australia, Canada, Slovenia and Sweden [12]. Moreover, treatment failure with dual therapy (500 mg ceftriaxone plus 1 g azithromycin) was reported in the UK in 2016 [13]. Few options are available for treating cephalosporin-resistant isolates owing to multidrug resistance. The WHO believes that gonorrhoea may once again have no treatment options [14].

The molecular mechanism of antimicrobial resistance in *N. gonorrhoeae* is a complex process that includes the destruction or modification of antibiotics, changes in the antibiotic target, reduced antibiotic concentrations in cells and increased efflux [2,15]. Many studies have shown that the mechanism of ESC resistance appears to be related to alterations in the *penA* gene encoding penicillin-binding protein type 2 (PBP2). Most *N. gonorrhoeae* isolates with reduced susceptibility to ESCs have a mosaic *penA* [16]. Incorporation of three mutations (A311V, T316P and T483S) in the mosaic *penA35* allele account for high-level ceftriaxone resistance (MICs of 2–4 mg/L) in *N. gonorrhoeae* strain H041 from Japan [17]. The changes in these residues have been observed in other highly resistant strains, namely strains F89 and A886, isolated from France and Australia, respectively [18]. However, recent studies have suggested that not all *N. gonorrhoeae* strains with reduced susceptibility to ESCs have a mosaic *penA* gene. Some important non-mosaic mutations have been identified [19]. In a study in South Korea, the PBP2 pattern containing a substitution at position 501 (Ala501 → Val) was identified and is considered to cause reduced susceptibility to cefixime without requiring additional mutations [20]. Previous studies have shown that the reduced susceptibilities of *N. gonorrhoeae* to cefixime or ceftriaxone are associated with mutations in *penA*, *mtrR*, *porB* and *ponA* genes [21–23]. In addition, overexpression of the MtrC–MtrD–MtrE efflux pump caused by mutation in the *mtrR* gene increases the level of antibiotic efflux, and alteration of *porB* leads to the inhibition of the entry of antibiotics into the cells [24]. Mutation of a gene encoding a subunit of an ATP-binding cassette (ABC) transporter results in an additional mutation in the FtsX protein at position 251 (R251H) that is involved in cephalosporin resistance [25]. However, these may not be the only factors involved in ESC resistance because the molecular mechanisms of these antibiotics are not well understood.

Recently, RNA sequencing (RNA-Seq) has become a powerful tool for investigating the transcriptional gene expression profile of an organism [26]. Differentially expressed genes (DEGs) are determined by RNA-Seq, and expression analysis of different isolates and the functional genes are analysed by functional enrichment analysis. RNA-Seq has been used to improve gene model predictions, to discover new transcripts, to measure transcript expression levels and to investigate molecular mechanisms [27]. Furthermore, RNA-Seq is more sensitive and efficient than previous microarrays for comparing gene expression profiles [28].

In the present study, clinical gonococci with decreased susceptibility to ceftriaxone (MIC ≥ 0.125 mg/L) were detected and RNA-Seq was employed to identify DEGs between susceptible (MIC ≤ 0.004 mg/L) and decreased susceptible isolates. The presence of DEGs was further verified by quantitative reverse transcription PCR (qRT-PCR) to detect clinical isolates with or without decreased susceptibility to ceftriaxone. Gene Ontology (GO) and KEGG pathway analyses were performed to identify the biological function of DEGs and to explore novel genes that might be related to ceftriaxone resistance in order to ultimately provide new clues for investigating molecular mechanisms.

2. Materials and methods

2.1. Ethical approval

This study was approved by the Medical Ethics Committee of Guangdong Provincial Centre for Skin Diseases and STD Control (China). The isolates used in the study were all residual clinical specimens, and no personal information was collected.

2.2. Isolation and culture of N. gonorrhoeae

Clinical isolates of *N. gonorrhoeae* were collected from outpatients with gonorrhoea attending sexually transmitted diseases clinics in Guangdong Province during 2016–2017. Gonococci were isolated in selective Thayer–Martin agar medium and their identity was confirmed by Gram staining, oxidase and catalase assays, and sugar fermentation test. All strains were preserved in skim milk and were stored at −70 °C.

2.3. Antimicrobial susceptibility testing

All isolates were assessed for susceptibility to ceftriaxone using the agar plate dilution method recommended by the WHO Western Pacific *N. gonorrhoeae* monitoring programme. Clinical isolates and WHO reference strains were suspended in 0.9% saline and were adjusted to ca. 10^8 CFU/mL (0.5 McFarland standard) as inoculum. A multipoint inoculator was used to inoculate the suspension into medium with ceftriaxone ranging from 0.002– 1 mg/L. MICs were determined following overnight incubation at 35 °C in a CO_2 incubator.

2.4. Construction of RNA-Seq library

Three clinical isolates with ceftriaxone MICs of ≤0.004 mg/L (susceptibility to ceftriaxone) were used as controls and seven clinical isolates with MICs of ≥0.125 mg/L (decreased susceptibility to ceftriaxone) were randomly selected for RNA-Seq.

To ensure the quality of the sequencing sample, an Agilent 2100 Bioanalyzer and Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA) were employed to detect total RNA [RNA concentration, RNA integrity number (RIN) value, 28S/18S ratio and fragment length], and RNA-Seq library construction was performed using BGISEQ-500 (BGI, Guangzhou, China). For subsequent analysis, high-quality sequencing reads were ensured by filtering the reads using SOAPnuke (BGI). After filtering, the remaining reads were called 'clean reads'.

Hierarchical indexing for spliced alignment of transcripts (HISAT) was then applied to obtain a genome map [29], and Bowtie2 was used to map the clean reads to the reference sequence and to calculate the gene alignment rate [30]. Next, the gene and transcript expression levels [fragments per kilobase (FPKM) value] were calculated using RSEM [31], and the function 'cor' was used to calculate the Pearson correlation coefficient between every two samples for RSEM. Finally, hierarchical clustering and 'princomp' were performed for principal component analysis.

