Analysis of Drug Resistance in *Helicobacter Pylori* by Complete Genome Sequencing in Bama County, Guangxi, China

yanqiang huang (hyq77615@163.com)
Research Center for the prevention and treatment of drug resistant microbial infecting https://orcid.org/0000-0002-3457-362X

Xiao-Hua Li
Youjiang Medical University for Nationalities

Yong-Yi Huang
Youjiang Medical University for Nationalities

Xian-ke Luo
Gastroenterology department

Yan-Chun Qin
Youjiang Medical University for Nationalities

Yuan-Yuan Dai
Youjiang Medical University for Nationalities

Chun Qin
Youjiang Medical University for Nationalities

Ru-Jia Li
Youjiang Medical University for Nationalities

Hao Chen
Wannan Medical College

Research

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Abstract

Background: The resistance rate of clinical *Helicobacter pylori* (Hp) isolates has increased, however, the mechanism of drug resistance is unclear. In this study, we isolated drug-resistant Hp strains isolated from different areas and different populations of China for genomic analysis.

Objectives: The aim of this study was to investigate drug resistance in Hp from Bama County, Guangxi, China.

Methods: Minimal inhibitory concentrations (MICs) of clarithromycin, metronidazole and levofloxacin were determined and complete genome sequencing was performed with annotation. The presence of hp1181 and hp1184 genes was detected by RT-PCR. The relationships between hp1181, hp1184 and clarithromycin resistance were confirmed by gene mutation and drug-resistant strains.

Results: Three drug-resistant Hp strains were isolated from patients with gastritis in Bama County. The strains showed a high degree of homology with hp26695 through complete genome detection and identification. Differences in genome sequences, gene quantity and gene characteristics were detected amongst the three strains. Prediction and analysis of the function on drug-resistant genes indicated that the RNA expression of hp1181 and hp1184 increased in the three strains that were the same in the artificially induced clarithromycin-resistant bacteria. After gene knockout, the drug sensitivity of the strains increased significantly.

Conclusions: The expressions of the genes hp1184 and hp1181 were associated with clarithromycin resistance in the Hp from Bama, Guangxi.

1. Background

Helicobacter pylori (Hp) is recognized as an important human pathogen that colonizes the gastric mucus resulting in superficial gastritis, atrophic gastritis and gastric cancer[1–3]. Present treatments for Hp infection include proton pump inhibitors or bismuth in combination with amoxicillin or metronidazole and clarithromycin[4–5]. The rate of drug resistance is increasing due to a wide range of use of antibiotics and high resistance rates to clarithromycin, metronidazole and levofloxacin are associated with the failure of Hp eradication[6–8]. The World Health Organization (WHO) gave clarithromycin-resistant Hp as a high priority bacterium for antibiotic research and development[9].

At present, the mechanism of antibiotic resistance of Hp is not completely understood[10–11]. It is widely accepted that the resistance to these antimicrobials is related to mutations in Hp gene: clarithromycin-resistant strains present three-point mutations in the region of domain V of 23S ribosomal RNA (rRNA): A2142G, A2142C and A2143G[12–13]. In addition to the mutations, the efflux pump cluster is also involved in the development of resistance to clarithromycin[14–15]. Moreover, there may be gene mutation sites that are not yet known and so the mechanism of drug resistance warrants further study.

We isolated, cultured Hp from the population in Bama County, which is a township known for the longevity of its residents in Guangxi, and randomly selected three strains of multiple drug-resistant Hp with resistance to clarithromycin. Complete genome sequences were analyzed to ascertain the genomic characteristics of the strains and to reveal the underlying mechanism of drug resistance in Hp.

2. Materials And Methods

2.1 Isolation and culture of Hp

This study had received a strict medical ethics review from Youjiang Medical University for Nationalities. Written informed consent was obtained from each patient. Gastric mucosa tissue samples were collected from the People's Hospital of Bama Yao Autonomous County in patients' gastric body and pylorus with gastritis or gastric ulcers. Isolation and culture of Hp were performed at the Prevention and treatment Research Center for Resistance Microbiological, Youjiang Medical University for Nationalities. Patients investigated had not taken any antibiotics for at least four weeks before examination. The isolation and identification of Hp were performed as previously described [16-17]. The culture conditions used Columbia agar plates containing 5% fresh defibrinated sheep blood. The microaerophilic conditions included 5% O2, 10% CO2 and 85% N2 at 37 °C for 3~5 days. Suspicious colonies were confirmed by Gram stain, urease, oxidase, catalase activity testing and urease gene PCR testing.

