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Relationship between drug resistance and the clustered, regularly interspaced, short, palindromic repeat-associated protein genes *cas1* and *cas2* in *Shigella* from giant panda dung

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Abstract

Background: To detect drug resistance in *Shigella* obtained from the dung of the giant panda, explore the factors leading to drug resistance in *Shigella*, understand the characteristics of clustered, regularly interspaced, short, palindromic repeats (CRISPR), and assess the relationship between CRISPR and drug resistance.

Methods: We collected fresh feces from 27 healthy giant pandas in the Giant Panda Conservation base (Wolong, China). We identified the strains of *Shigella* in the samples by using nucleotide sequence analysis. Further, the Kirby-Bauer paper method was used to determine drug sensitivity of the *Shigella* strains. CRISPR-associated protein genes *cas1* and *cas2* in *Shigella* were detected by polymerase chain reaction (PCR), and the PCR products were sequenced and compared.

Results: We isolated and identified 17 strains of *Shigella* from 27 samples, including 14 strains of *Shigella flexneri*, 2 strains of *Shigella sonnei,* and 1 strain of *Shigella dysenteriae.* Further, drug resistance to cefazolin, imipenem, and amoxicillin–clavulanic acid was identified as a serious problem, as multidrug-resistant strains were detected. Further, cas1 and cas2 showed different degrees of point mutations.

Conclusion: The CRISPR system widely exists in *Shigella* and shares homology with that in *Escherichia coli.* The *cas1* and *cas 2* mutations contribute to the different levels of resistance. Point mutations at sites 3176455, 3176590, and 3176465 in *cas1 (a)*; sites 3176989, 3176992, and 3176995 in *cas1 (b)*; sites 3176156 and 3176236 in *cas2* may affect the resistance of bacteria, cause emergence of multidrug resistance, and increase the types of drug resistance.

Abbreviations: CRISPR = clustered, regularly interspaced, short, palindromic repeats, KIA = Kligler Iron Agar, MIU = Motility Indole Urea, NCBI = National Center for Biotechnology Information Species, PCR = polymerase chain reaction.

Keywords: clustered, drug resistance, giant panda, palindromic repeat, regularly interspaced, *Shigella*, short

1. Introduction

Giant panda is a unique and rare wild animal.[1] Disease is one of the main causes of death among giant pandas, and intestinal disease is the most serious of them.[2,3] Among intestinal infectious diseases in giant pandas, bacterial dysentery caused by *Shigella* is common.[4–6] Currently, the treatment of bacterial dysentery is mainly with antibiotics. However, with the growing abuse of antibiotics, the drug resistance of bacteria is becoming a more serious problem and increasing the risk of bacterial dysentery.[7] In a clinical setting, drug-resistant strains of bacteria can be produced by obtaining the exogenous gene and performing a horizontal transfer of the drug-resistance gene.[8–10] The clustered, regularly interspaced, short, palindromic repeats (CRISPR)-related protein gene family (Cas) is responsible for CRISPR transcription, processing, and degradation of foreign gene sequences.[11] Studies have shown that the *cas1* and *cas2* genes in from the Cas family are present in all CRISPR subtypes.[12,13] Therefore, they are often used as molecular markers for the identification of CRISPR systems. Previous studies have also shown that point mutations in *cas1* and *cas2* affect the resistance of bacteria by increasing the degree of drug resistance and leading to emergence of multidrug resistance strains, even among bacteria that are resistant to a drug due to a point mutation.[14–16] However, it is thus far unclear which specific mutations in these genes affect the resistance of bacteria. Therefore, in this study, we aimed to: isolate and identify *Shigella* strains from the feces of the fresh giant panda, collected from the Giant Panda Conservation base; study the relationship of drug resistance with *cas1* and *cas2* in bacteria; and identify genetic mutations that may lead to changes in drug resistance.

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2. Materials and methods

2.1. Sample

We collected fresh feces from 27 healthy giant pandas from the Giant Panda Conservation base (Wolong China).