2.5. Detection of differentially expressed genes

To identify DEGs between ceftriaxone-susceptible and decreased susceptibility *N. gonorrhoeae* isolates, DEGs were detected using DEseq2 and PossionDis. DEseq2 is based on the negative binomial distribution. Screening for DEGs with fold change ≥2 ($P ≤ 0.05$) was performed as described by Love et al. [32]. PossionDis is based on the Poisson distribution. Fold change ≥2 and false discovery rate (FDR) ≤0.001 were determined as described by Audic and Claverie [33]. Moreover, the fold change

between different samples was calculated based on expression of the gene. A smaller FDR and larger fold change indicated increased significance of expression of the difference between the samples.

2.6. Gene Ontology and KEGG pathway analyses of differentially expressed genes

To understand the biological function of DEGs, GO term and KEGG pathway were considered in the functional enrichment and were performed using phyper, a function of RSEM, with *N. gonorrhoeae* strain FA1090 as the reference set. The *P*-value calculation formula in the hypergeometric test is:

$$P = 1 - \sum_{i=0}^{n-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

(see wiki for details: https://en.wikipedia.org/wiki/Hypergeo-metric_distribution).

The FDR for each *P*-value was calculated, wherein FDR $\leq$ 0.01 is defined as significantly enriched values. Hence, genes that were involved in cephalosporin resistance were discovered. Moreover, DEGs were classified into different pathways that may affect bacterial resistance with the KEGG annotation result.

2.7. RNA extraction and qRT-PCR

To validate the findings of RNA-Seq data, all DEGs were selected to confirm their expression in ceftriaxone-susceptible or decreased susceptible isolates. A total of 46 *N. gonorrhoeae* strains from Guangdong Province isolated during 2016–2017 were selected, among which 25 were decreased susceptible and 21 were susceptible. Total RNA was extracted from each sample using the TRIzol method, and reverse transcription was performed on a T100™ Thermal Cycler (Bio-Rad, Hercules, CA) to obtain cDNA. The obtained cDNA was used as a template for qRT-PCR (CFX Maestro™; Bio-Rad). cDNA (1 μg) was added to the reaction mixture including 2× SYBR Green Master Mix (TIANGEN, Beijing, China), forward and reverse primer (primer sequences are listed in Table 1) and diethylpyrocarbonate (DEPC)-treated water (Bio-Sharp, Hefei, China) to reach a total volume of 20 μL. Thereafter,

qRT-PCR was performed under the following conditions: 94 °C for 3 min, followed by 40 cycles at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s. The plate read for SYBR melting curves was derived in every reaction. All target genes were performed using 16S rRNA as the reference, and each analysis was performed in triplicate. The relative expression levels were calculated using $2^{-\Delta\Delta}$Ct method.

3. Results

3.1. Isolate grouping for RNA-Seq

Clinical *N. gonorrhoeae* isolates were analysed for their ceftriaxone MIC and were divided into two categories: susceptible, MIC $\leq$ 0.004 mg/L; and decreased susceptible (MIC $\geq$ 0.125 mg/L). Three samples were randomly selected from the susceptible *N. gonorrhoeae* and were used in the control group (Ctr1, Ctr2 and Ctr3); and seven samples were randomly selected from the decreased susceptibility *N. gonorrhoeae* group and were used as the sample group (S051, S01251, S0251, S01252, S052, S006 and S01253) (Table 2).

3.2. Construction of RNA-Seq library

Ten sequencing samples were applied to the BGISEQ-500 platform and generated an average of 24.07 Mb reads per sample. A total of 1871 genes were assembled and exhibited an average mapping ratio of 90.54%; the average values of Q20 and Q30 were 96.42% and 87.17%, respectively. Clean reads were mapped to the reference genome using HISAT and an average of 90.54% reads were matched. To show the gene amounts under different FPKM value, the gene amounts under three different FPKM ranges ($\leq$1, 1–10 and $\geq$10) were calculated. All samples exhibited high levels of expression, indicating that the data could be used to analyse the DEGs.

3.3. Detection of differentially expressed genes

DEGs between the control and sample groups can be identified based on the gene expression level. There were 21 DEGs between the two groups with a fold change $\geq$2 ($P \leq$ 0.05), among which 11 were upregulated and 10 were downregulated. In Fig. 1a, red, blue and grey represent the upregulated, downregulated and non-DEGs, respectively. Moreover, a heatmap plot was used to show the DEG distribution. Hierarchical clustering of transcripts showed significantly upregulated and downregulated genes in the control and sample group based on the result of *t*-test with fold change $\geq$2 ($P \leq$ 0.05) (Fig. 1b). In addition, the distance of the relationship between each gene was shown by gene tree; the shortest distance

Table 1
Oligonucleotide primers used for quantitative reverse transcription PCR (qRT-PCR).