2.2 Antibiotic susceptibility testing

The antibiotic resistance of Hp was measured by dilution methods with reference to the protocols of the Clinical and Laboratory Standards Institute (Wayne, PA, USA)[18]. Briefly, the concentration of Hp was regulated to be 1×10^6 CFU/ml and incubated at 37 °C for 3~5 days under microaerophilic conditions. After incubation, the plates were visually examined and the MIC was determined to be the lowest concentration

that resulted in no turbidity. Metronidazole (Aladdin, d1707126), amoxicillin (Xiansheng pharmaceutical, 02-170404), levofloxacin (Shandong Lukang Pharmaceutical Group Saite Co., Ltd, 160608), clarithromycin (Yangzi River Pharmaceutical Group Co., Ltd 17111641) were also used.

2.3 Complete genome testing and analysis

Drug-resistant strains were selected and sent to the Shenzhen Huada Gene Co., Ltd. for complete genome analysis. After the DNA samples were delivered, the quality of the samples was tested and then used to construct a BSlibrary. The purified genomic DNA samples including genomic DNA, bacterial artificial chromosomes or long-length PCR products were sheared into smaller fragments by CovarisS/E210 or using a Bioruptor. The overhangs resulting from fragmentation were converted into blunt ends using T4 DNA polymerase, Klenow Fragment and T4 Polynucleotide Kinase. After adding an 'A' base to the 3' end of the blunt phosphorylated DNA fragments, adapters were ligated to the ends of the DNA fragments. The desired fragments were purified though gel-electrophoresis, selectively enriched and amplified by PCR. The index tag was introduced into the adapter at the PCR stage as appropriate and a library quality test was performed. Finally, the qualified BSlibrary was used for sequencing. Genomic component and gene function analyses were performed including gene prediction, tRNA, sRNA, genes annotation and the predicted open reading frames (ORFs) by GO.

2.4 Drug resistance gene detection

Drug resistance genes were predicted based on the results of the complete genome sequence analysis and selected for detection by RT-PCR. The reaction for cDNA synthesis was held at 25 ℃ for 10 min, 42 ℃ for 60 min and 99 ℃ for 5 min. The reaction consisted of 32 cycles with each cycle composed of 1 min at 95 ℃, 4 min at 56 ℃ and 7 min at 70 ℃. After a final extension of 15 min at 72 ℃, the RT-PCR products were visualized by electrophoresis on 1% agarose gel and 15% acrylamide gel with a 200-bp ladder size marker.

2.5 Knockout of mutant genes

HP1181 and HP 1184 knockout mutants were constructed by insertion of the KAN resistance cassette. Double-knockout mutants were made by natural transformation of the KAN resistance cassette with pBSII KS (as presented by Bi Hongkai, Laboratory of Nanjing Medical University) containing an internal fragment interrupted with a cat cassette from pAV35, with selection for both KAN- and CHL-resistant colonies. Insertion of the KAN and cat resistance cassette at the desired locations in the Hp putative efflux genes was confirmed by PCR.

2.6 Induction of drug resistance

The MIC of clarithromycin to Hp26695 was detected. Drug resistance was induced by 1/4MIC. The culture medium was changed every two days and MIC was detected every four days. The concentration of induced drug was changed with MIC.

3. Results

3.1 Bacterial resistance

Three drug-resistant strains isolated and identified by Gram stain, urease, oxidase, catalase activity testing, and urease gene PCR testing: the drug resistance information of these strains is summarized in Table 1.