2.2. Isolation and purification

Five grams of sample was collected from the feces under sterile conditions and diluted with sterile saline. The diluted samples were coated under aerobic conditions at 37 °C for 12 hours on the *Salmonella–Shigella* selective culture medium (Hangzhou Microbial Reagent Company).[17] Positive colonies were inoculated with Kligler Iron Agar (KIA) and Motility Indole Urea (MIU) culture media (Hangzhou Microbial Reagent Company) at 37 °C for 24 hours. Colonies that showed glucose fermentation, no lactose fermentation, no gas, and no H_2S production on the KIA medium and no motility, indole positivity, and no urinary enzyme on MIU media were suspected to be *Shigella*.[18] Thereafter, using nutrient agar to culture and purify the strains, Gram staining was performed on the purified colonies, following which the reserve was preserved.

2.3. DNA extraction

DNA from suspicious colonies was extracted using the TIANmap Bacteria DNA kit (TIANGEN) according to the manufacturer's instructions, and the DNA samples were stored at −20 °C.

2.4. 16S rRNA sequencing

The 16S rRNA gene from the DNA was amplified by polymerase chain reaction (PCR) using 2 universal primers – 27F and 1492R (F: 5′-AGAGTTTGATCCTGGCTCAG-3′; R: 5′-AAGGAGGGGATCCAGCC-3′). All reagents for the PCR were purchased from TaKaRa, Biological Engineering (Dalian) Co. After the amplification, 5 μL of product was run on a gel (1% agarose) for electrophoresis. The reaction conditions and system for 16S rRNA gene PCR are shown in Tables 1–2.

16S rRNA sequencing of the strains was performed by the TSINGKE Biological Technology Corp (Beijing). Similarity searches were conducted with the derived sequences, and the obtained sequences were compared with available sequences found in the National Center for Biotechnology Information Species (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree was constructed using DNAMAN and Megalign softwares.

Table 1

The reaction conditions for *16S rRNA* gene polymerase chain reaction (PCR).

Temperature	Time	Cycle
95 °C	10 minutes	1 cycle
95 °C	30 seconds	30 cycles
53 °C	30 seconds	
72 °C	90 seconds	

Table 2

The reaction system for *16S rRNA* gene polymerase chain reaction (PCR).

2xTaq PCR master mix	12.5 μL
Primer 1 (10 mM)	1.0 μL
Primer 2 (10 mM)	1.0 μL
DNA	2.0 μL
ddH_2O	7.5 μL
Total	25.0 μL

2.5. Biochemical characteristics

For biochemical identification of *Shigella*, the *Shigella* biochemical test tubes (15 types Hangzhou Microbial Reagent Company) were used for the suspected strains.

2.6. Serological identification

To determine the *Shigella* type, a tentative agglutination test with *Shigella* polyvalent diagnostic serum (Hangzhou Microbial Reagent Company) was conducted after biochemical identification. Thereafter, the aggregated strains from the tentative agglutination test were subjected to typing with intragroup factor serum.

2.7. Microbial sensitivity test

Sensitivity to various antibiotics was tested by the Kirby–Bauer method of disc diffusion, spreading bacterial suspensions on nutrient agar plates and applying filter paper disks containing different antibiotics (amount per disk: carbenicillin, 30 μg; ampicillin, 10 μg; sulfisoxazole, 30 μg; cefazolin, 30 μg; cefepime, 30 μg; amoxicillin-clavulanic acid, 10 μg; trimethoprim-sulfamethoxazole, 1.25/23.75 μg, 30 μg; ceftazidime, 30 μg; imipenem, 10 μg; gentamicin, 10 μg; tobramycin, 10 μg; amikacin, 30 μg; tetracycline, 30 μg; ciprofioxacin, 5 μg; norfloxacin, 10 μg; and chloramphenicol, 30 μg) (Hangzhou Microbial Reagent Company). For these assays, the strains obtained were incubated at 30 °C for 24 hours. The quality-control strain used was *Escherichia coli* ATCC 25922, which was stored at the Sichuan Agricultural University (Chengdu, China). The results were judged as per the Performance Standards for Antimicrobial Susceptibility testing approved by the Clinical and Laboratory Standards Institute.[19]

2.8. Analysis of cas

The sequences of *cas2*, *cas1 (a)*, and *cas1 (b)* were obtained from NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Table 3). Primers were designed using Primer 5.0 software and manufactured by TSINGKE Biological Technology Corp for amplification of the *cas* sequence of *Shigella*. After the amplification, 5 μL of product was run on a gel (2% agarose) for electrophoresis.[20] The reaction conditions and system for *cas1 (a)*, *cas1 (b)*, and *cas2* gene PCR in Tables 4–6.