Target gene	Orientation	Nucleotides	Size (bp)
16S rRNA	Forward	5′-CGAGTGTGTCAGAGGGAGGT-3′	210
	Reverse	5′-GCTACGCTACCAAGCAATCA-3′	
NGO_1117	Forward	5′-CCGTGTCCGTAAAGAGCAGG-3′	109
	Reverse	5′-CGCAATCCCTATGACTTCGC-3′	
NGO_1123	Forward	5′-CGGGTTGATTGAGATTGCC-3′	160
	Reverse	5′-CCGCTCGTCTATGGCGTAG-3′	
NGO_1118	Forward	5′-CAGGAACAAATCCAGCGACAAC-3′	108
	Reverse	5′-TTGGGAATGCCGATGTGATAG-3′	
NGO_0725	Forward	5′-TTATTGCCGATGCCGTCTG-3′	122
	Reverse	5′-AAACCGAAACCGAACGC −3′	
NGO_1073a	Forward	5′-TGTCAGCCGATTACGATGTC-3′	200
	Reverse	5′-GCTGTAGCTTACTTGATCGG-3′	
NGO_0983	Forward	5′-AGCTCCTGCTGCTGAG-3′	158
	Reverse	5′-CAGGTGCTTCGGTAGC-3′	
NGO_0854	Forward	5′-TCCGATGAATGAACGG-3′	72
	Reverse	5′-CGTTACTGATTTTTCTTACTGG-3′	
NGO_2131	Forward	5′-TCTGGGTAAACGAGCATA-3′	168
	Reverse	5′-GTCCGACAGGTATCCATA-3′	
NGO_1861	Forward	5′-AATTCCCCGCTGTTGGTA-3′	213
	Reverse	5′-TGAACCTTCAGACGGCAT-3′	
NGO_0950	Forward	5′-TGACAGAAACAAGCCGCT-3′	112
	Reverse	5′-GTTGGATTTGAGGGCAGG-3′	

Table 2
Basic information of RNA-Seq for *Neisseria gonorrhoeae*.

Group	Strain no.	Ceftriaxone MIC (mg/L)	RNA-Seq no.
Control			
Ctr1	GZ6	0.004	WHYR17109100_A
Ctr2	GZ89	0.002	WHYR17109101_A
Ctr3	GZ51	0.002	WHYR17109102_A
Sample			
S051	GZ69	0.5	WHYR17109093_A
S01251	GZ15	0.125	WHYR17109094_A
S0251	DG537	0.25	WHYR17109095_A
S01252	ZH552	0.125	WHYR17109096_A
S052	DG93	0.5	WHYR17109097_A
S006	GZ72	0.125	WHYR17109098_A
S01253	DG33	0.125	WHYR17109099_A

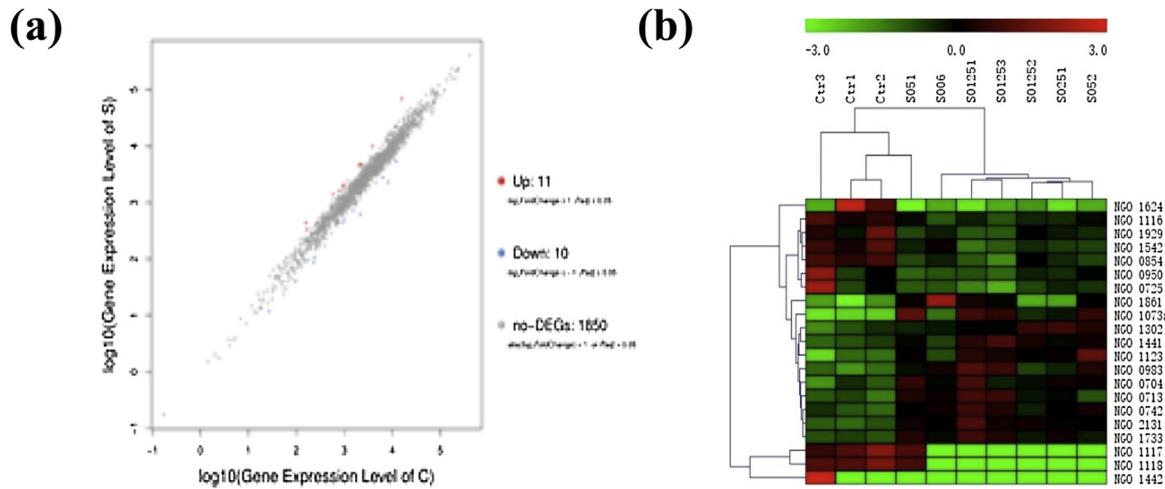MIC, minimum inhibitory concentration.

Fig. 1. Differentially expressed genes (DEGs) between control (C) group and sample (S) group of clinical *Neisseria gonorrhoeae* isolates. (a) The *x*- and *y*-axes represent $\log_{10}$-transformed gene expression levels. Red, blue and grey represent the upregulated, downregulated and non-DEGs, respectively. (b) Each row represents the relative expression of a single transcript, and each column represents a sample. Colours represent the $\log_{10}$-transformed gene expression level, with red and green representing high and low expression levels, respectively.

between nodes can represent the degree of difference between the two genes, which is same as sample tree. Obviously, the first seven genes and the last three genes showed low expression, whilst the other genes showed high expression compared with the control group. In addition, to better understand the function of the 21 DEGs, their annotations are listed in Table 3.

3.4. Gene Ontology analysis of differentially expressed genes

GO classification and functional enrichment with DEGs was performed. Nine GO biological processes were annotated for 16 genes, six GO cellular components had annotations for 15 genes, and three GO molecular functions contained 13 genes.

Table 3
Annotation of the 21 differentially expressed genes.