Table 1 Drug resistance characteristics of three drug-resistant strains (MIC: μg/ml)

strains	Metronidazole	Clarithromycin	Levofloxacin	Amoxicillin
Hpbs1	32	8	8	0.125
Hpbs2	16	8	0.125	0.125
Hpbs3	0.125	8	8	0.125

3.2 Bacterial sequence information

Based on the valid data from the previous sequencing platform, the CleanData could be assembled for each sample and the optimal assembly results were obtained after multiple adjustments. The assembly sequence was analyzed by correcting single base, circular judgment and plasmid comparison. The results of the genome assembly statistics of each sample are shown in Table 2. These three strains have been uploaded to the NCBI Biosample database: Hpbs1(https://www.ncbi.nlm.nih.gov/biosample/?term=SAMN10461767)

Hpbs2(https://www.ncbi.nlm.nih.gov/biosample/?term=SAMN10663081), and Hpbs3(https://www.ncbi.nlm.nih.gov/biosample/?term=SAMN10663175),

Table 2 Sequence information of three drug-resistant strains

Sample Name	ID Name	Sequence Type(#)	Sequence Topology	Sequence Number(#)	Total Length (bp)	GC Content (%)
Hpbs1	Chromosome1	Chromosome	circular	1	1,563,701	38.90
	All	All	-	1	1,563,701	38.90
Hpbs2	Chromosome1	Chromosome	circular	1	1,534,481	38.87
	All	All	-	1	1,534,481	38.87
Hpbs3	Chromosome1	Chromosome	circular	1	1,534,930	38.90
	All	All	-	1	1,534,930	38.90

Note: Sequence Type, chromosome or plasmid;Sequence Topology, circular or linear.

3.3 Gene information

Gene prediction was applied to determine gene composition. The statistics are shown in Table 3 below.

Table 3 The gene information of three drug-resistant strains

Sample Name (#)	Genome Size (#)	Total Number (#)	Total Length (bp)	Average Length (#)	Length / Genome Length (%)	GC Content (%)
Hpbs1	1,563,701	1,571	1,434,204	912.92	91.72	39.49
Hpbs2	1,534,481	1,792	1,395,399	778.68	90.94	39.44
Hpbs3	1,534,930	1,732	1,407,495	812.64	91.70	39.49

Note: Total Number,the count of genes; Total Length,total length of all genes; Average Length,average length of all genes; GC Content,the content of G and C in gene; Length/Genome Length, The proportion of gene length in genome.

3.4 Circular genome analysis

GC skew analysis was performed using (G-C) / (G + C) calculations based on Genomic sequences of sequenced strains. The results of gene distribution, ncRNA distribution and gene annotation are demonstrated in Fig. 1. Hpbs1 had 835 genes, 26 tRNAs, 6 rRNAs, 2 sRNAs in a positive chain. It also had 736 genes, 10 tRNAs, 0 rRNA, 5 sRNAs in negative chain and 157 repeats without positive or negative chain. There are 943 genes, 26 tRNAs, 6 rRNAs, 3 sRNAs, 849 genes, 10 tRNAs, 0 rRNA, 3 sRNAs, and 153 repeats in Hpbs2; 869 genes, 26 tRNAs, 6 rRNAs, 3 sRNAs, 863 genes,10 tRNAs, 0 rRNA, 3 sRNAs, 155 repeats in Hpbs3.

Fig. 1 The circular genome analysis of three resistant strains. A. Hpbs1; B. Hpbs2; C. Hpbs3.

3.5 Gene annotation

Functional annotation was accomplished by analysis of protein sequences. We aligned genes with databases to obtain their corresponding annotations. To demonstrate the biological meaning, the highest quality alignment result was chosen as a gene annotation. Functional annotation was completed by blasting genes with different databases. In this project we have finished P450, VFDB, ARDB, CAZY, SWISSPROT, NOG, COG, CARD, NR, DBCAN, T3SS, TREMBL, IPR, PHI, KEGG, GO, KOG...17 databases annotations. The annotation results are shown in Tables 4 and 5.

Table 4 Gene annotation statistics A

Sample Name (#)	Total (#)	P450 (#)	VFDB (#)	ARDB (#)	CAZY (#)	SWISSPROT (#)	NOG (#)	COG (#)	CARD (#)	NR (#)
Hpbs1	1,571	22 (1.4%)	196 (12.47%)	0 (0%)	14 (0.89%)	742 (47.23%)	67 (4.26%)	1,084 (69%)	14 (0.89%)	1,559 (99.23%)
Hpbs2	1,792	21 (1.17%)	177 (9.87%)	0 (0%)	14 (0.78%)	751 (41.9%)	125 (6.97%)	1,111 (61.99%)	13 (0.72%)	1,723 (96.14%)
Hpbs3	1,732	22 (1.27%)	174 (10.04%)	0 (0%)	13 (0.75%)	750 (43.3%)	97 (5.6%)	1,113 (64.26%)	15 (0.86%)	1,698 (98.03%)