Table 3

Clustered, regularly interspaced, short, palindromic repeat-associated protein gene primer sequence.

Name	Primer	Size, bp
cas1 (a)	F:5′-AATGGAATGGTCGCAAATAC-3′ R:5′-CGACAGGCTAATCTGACTTC-3′	280
cas1 (b)	F:5′-GCACTTCCATGATCTTCCTC-3′ R:5′-CCGCTTCACCGACCCAGA-3′	204
cas2	F:5′-TCGCAATCTGGCTACTGG-3′ R:5′-AACCCATCCAAATCCACC-3′	202

Table 4

The reaction conditions for *cas2* and *cas1 (a)* gene polymerase chain reaction (PCR).

Temperature	Time	Cycle
94 °C	5 minutes	1 cycle
94 °C	60 seconds	32 cycles
51 °C	45 seconds	
72 °C	60 seconds	
72 °C	10 minutes	1 cycle

Table 5

The reaction conditions for *cas1 (b)* gene polymerase chain reaction (PCR).

Temperature	Time	Cycle
94 °C	5 minutes	1 cycle
94 °C	60 seconds	40 cycles
61 °C	45 seconds	
72 °C	60 seconds	
72 °C	10 minutes	1 cycle

Table 6

The reaction system for *cas1 (a)*, *cas1 (b)*, and *cas2* gene polymerase chain reaction (PCR).

2xTaq PCR master mix	12.5 μL
FPrimer 1	1.0 μL
RPrimer 2	1.0 μL
DNA	2.0 μL
ddH_2O	7.5 μL
Total	25.0 μL

3. Results

3.1. Bacterial morphology

Colonies grown on the *Salmonella–Shigella* agar medium were circular, smooth with entire edges, translucent, light beige, and approximately 1 to 2 mm in size. The KIA culture slant was red with a yellow bottom and showed no gas or H_2S production.

Table 7

Serotype distribution and configuration of the 17 *Shigella* strains identified.

Sero-group	Serotype	Number	Percent, %
A	*Dysenteriae*-8	1	5.88
B	*Flexneri*-1a	2	11.77
	Flexneri-1b	1	5.88
	Flexneri-2a	1	5.88
	Flexneri-2b	3	17.64
	Flexneri-3a	2	11.77
	Flexneri-4a	2	11.77
	Flexneri-5b	1	5.88
	Flexneri-6	2	11.77
D	*Sonnei*-1	1	5.88
	Sonnei-2	1	5.88

Further, the MIU culture was nonmotile, indole positive, and urinary enzyme negative. Microscopic examination showed that the strains were aerobic, gram-negative, asporous, and non-capsulated. Ultimately, from the 28 samples, we purified 17 strains with typical *Shigella* colony characteristics.

3.2. 16S rRNA sequencing

16S rRNA sequencing analysis of the 17 strains revealed 14 strains of *Shigella flexneri*, 2 strains of *Shigella sonnei*, and 1 strain of *Shigella dysenteriae*.

3.3. Biochemical characteristics

The strains were negative for urea, lysine decarboxylase, salicylic acid, esculin hydrate, glucosamine, and Simmons' citrate, but positive for β-galactose acid, ornithine decarboxylase, indole, mannitol, raffinose, glycerin, and mucate. Comparing with the typical *Shigella* characteristics, the strains were confirmed as *Shigella*.

3.4. Serological identification

The tentative agglutination test with *Shigella* polyvalent diagnostic sera was positive for the whole strains. The results of the subsequent agglutination test with cluster factor serum used to determine the type of *Shigella* are presented in Table 7.

Table 8

Drug-sensitivity testing.