Gene ID	Cellular component	Molecular function	Biological process	Nr description
NGO_1073a	Integral component of membrane	Porin activity	Transmembrane transport	Opacity-associated protein
NGO_1123	NA	NA	NA	Phage-associated protein
NGO_0983	NA	NA	NA	Outer membrane protein H.8
NGO_2131	NA	Peptidase activity	Proteolysis	Stringent starvation protein B
NGO_1861	NA	NA	NA	Outer membrane preprotein PIIc
NGO_1302	NA	NA	NA	IS*1016* transposase
NGO_0704	NA	3,4-Dihydroxy-2-butanone-4-phosphate synthase activity; metal ion binding	Riboflavin biosynthetic process	3,4-Dihydroxy-2-butanone-4-phosphate synthase
NGO_0713	NA	2-Dehydro-3-deoxy-phosphogluconate aldolase activity; 4-hydroxy-2-oxoglutarate aldolase activity	Metabolic process	Ketohydroxyglutarate aldolase
NGO_1441	Integral component of membrane	NA	NA	Pilus assembly protein PilE
NGO_0742	Bacterial nucleoid; GO: cytoplasm	DNA binding	NA	DNA-binding protein
NGO_1733	NA	Metal ion binding; GTP binding; GTPase activity	NA	Ribosome small subunit-dependent GTPase
NGO_1116	NA	Sequence-specific DNA binding	NA	Transcriptional regulator
NGO_1929	NA	NA	NA	Hypothetical protein
NGO_0950	Integral component of membrane	NA	NA	Hypothetical protein
NGO_1542	Integral component of membrane; plasma membrane	Catalytic activity; penicillin binding	Peptidoglycan biosynthetic process; response to antibiotic; cell division; cell wall organisation; regulation of cell shape; cell cycle	Penicillin-binding protein
NGO_1624	NA	NA	NA	Phage-associated protein
NGO_1442	NA	Alcohol dehydrogenase (NAD) activity; zinc ion binding	Oxidation–reduction process	Alcohol dehydrogenase
NGO_0854	Membrane	NA	NA	Hypothetical protein
NGO_0725	NA	NA	NA	Phage-associated protein
NGO_1118	NA	NA	NA	Phage-associated protein
NGO_1117	NA	NA	NA	Phage-associated protein

NA, no annotation.

Bacterial resistance is closely related to the cell membrane, thus we focused on analysis of the cellular component. The integral components of the membrane (GO: 0016021) enrichment included YP_8053, YP_4853, YP_8495 and YP_8590; membrane (GO: 0016020) enrichment included YP_7970; plasma membrane (GO: 0005886) enrichment included YP_8590; and YP_7871 belonged to cytoplasm (GO:0005737), bacterial nucleoid (GO: 0043590) and nucleoid (GO: 0009295). The integral component of the membrane had the highest degree of enrichment, and these clustered DEGs may have a high correlation with resistance.

3.5. KEGG pathway analysis of differentially expressed genes

KEGG pathway classification and functional enrichment with DEGs was performed. The top 16 most differentially expressed pathways are listed in Table 4. One of the DEGs belonged to the biosynthesis of antibiotics, which may have an important function in ceftriaxone resistance. The amount of upregulated and downregulated genes are represented by red and blue, respectively, in Fig. 2a, and the number of DEGs in metabolic pathways is the largest group.

Rich factor was used to analyse the significant enrichment of DEGs. Rich factor refers to the enrichment factor value, which is the quotient of foreground value (number of DEGs) and background value (number of all genes). A larger Rich factor denotes a more significant enrichment. In Fig. 2b, the high and low q-values are represented by white and blue, respectively. A lower q-value indicates a more significant enrichment. The DEG number is represented by the point size in the diagram, wherein bigger dots refer to larger amounts. As shown in Fig. 2b, naphthalene and chloroalkene degradation were significantly enriched. Only one gene from the genome was regulated in these pathways. Moreover, the gene had different expression in the control and sample groups, indicating that naphthalene and chloroalkane degradation may have important roles in bacterial resistance.

3.6. qRT-PCR validation

qRT-PCR was performed for the top ten DEGs (NGO_1117, NGO_1123, NGO_1118, NGO_0725, NGO_1073a, NGO_0983, NGO_0854, NGO_2131, NGO_1861 and NGO_0950) in the susceptible and decreased susceptible N. gonorrhoeae isolates. Relative expression levels were calculated using $2^{-\Delta\Delta}Ct$, and a two-sample t-test was used to verify the significant difference ($P \leq 0.05$). Compared with susceptible N. gonorrhoeae, NGO_1123, NGO_1073a, NGO_1861 and NGO_0950 were upregulated, whereas NGO_1117, NGO_1118, NGO_0725, NGO_0983, NGO_0854 and NGO_2131 were downregulated (Fig. 3). However, NGO_0950 and NGO_0983 were not in agreement with the RNA-Seq data and require further research.

4. Discussion

This study is the first to employ RNA-Seq for transcriptional analysis between ceftriaxone-susceptible N. gonorrhoeae isolates and isolates with decreased susceptible to ceftriaxone, and 21 DEGs were found. Functional enrichment with GO and KEGG pathway analyses of these DEGs were performed, focusing on the DEGs in the cellular components. Changes in gonococcal biofilms contribute to the development of multidrug-resistant strains [34]. We focused on the pathways that had more clustering genes and high enrichment in KEGG.

The RNA-Seq data demonstrated that the expression levels of genes associated with cellular component were upregulated, such as NGO_1441, NGO_1073a and NGO_0742, whereas NGO_0950, NGO_1542 and NGO_0854 were downregulated. Variability in the pilin protein is one of the important causes of bacterial resistance [34,35]. The YP_8495 encoded by NGO_1441 has type IV pilus assembly protein PilE function that modulates PilE and can significantly alter the serum resistance of N. gonorrhoeae [36]. Upregulation of NGO_1441 leads to variability in PilE and causes cephalosporin resistance. Porin mutation causes moderate levels of resistance to penicillin and tetracycline in N. gonorrhoeae and increases the expression of the MtrC-MtrD-MtrE efflux pump that can lead to a new resistant phenotype [37]. NGO_1073a encodes YP_4853, which has functions in porin activity and transmembrane transport. High expression of NGO_1073a could regulate this function and cause resistance. We did not expect NGO_0742 encoding YP_7871, which is an uncharacteristic protein, to exhibit the function of DNA binding. Furthermore, recent research has reported that susceptibility to cephalosporins is related to penA, mtrR, penB and ponA genes in N. gonorrhoeae [18,38]. Possibly YP_7871 can bind and regulate the expression of these genes.

Table 4
Top 16 most abundant differentially expressed gene (DEG) pathways.