Table 5 Gene annotation statistics B

Sample Name (#)	DBCAN (#)	T3SS (#)	TREMBL (#)	IPR (#)	PHI (#)	KEGG (#)	GO (#)	KOG (#)	OverAll (#)
Hpbs1	30 (1.9%)	175 (11.13%)	1,557 (99.1%)	1,234 (78.54%)	54 (3.43%)	1,026 (65.3%)	957 (60.91%)	142 (9.03%)	1,563 (99.49%)
Hpbs2	29 (1.61%)	197 (10.99%)	1,706 (95.2%)	1,372 (76.56%)	52 (2.9%)	1,078 (60.15%)	1,056 (58.92%)	144 (8.03%)	1,750 (97.65%)
Hpbs3	30 (1.73%)	209 (12.06%)	1,688 (97.45%)	1,340 (77.36%)	51 (2.94%)	1,067 (61.6%)	1,030 (59.46%)	139 (8.02%)	1,711 (98.78%)

3.6 Analysis of drug resistance gene database

The drug resistance gene numbers of three strains were different in the CARD drug resistance database, which are 14, 13 and 15 genes, respectively. However, after sorting, it was found that some genes were repetitive. The specific numbers and characteristics of genes are shown

in the Tables 6 and 7. NP_207975.1 and NP_207972.1 were efflux pump genes of 26695 strain, i.e. hp1181 and hp1184 genes. Their drug resistance was verified by RT-PCR as illustrated in Fig. 2. After knocking out the drug resistance gene, drug sensitivity was significantly improved as shown in Fig. 3.

Table 6 Analysis of gene resistance in CARD

Gene_id	Subject_id	Align_length	Mismatch	Gap	Gene_start	Gene_end	Subject_start	Subject_end	E_value
GL000175	YP_208874.1	97	39	0	2	98	4	100	6.00E-40
GL000286	YP_006374661.1	398	88	2	1	397	29	421	0
GL000295	NP_312937.1	1389	658	21	8	1371	8	1339	0
GL000296	AAK44936.1	124	35	0	1	124	1	124	4.00E-63
GL000306	NP_207975.1	459	16	0	1	459	1	459	0
GL000309	NP_207972.1	443	10	0	1	443	1	443	0
GL000772	AIL15701	421	220	3	1	420	1	417	4.00E-126
GL000822	YP_002344422.1	853	293	6	3	818	2	851	0
GL000911	NP_415611.1	247	130	2	1	247	1	243	2.00E-66
GL000972	WP_005768149.1	810	390	18	3	773	12	809	0
GL001063	AJF83452.1	287	164	2	1	283	2	288	1.00E-71
GL001265	NP_415804.1	262	141	1	1	261	1	262	2.00E-80
GL001295	YP_001332362.1	222	123	4	1	221	1	216	7.00E-51
GL001455	AJF82049.1	254	141	2	4	255	7	260	2.00E-62