Antibiotic class	Antibacterial drugs	*Shigella* (n = 17)		*Flexneri Shigella* (n = 14)		*Sonnei Shigella* (n = 2)		*Dysenteriae Shigella* (n = 1)	
		Number	Percent, %	Number	Percent, %	Number	Percent, %	Number	Percent, %
Tetracycline	Tetracycline	1	5.88	0	0.00	1	50	0	0.00
Chloramphenicol	Chloramphenicol	2	11.76	1	7.14	1	50	0	0.00
Aminoglycosides	Gentamicin	2	11.76	2	14.29	0	0.00	0	0.00
Quinolone	Amikacin	0	0.00	0	0.00	0	0.00	0	0.00
	Tobramycin	2	11.76	2	14.29	0	0.00	0	0.00
	Norfloxacin	1	5.88	0	0.00	0	0.00	1	100
β-Lactam	Ciprofioxacin	1	5.88	0	0.00	0	0.00	1	100
	Carbenicillin	3	17.65	1	7.14	1	50	1	100
Carbapenems	Ampicillin	3	17.65	1	7.14	1	50	1	100
	Amoxicillin clavulanic acid	4	23.53	2	14.29	1	50	1	100
	Cefepime	1	5.88	1	7.14	0	0.00	0	0.00
	Ceftazidime	2	11.76	1	7.14	1	50	0	0.00
	Cefazolin	6	35.29	3	21.43	2	100	1	100
	Imipenem	5	29.41	5	35.71	0	0.00	0	0.00
Sulfanilamide	Sulfisoxazole	3	17.65	2	14.29	1	50	0	0.00
Folicacid metabolic inhibitor	Trimethoprim sulfamethoxazole	2	11.76	1	7.14	1	50	0	0.00

3.5. Microbial sensitivity test

The analyzed strains showed maximum drug resistance to cefazolin (35.29%), imipenem (29.41%), and amoxicillin–clavulanic acid (23.53%). Furthermore, the strains showed resistance to all the antibiotics except amikacin. Three strains – 2 strains of *S flexneri* and 1 strain of *S dysenteriae* – showed resistance to 2 categories of drugs and the β-lactam antibiotics; 2 strains – *S flexneri* and *S sonnei* – showed resistance to