Pathway ID	Pathway	DEGs[a] (9)	All-gene[b] (1383)	P-value	q-value
ko00626	Naphthalene degradation	1	1	0.000650759	0.005206074
ko00625	Chloroalkane and chloroalkene degradation	1	1	0.000650759	0.005206074
ko01220	Degradation of aromatic compounds	1	5	0.003216316	0.011938325
ko00071	Fatty acid degradation	1	5	0.003216316	0.011938325
ko00740	Riboflavin metabolism	1	7	0.004476872	0.011938325
ko00350	Tyrosine metabolism	1	7	0.004476872	0.011938325
ko00730	Thiamine metabolism	1	12	0.007564645	0.017074095
ko00550	Peptidoglycan biosynthesis	1	17	0.01056338	0.017074095
ko00030	Pentose phosphate pathway	1	18	0.01115264	0.017074095
ko00630	Glyoxylate and dicarboxylate metabolism	1	18	0.01115264	0.017074095
ko00010	Glycolysis/gluconeogenesis	1	19	0.01173844	0.017074095
ko01120	Microbial metabolism in diverse environments	2	100	0.01336279	0.017817053
ko01100	Metabolic pathways	4	427	0.02901409	0.035709649
ko01200	Carbon metabolism	1	69	0.03699681	0.040205888
ko01110	Biosynthesis of secondary metabolites	2	198	0.03769302	0.040205888
ko01130	Biosynthesis of antibiotics	1	142	0.06239486	0.06239486

[a] Number of DEGs that annotated to specific pathway.
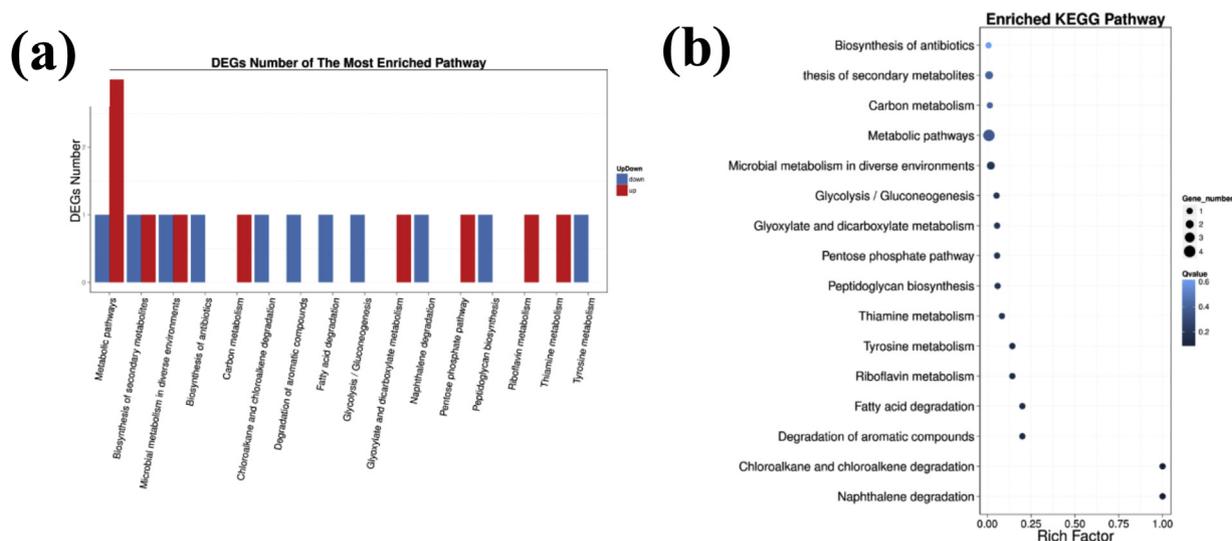[b] Number of genes that annotated to specific pathway.

Fig. 2. KEGG pathway classification and functional enrichment of differentially expressed genes (DEGs). (a) The *x*-axis represents the terms of the pathway and the *y*-axis represents the number of upregulated or downregulated genes. (b) The *x*-axis represents the enrichment factor and the *y*-axis represents the pathway name.

YP_8590 is among the DEGs with low expression encoded by NGO_1542 and is a penicillin-binding protein with catalytic activity that can affect the biosynthesis of peptidoglycan and regulate the cell division protein FtsI (PBP3). The resistance of *N. gonorrhoeae* is currently related to PBP1 and PBP2 [39]; however, no reports have been found on PBP3. No explicit functional annotation and classification have been observed for YP_8053 and YP_7970, which are encoded by NGO_0950 and NGO_0854, thereby implying the need for further functional verification. In addition to DEGs being annotated by cellular component, other DEGs may be involved in the regulation of cephalosporin resistance. For example, NGO_1116 encodes YP_8195, which has a repressive effect and can specifically bind to DNA. Duffin and Barber found that DprA, a DNA processing enzyme, affects the DNA transformation process and causes mutation of gonococcal pilin [40]. However, the particular DNA-bound protein of YP_8195 has not been determined and could be PenA, MtrR, PenB or PonA, which can all regulate cephalosporin resistance [18,38]. Furthermore, NGO_1861 is an outer membrane preprotein PIIc encoded by YP_8894. The outer membrane proteins of *N. gonorrhoeae* show an influence on multidrug resistance, specifically the variation of porin, pilus secretion protein and enzymes [35]. Other proteins encoded by DEGs can also regulate cephalosporin activities, which can be subjected to further validation and future studies.

Many studies have shown that mutations in *penA*, *mtrR*, *porB* and *ponA* genes are associated with the reduced susceptibility of *N. gonorrhoeae* isolates to cefixime or ceftriaxone [21–23]. In the current study, *penA* mosaic alleles (XXX or XXXIV pattern) altered the structure of PBP2 and affected the activity of cephalosporins. Moreover, an additional mutation site (A501V substitution) was found, which may be more meaningful than mosaic structures in ceftriaxone resistance [41]. Mutations of MtrR (A39T/H105Y substitution) promote overexpression of the efflux pump MtrCDE and enhance tolerance to antibiotics [42], and mutation in PorB (G120K/A121D substation) reduces the permeability of hydrophilic antibiotics. Thus, ceftriaxone resistance could emerge due to overexpression of MtrCDE and variation in the PorB1b protein working together [21]. In addition, an L421P substitution was found in PonA and reduced the acylation rate of β-lactam antibiotics because of the change in PBP1 [25]. However,

in the current data no significant differences in the expression in the four genes between the control and sample groups were observed.