Table 7 Characteristics of drug resistance genes in CARD

Subject_id	ARO_number	Definition of Term
YP_208874.1	Neisseria gonorrhoeae FA 1090	rpsJ is a tetracycline resistance protein identified in Neisseria gonorrhoeae. Tetracycline resistance is conferred by binding to the ribosome as a 30S ribosomal protection protein. [PMID:2750660 5]
YP_006374661.1	Enterococcus faecium DO	Sequence variants of Enterococcus faecium elongation factor Tu that confer resistance to GE2270A [PMID:7989561]
NP_312937.1	Escherichia coli O157:H7 str. Sakai	Point mutations that occurs in Escherichia coli rpoB resulting in resistance to rifampicin [PMID:3050121]
AAK44936.1	Mycobacterium tuberculosis CDC1551	Ribosomal protein S12 stabilizes the highly conserved rpsL pseudoknot structure formed by 16S rRNA. Amino acid insubstitutions in RpsL affect the higher-order structure of 16S rRNA and confer streptomycin resistance by disrupting interactions between 16S rRNA and streptomycin. [PMID:22943573, PMID:7934937, PMID:17238915, PMID:8849220, PMID:7968530, PMID:15305490]
NP_207975.1	Helicobacter pylori 26695	hp1184 is a translocase that belongs to the MATE efflux pump family. It is found in H. pylori and is involved in the active efflux of antibiotics. [PMID:27303615, PMID:15793129]
NP_207972.1	Helicobacter pylori 26695	hp1181 is a translocase that is part of the MFS efflux pump family. It is found in H. pylori and has role in the active efflux of antibiotics. [PMID:27303615]
AIL15701	Escherichia coli ATCC 25922	murA or UDP-N-acetylglucosamine enolpyruvyl transferase catalyses the initial step in peptidoglycan biosynthesis and is inhibited by fosfomycin. Overexpression of murA through mutations such as Asp369Asn and Leu370Ile confers fosfomycin resistance. Extensive evidence has shown the significance of C115 mutations in conferring fosfomycin resistance since this residue represents a primary binding site for the antibiotic across many species. [PMID:8281938, PMID:8664284, PMID:23143172, PMID:20071153]
YP_002344422.1	Campylobacter jejuni subsp. jejuni NCTC 11168	Campylobacter jejuni is a major bacterial infectious agent associated with gastroenteritis. Quinolone resistance is reportedly conferred by a single C-257-T nucleotide substitution in the gyrA gene. [PMID:26658311]
NP_415611.1	Escherichia coli str. K-12 substr. MG1655	fabG is a 3-oxoacyl-acyl carrier protein reductase involved in lipid metabolism and fatty acid biosynthesis. The bacterial biocide Triclosan blocks the final reduction step in fatty acid elongation, inhibiting biosynthesis. Point mutations in fabG can confer resistance to Triclosan. [PMID:27577999]
WP_005768149.1	Bartonella bacilliformis KC583	Point mutation in Bartonella bacilliformis resulting in aminocoumarin resistance [PMID:9797224]
AJF83452.1	Acinetobacter baumannii	The LpxC gene is widely known to be involved in the biosynthesis of lipid A in Gram-negative bacteria and mutations to this gene may cause resistance to antimicrobial peptides that target the outer membrane [PMID:20855724, PMID:24189257]
NP_415804.1	Escherichia coli str. K-12 substr. MG1655	fabI is a enoyl-acyl carrier reductase used in lipid metabolism and fatty acid biosynthesis. The bacterial biocide Triclosan blocks the final reduction step in fatty acid elongation, inhibiting biosynthesis. Point mutations in fabI can confer resistance to Triclosan and Isoniazid [PMID:27577999]
YP_001332362.1	Staphylococcus aureus subsp. aureus str. Newman	ArlR is a response regulator that binds to the norA promoter to activate expression. ArlR must first be phosphorylated by ArlS. [PMID:10633099]
AJF82049.1	Acinetobacter baumannii	The LpxA gene is widely known to be involved in the biosynthesis of lipid A in Gram-negative bacteria and mutations to this gene may cause resistance to antimicrobial peptides that target the outer membrane [PMID:24189257, PMID:20855724]

Fig. 2. hp1181 and hp1184 gene expressions in drug-resistant strains. A. hp1181; B. hp1184.

Fig. 3. Drug sensitivity was improved after knockout of the drug resistance genes. A. hp1181 knocked out; B. MIC after hp1181 knocked out; C. hp1184 knocked out; D. MIC after hp1184 knocked out.

3.7 Identification of 23S rRNA gene mutations

Three strains were resistant to clarithromycin, so we analyzed and identified the sites of clarithromycin-resistant mutations. We found that three strains had mutations in A2142G, A2143G, G2144T, and some had mutations in other sites, as shown in Table 8.

Table 8 Mutations in the 23S rRNA genes of Hp strains

3.8 Gene mutation induced in drug-resistant strains

After induction with clarithromycin, Hp26695 drug resistance was enhanced on the 12th day, reached the highest level on day 16 and increased to 8μg/ml on the 24th day. The expressions of hp1181 and hp1184 were also increased with increasing clarithromycin resistance, especially hp1184, as shown in Fig. 4. Only A2142G and A 2143G mutations were detected in 23S RNA, with no other mutation sites being found. These data indicated that these two genes may be involved early in the regulation of clarithromycin resistance.