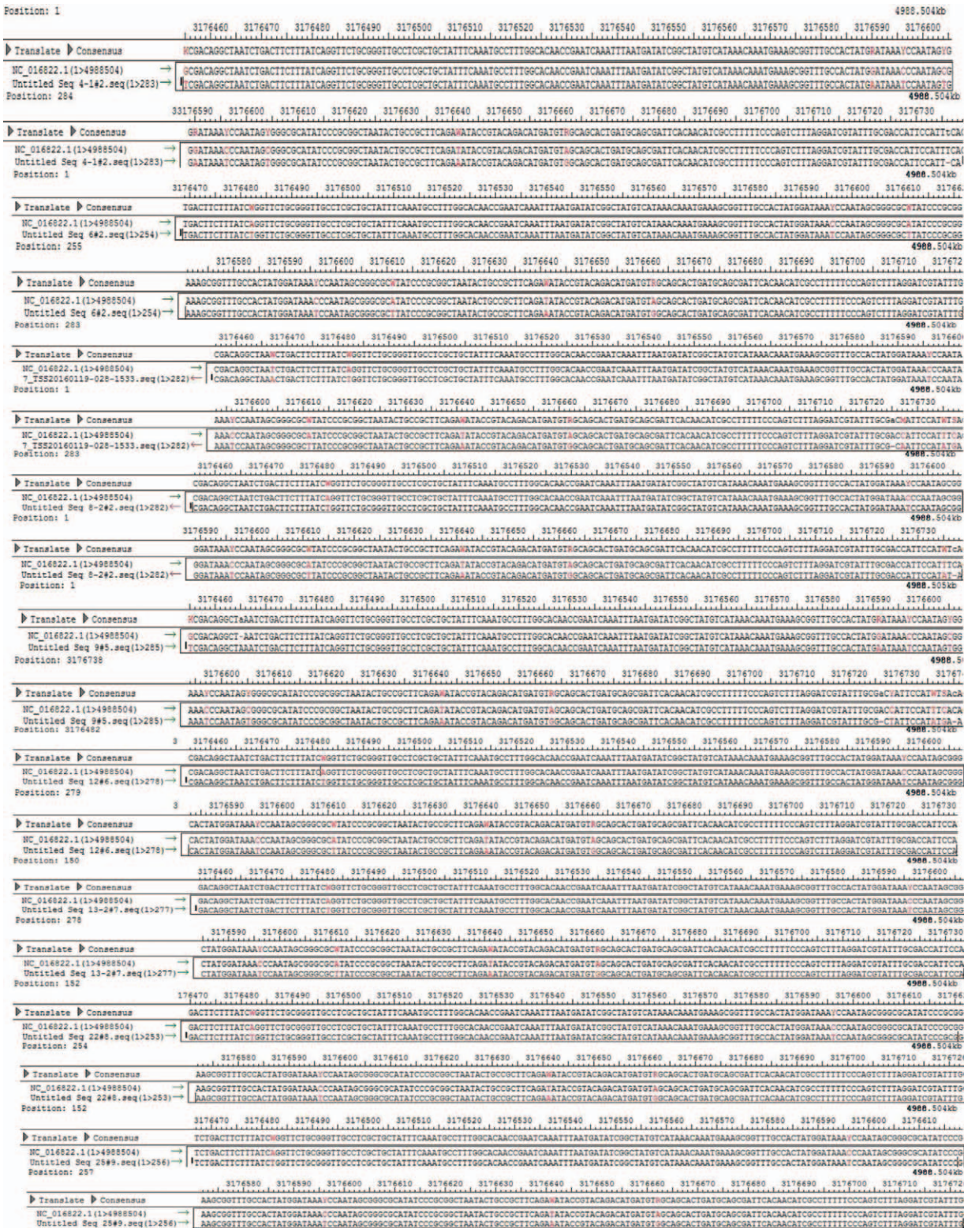

Figure 1. *cas1 (a)* gene mutation analysis. The top-most sequence is the standard sequence, followed by the detected sequences. The red letters indicate a change of base.

5 categories of drugs including chloramphenicol, β-lactam antibiotics, sulfonamides, and trimethoprim–sulfamethoxazole; and 5 strains of *S flexneri* showed sensitivity to all types of antibacterial drugs used (Table 8).

3.6. Analyses of cas

The Ssequencesd of *cas1 (a)*, *cas1 (b)*, and *cas2* of the 17 identified *Shigella* strains were compared with the published sequences of *Shigella S sonnei 53G* from NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) by SeqMan software. The results showed that *cas1 (a)*, *cas1 (b)*, and *cas2* have different degrees of mutation.

A total of 13 strains contained *cas1 (a)*, including 4 strains of *E coli* and 9 strains of *Shigella* (4–1, 6, 7, 8–2, 9, 12, 13–2, 22, and 25–2) and showed similarity >90%. Except for strain no. 9, the strains showed point mutations in sites 3176596 (C→T), 3176641 (T→A), and 3176662 (A→G). Furthermore, except strain nos. 4–1 and 9, all strains showed point mutations in site 3176482 (A→T), and strain nos. 6, 7, 8–2, 12, and 13–2 showed point mutations in site 3176611 (A→T). Additionally, strain nos. 7 and 9 showed a base A deletion at position 3176726, and strain no. 9 showed a base A insertion at position 3176465 (Fig. 1).

A total of 7 strains containing *cas1 (b)*, including 2 strains of *E coli* and 5 strains of *Shigella* (4–1, 6, 9, 22, and 25–2), showed a similarity >90%. All the strains showed point mutations in sites 3177016 (G→C), 3177019 (T→C), 3177037 (T→C), 3177076 (T→C), and 3177082 (A→G). Except for strain nos. 4–1, the remaining strains showed point mutations in 3177171 (G→C). Strain no. 6 showed a base T insertion at position 3177159 (Fig. 2).