In the KEGG pathway, many pathways have more clustering genes. We focused on the biosynthesis of antibiotics and a pathway associated with gonococcal resistance, wherein only one gene (NGO_1442) was found. YP_8496 is an alcohol dehydrogenase and one of the regulatory factors of PerR. PerR can enhance the antioxidant capacity of *N. gonorrhoeae* by regulating the effects of the ABC transporter MntABC on manganese and improve the intracellular survival rate of *N. gonorrhoeae* [43]. Furthermore, YP_8496 can regulate microbial metabolism in diverse environments. NGO_1442 has an unpredictable role in cephalosporin resistance as well as in metabolic pathways, biosynthesis of secondary metabolites, naphthalene degradation and chloroalkene degradation.

To confirm the RNA-Seq data, functional DEGs were selected for qRT-PCR. It was found that NGO_1123, NGO_1073a and NGO_0950 were upregulated in decreased susceptibility isolates, whilst NGO_1117, NGO_1118, NGO_0983 and NGO_0854 were downregulated. The qRT-PCR results of NGO_1123, NGO_1073a, NGO_1117, NGO_1118 and NGO_0854 did not have the same trend as the RNA-Seq data, as well as the NGO_0950 and NGO_0983. The proteins encoded by NGO_1123, NGO_1117 and NGO_1118 belong to phage-associated proteins. Proença et al. found that a phage-associated protein (EC300) exhibited a lytic enzyme activity and enhanced the killing efficacy against *Enterococcus faecalis* exhibiting a high level of antibiotic resistance [44]. The identical phage-associated protein may have a function in gonococcal resistance to a certain extent. No specific functional annotations exist for the proteins encoded by NGO_0983, NGO_0950 and NGO_0854. However, the significant difference between the actual expression of ceftriaxone susceptible and decreased susceptibility *N. gonorrhoeae* isolates requires further research.

In summary, these data indicated that 12 of the DEGs may be related to decreased susceptibility of *N. gonorrhoeae* to cephalosporins, namely NGO_0950, NGO_1073a, NGO_1441, NGO_0854, NGO_0742, NGO_1861, NGO_1116, NGO_1442, NGO_1123, NGO_0983, NGO_1118 and NGO_1117. Further characterisation of these genes may provide new targets for reducing ceftriaxone resistance and controlling the spread of gonorrhoea.
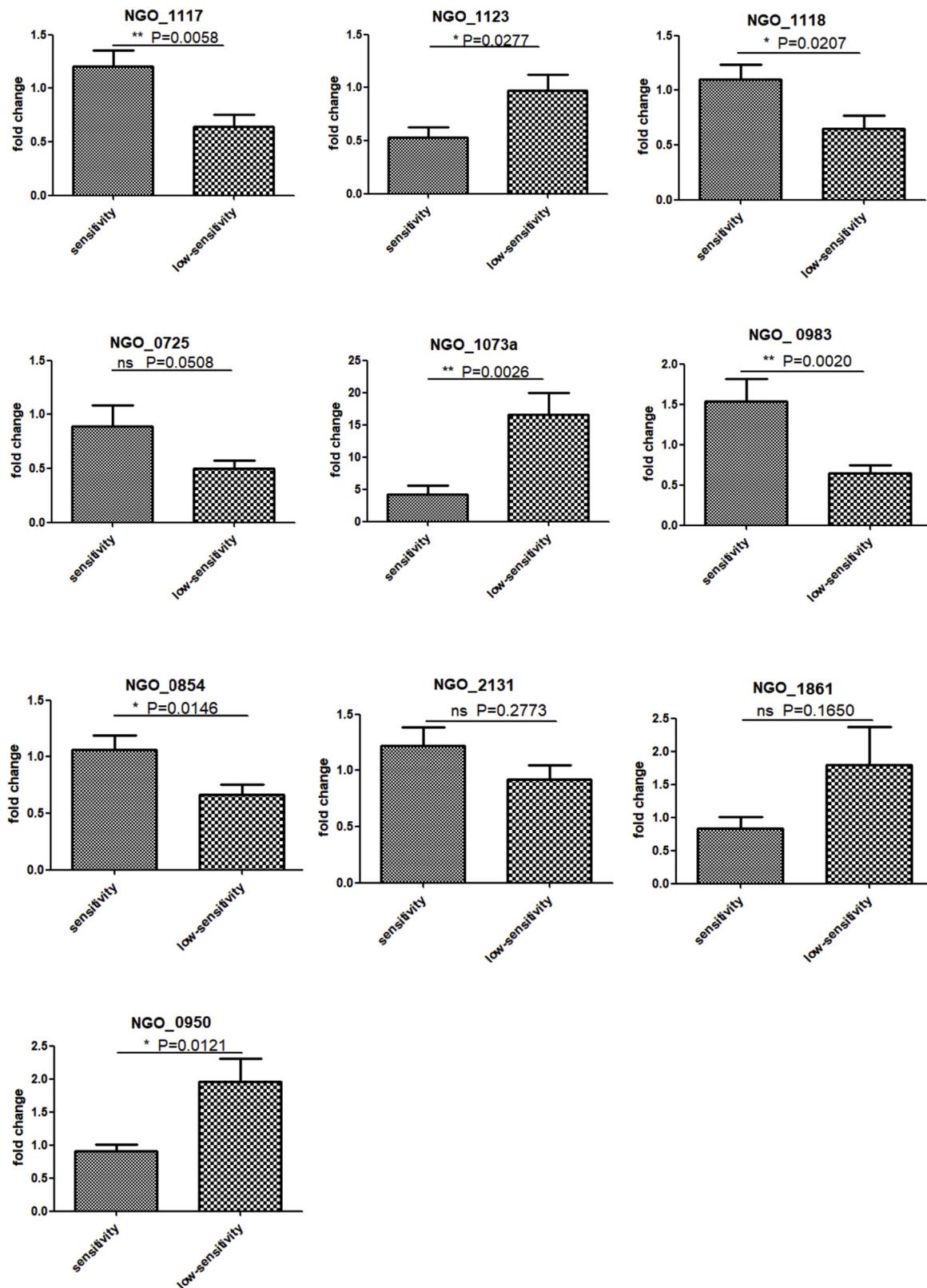
Fig. 3. Quantitative reverse transcription PCR (qRT-PCR) analysis data for the top 10 differentially expressed genes in *Neisseria gonorrhoeae* isolates susceptibility or decreased susceptibility to ceftriaxone. The *x*-axis represents the groups and the *y*-axis represents the fold change. The height of the box represents the mean of $2^{-\Delta\Delta Ct}$ values, and associated error bars denote the standard error of the mean.