Fig. 4. Induction of resistance to clarithromycin and expression of drug resistance genes in Hp. A. Induction of clarithromycin resistance; B. Expression of drug resistance gene

Table 8 23S rRNA mutations of Hp strains

4. Discussion

The treatment of Hp remains based on bismuth tetralogy at present. Hp is eradicated clinically using common antibiotics including clarithromycin, amoxicillin, metronidazole, tetracycline and levofloxacin star. However, in recent years, the growing rate of antibiotic resistance has resulted in the failure of Hp eradication[19–20]. The most serious resistance has developed to drugs including metronidazole, clarithromycin, and levofloxacin star. The common mechanisms of bacterial resistance are the production of inactivated enzymes, change in the target position of antibacterial drugs, change in the permeability of bacterial outer membrane, effects on the active outflow system, formation of bacterial biofilm and cross resistance[21–23]. There are some differences in the mechanisms of drug resistance of each kind of bacteria, however, the same kind of bacteria still have different resistances to the same antibiotic in different areas[24]. The mechanism of drug resistance of Hp remains unclear and needs further study.

We selected drug-resistant strains using metronidazole, clarithromycin and levofloxacin for genome sequencing analysis. We found that there were no significant differences from the number of drug-resistant genes in the CARD database. This may be because two kinds of antibiotic resistance can develop and the drug-resistant genes in Hp are mainly hp1181 and hp1184. hp1181 is putative NDA translocase, related to the major facilitator superfamily, and is an integral membrane protein; hp1184 belongs to the MATE family, another translocase, resulting in the aforementioned susceptibility. These can contribute to resistance via a multi-drug resistance efflux protein, active-efflux of antibiotics and other efflux pump genes, such as HefA. After knockout of these two genes, the MICs of the drugs were significantly decreased and the sensitivity increased. It is noteworthy that in addition to these two genes, the GE2270A gene of *Enterococcus* and Mura gene of *E. coli* also show a correlation. It is likely that the drug-resistant plasmids of other strains invade Hp through transformation or other mechanisms. Bacteria other than Hp in the gastric mucosa of patients indirectly confirm this view. The main reason for this may be long-term acid resistant treatment, gastric erosion, or intestinal bacterial reflux. This will lead to drug resistance becoming more difficult to prevent and control, so we must attach great importance thereto. In addition, all three strains have clarithromycin resistance. The mechanism of resistance to clarithromycin is mainly reflected in the mutations A2142G, A2143G, G2144T. In addition, it is common that there are several mutations in the same strain.

hp1181 and hp1184 are related to multi-drug resistance and also to clarithromycin resistance, which has been previously reported in the literature[25–26]. The RNA expressions of hp1181 and hp1184 were increased with the emergence of clarithromycin resistance with hp1184 showing the fastest increase. Therefore, these genes are also involved in the regulation of drug resistance and may be one of the mechanisms of Hp resistance to clarithromycin. Compared with the clinical isolates, 23S RNA mutation sites of Hp were less frequent in artificially induced strains that had only A2142G and A2143G mutations. These may be due to the single factor of artificial induction that is not as complex as human stomach environment. More importantly, hp1184 mutation may be the earliest and most persistent response to clarithromycin resistance, and it may be the main target gene for the prevention and treatment of clarithromycin resistance.

The genetic characteristics of multi-drug-resistant strains in this area were preliminarily identified: the relationship between hp1181, hp1184 and clarithromycin resistance was demonstrated through genome sequencing analysis and gene function identification to drug-resistant Hp from Bama County, Guangxi Province. Our study further provided an improved experimental basis for the prevention and treatment of drug resistance of Hp.

Declarations:

λ Ethics approval and consent to participate:

λ Not applicable

λ Consent for publication:

λ Not applicable

λ Availability of data and material:

λ The datasets generated during and/or analyses during the current study are available in the Pubmed and CNKI (China National Knowledge Infrastructure)

λ Competing interests:

λ The authors declare that they have no competing interests.