A total of 11 strains containing *cas2*, including 3 strains of *E coli* and 8 strains of *Shigella* (4–1, 6, 7, 9, 12, 13–2, 22, and 25–2), showed similarity >90%. All the strains showed point mutations in sites 3176096 (C→T) and 3176100 (A→G). In addition, strain nos. 4–1 and 9 showed point mutations in site 3176156 (G→T), whereas the rest of the strains showed point mutations in sites 3176063 (G→A), 3176120 (C→T), 317138 (A→T), 3176147 (G→A), 3176149 (T→A), 317150 (A→G), 3176183 (T→G), 3176186 (T→C), 3176192 (A→T), 31769195 (A→G), and 3176201 (C→A) (Fig. 3).

4. Discussion

The main habitats of the giant panda are the wild, the Wolong National Nature Reserve, the Bifengxia Panda Reserve, and the Beijing zoo, they are currently also kept captive at the Giant Panda Conservation base. Once used the cefazolin for the ailing giant panda. Studies[21,22] have shown that resistant strains can result from the horizontal transfer of drug resistance genes. In this study, the results of drug-sensitivity tests showed that the growing resistance to β-lactam in *Shigella* is a very serious issue, and the degree of resistance to cefazolin (35.29%) and augmentin (23.53%) are higher than other drug. Although *Shigella* is known to be sensitive to amikacin, *S flexneri* showed strong resistance to imipenem (35.71%). This could be due to the following reasons:

(1) China has a high incidence of bacterial dysentery and uses antibiotics on a large scale; as a result, the problem of bacterial drug resistance has become serious.[23,24] In