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Competing interests

None declared.

Ethical approval

This study was approved by the Medical Ethics Committee of Guangdong Provincial Centre for Skin Diseases and STD Control (China).

References

[1] Wi T, Lahra MM, Ndowa F, Bala M, Dillon JR, Ramon-Pardo P, et al. Antimicrobial resistance in *Neisseria gonorrhoeae*: global surveillance and a call for international collaborative action. PLoS Med 2017;14:e1002344.

[2] Suay-Garcia B, Perez-Gracia MT. Drug-resistant *Neisseria gonorrhoeae*: latest developments. Eur J Clin Microbiol Infect Dis 2017;36:1065–71.

[3] Costa-Lourenço A, Barros Dos Santos KT, Moreira BM, Fracalanzza SEL, Bonelli RR. Antimicrobial resistance in *Neisseria gonorrhoeae*: history, molecular mechanisms and epidemiological aspects of an emerging global threat. Braz J Microbiol 2017;48:617–28.

[4] World Health Organization. WHO guidelines for the treatment of *Neisseria gonorrhoeae*. Geneva, Switzerland: WHO; 2016.

[5] US Centers for Disease Control and Prevention (CDC). 2016 Sexually transmitted diseases surveillance. Atlanta, GA: CDC; 2016 https://www.cdc.gov/std/stats16/. [Accessed 21 January 2019].

[6] National Health and Family Planning Commission of China (NHFPC). Overview of the national legal infectious disease epidemic in 2017. NHFPC; 2018 http://www.nhfpc.gov.cn/jkj/new_index.shtml. [Accessed 21 January 2019].

[7] World Health Organization. Global health sector strategy on sexually transmitted infections 2016–2021: towards ending STIs. Geneva, Switzerland: WHO; 2016.

[8] Unemo M, Shafer WM. Antimicrobial resistance in *Neisseria gonorrhoeae* in the 21st century: past, evolution, and future. Clin Microbiol Rev 2014;27:587–613.

[9] Deguchi T, Yasuda M, Yokoi S, Ishida K, Ito M, Ishihara S, et al. Treatment of uncomplicated gonococcal urethritis by double-dosing of 200 mg cefixime at a 6-h interval. J Infect Chemother 2003;9:35–9.

[10] Unemo M. Current and future antimicrobial treatment of gonorrhoea—the rapidly evolving *Neisseria gonorrhoeae* continues to challenge. BMC Infect Dis 2015;15:364.

[11] Ohnishi M, Golparian D, Shimuta K, Saika T, Hoshina S, Iwasaku K, et al. Is *Neisseria gonorrhoeae* initiating a future era of untreatable gonorrhea?: detailed characterization of the first strain with high-level resistance to ceftriaxone. Antimicrob Agents Chemother 2011;55:3538–45.

[12] Unemo M, Jensen JS. Antimicrobial-resistant sexually transmitted infections: gonorrhoea and *Mycoplasma genitalium*. Nat Rev Urol 2017;14:139–52.

[13] Fifer H, Natarajan U, Jones L, Alexander S, Hughes G, Golparian D. Failure of dual antimicrobial therapy in treatment of gonorrhea. N Engl J Med 2016;374:2504–6.

[14] World Health Organization. Global action plan to control the spread and impact of antimicrobial resistance in *Neisseria gonorrhoeae*. Geneva, Switzerland: WHO; 2012.

[15] Yelin I, Kishony R. Antibiotic resistance. Cell 2018;172: 1136–1136.e1.

[16] Abrams AJ, Kirkcaldy RD, Pettus K, Fox JL, Kubin G, Trees DL. A case of decreased susceptibility to ceftriaxone in *Neisseria gonorrhoeae* in the absence of a mosaic penicillin-binding protein 2 (*penA*) allele. Sex Transm Dis 2017;44:492–4.

[17] Tomberg J, Unemo M, Ohnishi M, Davies C, Nicholas RA. Identification of amino acids conferring high-level resistance to expanded-spectrum cephalosporins in the *penA* gene from *Neisseria gonorrhoeae* strain H041. Antimicrob Agents Chemother 2013;57:3029–36.

[18] Unemo M, Golparian D, Nicholas R, Ohnishi M, Gallay A, Sednaoui P. High-level cefixime- and ceftriaxone-resistant *Neisseria gonorrhoeae* in France: novel *penA* mosaic allele in a successful international clone causes treatment failure. Antimicrob Agents Chemother 2012;56:1273–80.