Declarations

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Figures

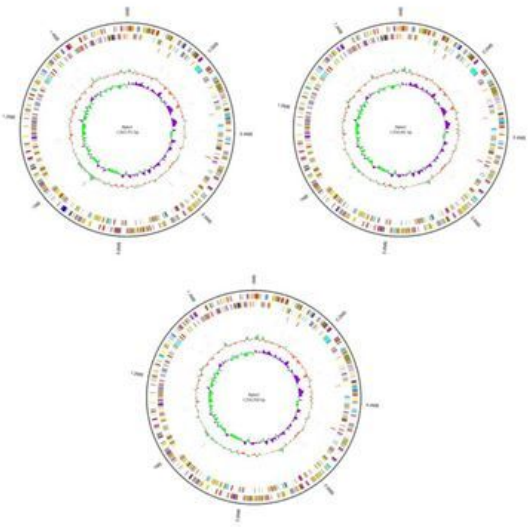

Figure 1

The circular genome analysis of three resistant strains. A. Hpbs1; B. Hpbs2; C. Hpbs3.

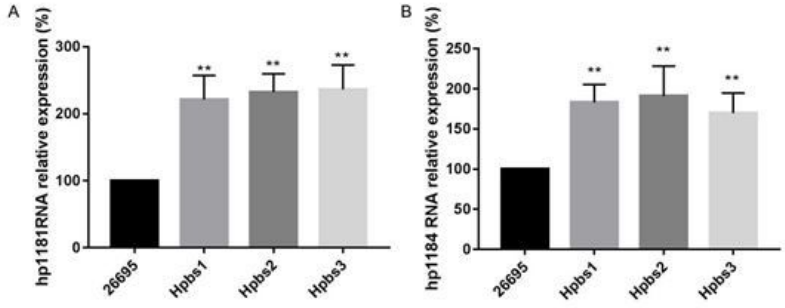

Figure 2

hp1181 and hp1184 gene expressions in drug-resistant strains. A. hp1181; B. hp1184.

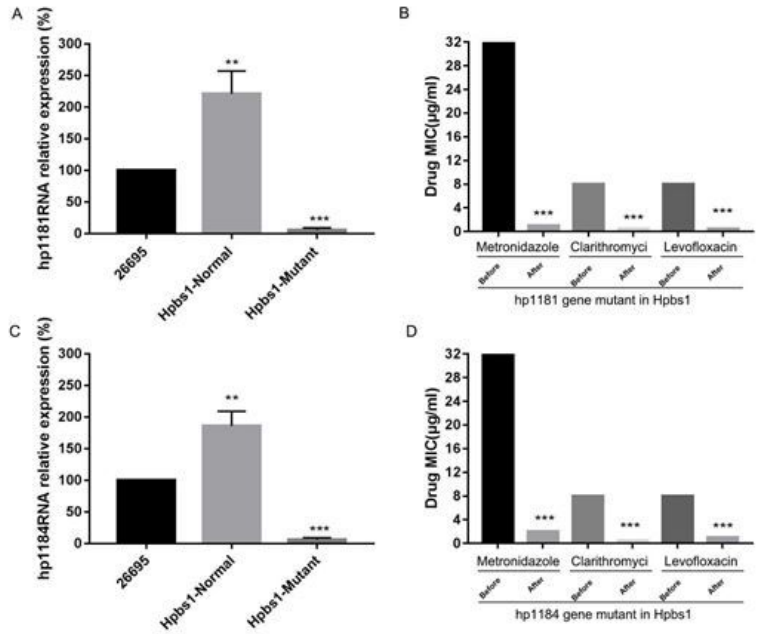

Figure 3

Drug sensitivity was improved after knockout of the drug resistance genes. A. hp1181 knocked out; B. MIC after hp1181 knocked out; C. hp1184 knocked out; D. MIC after hp1184 knocked out.

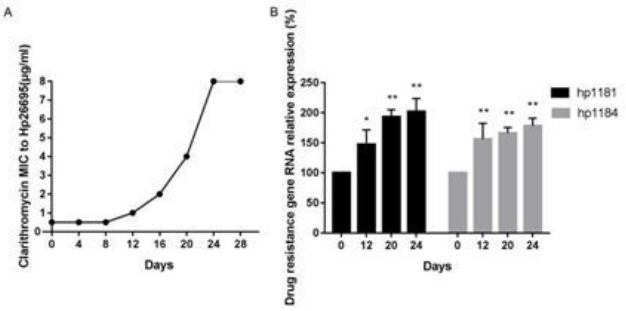

Figure 4

Induction of resistance to clarithromycin and expression of drug resistance genes in Hp. A. Induction of clarithromycin resistance; B. Expression of drug resistance gene