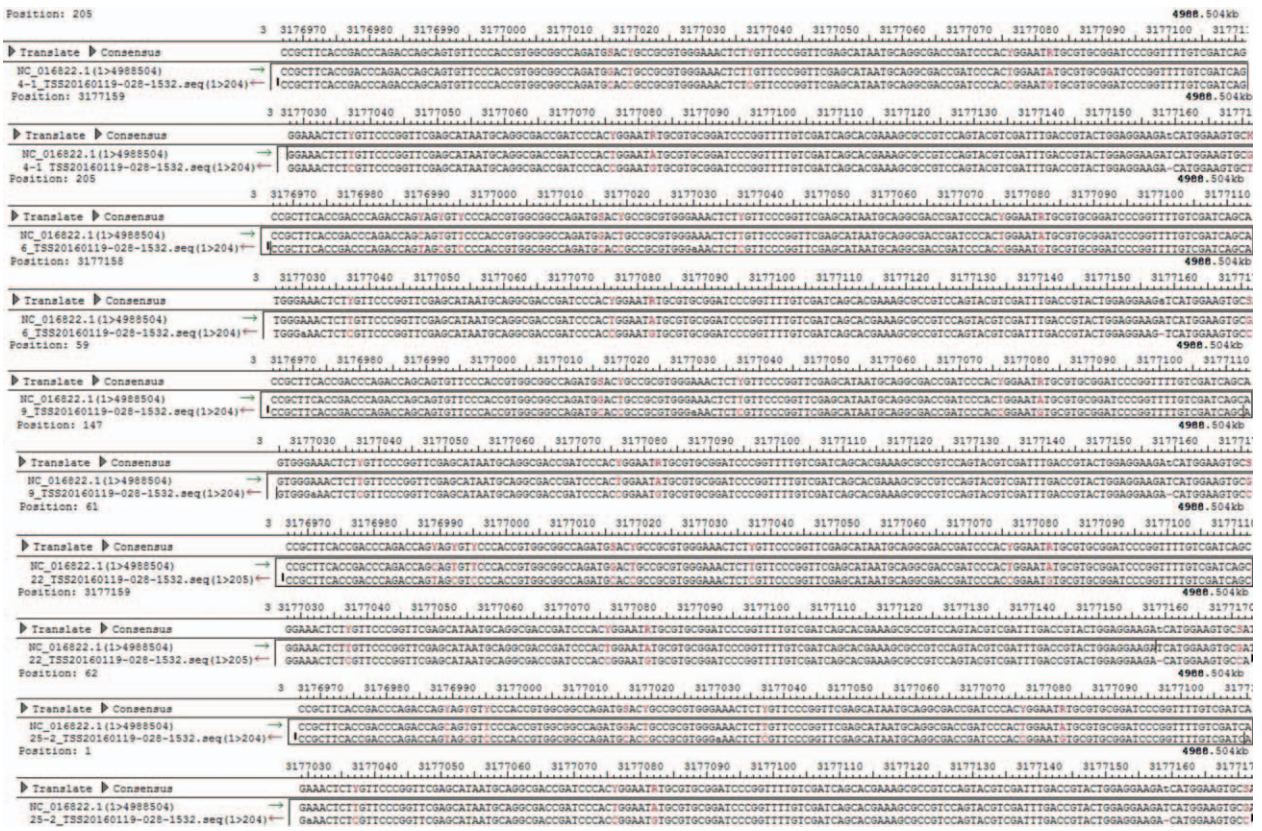

Figure 2. *cas1 (b)* gene mutation analysis. The top-most sequence is the standard sequence, followed by the detected sequences. The red letters indicate a change of base.