[19] Lee SG, Lee H, Jeong SH, Yong D, Chung GT, Lee YS, et al. Various *penA* mutations together with *mtrR*, *porB* and *ponA* mutations in *Neisseria gonorrhoeae* isolates with reduced susceptibility to cefixime or ceftriaxone. J Antimicrob Chemother 2010;65:669–75.

[20] Osaka K, Takakura T, Narukawa K, Takahata M, Endo K, Kiyota H, et al. Analysis of amino acid sequences of penicillin-binding protein 2 in clinical isolates of *Neisseria gonorrhoeae* with reduced susceptibility to cefixime and ceftriaxone. J Infect Chemother 2008;14:195–203.

[21] Lindberg R, Fredlund H, Nicholas R, Unemo M. *Neisseria gonorrhoeae* isolates with reduced susceptibility to cefixime and ceftriaxone: association with genetic polymorphisms in *penA*, *mtrR*, *porB1b*, and *ponA*. Antimicrob Agents Chemother 2007;51:2117–22.

[22] Zhao S, Duncan M, Tomberg J, Davies C, Unemo M, Nicholas RA, et al. Genetics of chromosomally mediated intermediate resistance to ceftriaxone and cefixime in *Neisseria gonorrhoeae*. Antimicrob Agents Chemother 2009;53:3744–51.

[23] Tanaka M, Nakayama H, Huruya K, Konomi I, Irie S, Kanayama A, et al. Analysis of mutations within multiple genes associated with resistance in a clinical isolate of *Neisseria gonorrhoeae* with reduced ceftriaxone susceptibility that shows a multidrug-resistant phenotype. Int J Antimicrob Agents 2006;27:20–6.

[24] Osawa K, Shigemura K, Nukata Y, Kitagawa K, Yamamichi F, Yoshida H, et al. *penA*, *ponA*, *porB1*, and *mtrR* mutations and molecular epidemiological typing of *Neisseria gonorrhoeae* with decreased susceptibility to cephalosporins. Antimicrob Agents Chemother 2017;61:e01174-17.

[25] de Curraize C, Kumanski S, Micaëlo M, Fournet N, La Ruche G, Meunier F, et al. Ceftriaxone-resistant *Neisseria gonorrhoeae* isolates (2010 to 2014) in France characterized by using whole-genome sequencing. Antimicrob Agents Chemother 2016;60:6962–4.

[26] Truong AD, Hong YH, Lillehoj HS. RNA-Seq profiles of immune related genes in the spleen of necrotic enteritis-afflicted chicken lines. Asian Australas J Anim Sci 2015;28:1496–511.

[27] Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, et al. Differential gene and transcript expression analysis of RNA-Seq experiments with TopHat and Cufflinks. Nat Protoc 2012;7:562–78.

[28] Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat Methods 2008;5:621–8.

[29] Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. Nat Methods 2015;12:357–60.

[30] Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods 2012;9:357–9.

[31] Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics 2011;12:323.

[32] Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-Seq data with DESeq2. Genome Biol 2014;15:550.

[33] Audic S, Claverie J. The significance of digital gene expression profiles. Genome Res 1997;7:986–95.

[34] Kouzel N, Oldewurtel ER, Maier B. Gene transfer efficiency in gonococcal biofilms: role of biofilm age, architecture, and pilin antigenic variation. J Bacteriol 2015;197:2422–31.

[35] Yoo JS, Seong WK, Kim TS, Park YK, Oh HB, Yoo CK. Comparative proteome analysis of the outer membrane proteins of in vitro-induced multi-drug resistant *Neisseria gonorrhoeae*. Microbiol Immunol 2007;51:1171–7.

[36] Matthias KA, Rest RF. Control of pili and sialyltransferase expression in *Neisseria gonorrhoeae* is mediated by the transcriptional regulator CrgA. Mol Microbiol 2014;91:1120–35.

[37] Olesky M, Zhao S, Rosenberg RL, Nicholas RA. Porin-mediated antibiotic resistance in *Neisseria gonorrhoeae*: ion, solute, and antibiotic permeation through PIB proteins with *penB* mutations. J Bacteriol 2006;188:2300–8.

[38] Barry PM, Klausner JD. The use of cephalosporins for gonorrhea: the impending problem of resistance. Expert Opin Pharmacother 2009;10:555–77.

[39] Takahashi S, Kurimura Y, Hashimoto J, Uehara T, Hiyama Y, Iwasawa A, et al. Antimicrobial susceptibility and penicillin-binding protein 1 and 2 mutations in *Neisseria gonorrhoeae* isolated from male urethritis in Sapporo, Japan. J Infect Chemother 2013;19:50–6.

[40] Duffin PM, Barber DA. DprA is required for natural transformation and affects pilin variation in *Neisseria gonorrhoeae*. Microbiology 2016;162:1620–8.

[41] Takahata S, Senju N, Osaki Y, Yoshida T, Ida T. Amino acid substitutions in mosaic penicillin-binding protein 2 associated with reduced susceptibility to cefixime in clinical isolates of *Neisseria gonorrhoeae*. Antimicrob Agents Chemother 2006;50:3638–45.

[42] Zarantonelli L, Borthagaray G, Lee EH, Veal W, Shafer WM. Decreased susceptibility to azithromycin and erythromycin mediated by a novel *mtr(R)* promoter mutation in *Neisseria gonorrhoeae*. J Antimicrob Chemother 2001;47:651–4.

[43] Wu HJ, Seib KL, Srikhanta YN, Kidd SP, Edwards JL, Maguire TL, et al. PerR controls Mn-dependent resistance to oxidative stress in *Neisseria gonorrhoeae*. Mol Microbiol 2006;60:401–16.

[44] Proença D, Leandro C, Garcia M, Pimentel M, São-José C. EC300: a phage-based, bacteriolysin-like protein with enhanced antibacterial activity against *Enterococcus faecalis*. Appl Microbiol Biotechnol 2015;99:5137–49.