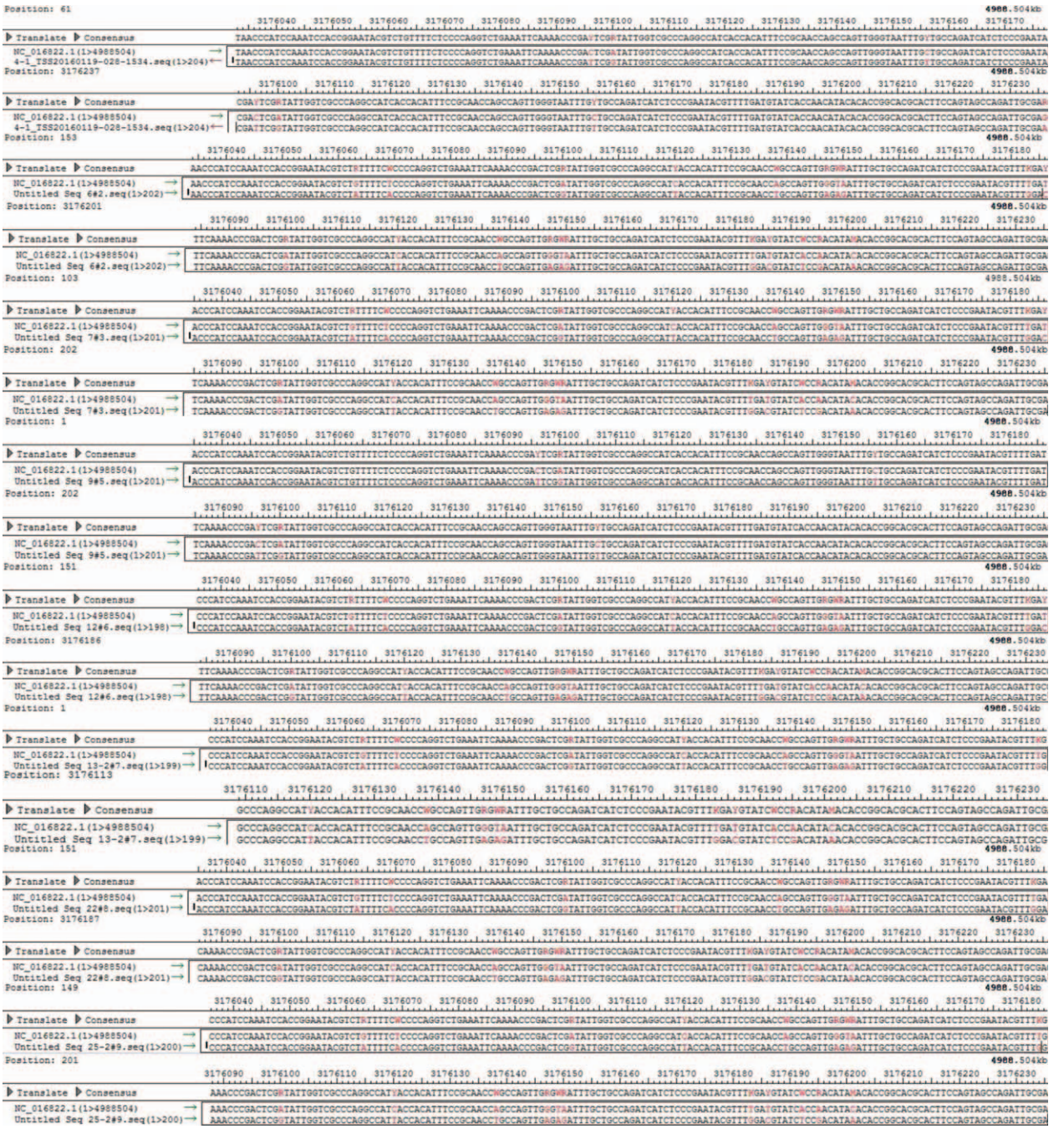

Figure 3. *cas2* gene mutation analysis. The top-most sequence is the standard sequence, followed by the detected sequences. The red letters indicate a change of base.

addition, tourists or breeders might transfer their own resistance genes to the giant panda, leading to drug resistance among the pandas.

(2) Cefazolin has previously been used for the treatment of the giant panda and may have caused drug resistance.

(3) The habitat of giant panda is complex, and as such, drug resistance among the pandas might be due to cross contamination.

Thus, for clinical treatment of bacterial dysentery in giant pandas, amikacin should be used, all sensitive drugs should be replaced at regular intervals, direct contact between tourists and the giant panda should be reduced, the different sources of panda polyculture should be avoided, and cross contamination should be prevented.

The distribution of *cas1* and *cas2* in *Shigella* indicated that the CRISPR system widely exists in *Shigella*. The results show that the similarity of *cas1* and *cas2* between *E coli* and *Shigella* was >90%, which is consistent with the results of a previous study.[25] The CRISPR sequences in *Shigella* and *E coli* are homologous, but still show some differences, which may be due to the transfer of the CRISPR sequence from *E coli* to *Shigella*. Due to gradual delivery or bacterial evolution, the sequence may have changed. Sequence variation may have contributed to the different degrees of resistance between *E coli* and *Shigella*.

Our study on the association between *cas1 (a)*, *cas1 (b)*, and *cas2* gene mutations and drug resistance showed that strain nos. 4–1 and 9 are multidrug resistant, strain nos. 4–1 is resistant to 5 categories of drugs, strain no. 9 is resistant to 2 types of drugs, strain nos. 6, 7, 8–2, 12, and 22 are sensitive to all drugs, and strain nos. 13–2 and 25–2 are only resistant to 1 type of drugs. Analysis of *cas1 (a)* mutation sites showed that the base A deletion in site 3176726 may not be related to the degree of drug resistance, and the point mutation in site 3176455 (G→T) and 3176590 (G→A) may be one of the causes of multiple drug resistance. Further, because of the base A insertion in site 3176465, the point mutation in strain no. 9 changed from position 3176590 to 3176591. That may be caused the type and the number of drug-resistance of no. 9 is lower than no. 4–1. Analysis of *cas1 (b)* mutation sites showed that the point mutation in sites 3176989 (Cmu), 3176992 (T,3), and 3176995 (Tan) may have reduced the degree of drug resistance. Finally, analysis of *cas2* mutation sites showed that the point mutation in site 3176156 (C17) may be contribute to multiple drug resistance and the point mutation in 3176236 (G17) may be caused the type and the number of drug-resistance of no. 9 is lower than no. 4–1.

5. Conclusion

This study found that the mutations of CRISPR-related protein genes *cas1* and *cas2* are related to the degree of drug resistance, which is consistent with the results of previous studies.[2,5] Although the CRISPR degrades foreign gene sequences, owing to the use of antibiotics and the evolution of bacteria, the function of the CRISPR/Cas system may change and affect the degree of bacterial resistance. In this study, we found that that the point mutations in sites 3176455, 3176590, and 3176465 of *cas1 (a)*; sites 3176989, 3176992, and 3176995 of *cas1 (b)*; and sites 3176156 and 3176236 of *cas2* may affect the degree of drug resistance, cause emergence of multidrug resistant strains, and cause variation in drug resistance. However, it is currently unclear whether the point mutations at these sites affect the mechanism of resistance of *Shigella*, and therefore, this topic needs further research.

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