



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 *cas2* 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

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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.

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 H₂S 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, $5 \,\mu$ L 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

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 ₂ O	7.5 μL
Total	25.0 µL

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.

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, 30µg; ampicillin, 10µg; sulfisoxazole, 30µg; cefazolin, 30µg; cefepime, 30 µg; amoxicillin-clavulanic acid, 10 µg; trimethoprimsulfamethoxazole, 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, $5 \,\mu$ L 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'	280
	R:5'-CGACAGGCTAATCTGACTTC-3'	
cas1 (b)	F:5'-GCACTTCCATGATCTTCCTC-3'	204
	R:5'-CCGCTTCACCGACCCAGA-3'	
cas2	F:5'-TCGCAATCTGGCTACTGG-3'	202
	R:5'-AACCCATCCAAATCCACC-3'	

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 ₂ O	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 H₂S production.

Table 8

Drug-sensitivity testing.

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Table 7

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

Sero-group	Serotype	Number	Percent, %
A	Dysenteriae-8	1	5.88
В	<i>Flexneri</i> -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.

		Shige	<i>lla</i> (n=17)	Flexneri S	higella (n=14)	Sonnei S	<i>higella</i> (n=2)	Dysenteriae	Shigella (n=1)
Antibiotic class	Antibacterial drugs	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	4	23.53	2	14.29	1	50	1	100
	ciavuianic acid		5.00		7 4 4	0	0.00	0	0.00
	Cetepime	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 amoxicillinclavulanic 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

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Untitled Seq 13-2#7.seq(1>27 Position: 152	↑)→ CIAIGGAIAAACCAAIAGOGGGGETIAICCCCCCCCTIAAIACIGCCCCICAAAIAACCGIACGAGGAIGAIGAGACGAGAIGAGAGAAIAGAAACCACACCCCITTICCCAACCITTICCCAACCITTICGAGACGAITTICCCAACCITTICCCAACCITTICGAGACGAITTICCCAACCITTICCCAACCITTICGAGACGAITTICCCAACCITTICCCAACCITTICGAGACGAITTICCCAACCITTICCCAACCITTICGAGACGAITTICCCAACCITTICCCAACCITTICGAGACGAITTICCCAACCITTICCCAACCITTICGAGACGAITTICCCAACCITTICCCAACCITTICGAGACGAITTICCCAACCITTICCCAACCITTICGAGACGAITTICCCAACCITTICCCAACCITTICGAGACGAITTICCCAACCITTICCCAACCITTICGAGACGAITTICCCAACCITTICCCAACCITTICGAGACGAITTICCCAACCITTICCCAACCITTICGAGACGAITTICCCAACCITTICCCAACCITTICGAGACGAITTICCCAACCITTICCCAACCITTICGAGACGAITTICCCAACCITTICCCAACCITTICGAGACGAITTICCCAACCITTICCCAACCITTICGAGACGAITTICCCAACCITTICCCAACCITTICGAGACGAITTICCCAACCITTICCCAACCITTICGAGACGAITTICCCAACCITTICCCAACCITTICCCAACCITTICGAGACGAITTICCCAACCITTICCCAACCITTICCCAACCITTICGAGACGAITTICCCAACCITTICCCAACCITTICGAGACGAITTICCCAACCITTICCCAACCITTICCCAACCITTICCCAACCITTICCCAACCITAGAACGAACCAACCIAACCI
🕈 Translate 🎙 Consensus	GACTICITIATCH000TIC1020000TIG0CT00CT0CTATTCAAATG0CTIT100CACC00ACC0AACC0AATTAATGATAACGAATGAAAC0AATGAAAG0000TIG0CACTAT90ATAAAC0CAATAG009000CATATG0000TI
NC_016822.1(1>4988504) Untitled Seg 22#8.seg(1>253) Position: 254	GRCTICTITATCH001TC100090T100CT00CT0CT0CT0CTCATTAAATCCATTC000CTAT0CCAAATTAATGAATCOACCAATT0AACGAATT0AAACG00T1T0CCACTAT000ATTAACCCATAT000090CCATATCCC GRCTICTITATC100TC1000B0T10CCCCCT0CT0CTATAACG0ATCOAATT0AAT0AA0CG0TT0CCACTAT000AACG00T10CCACTAT000090CCATATCCC GRCTICTTTATC100TC100B0T10CCCCT0CT0CTATATCCAATT0AAT0AA0CG0ATTCAAATTAAT0ATC0AAT0A00000CTAT0CCACTAT0000FCCATATC0 GRCTICTTTATC100TC100B0T10CCCCT0CT0CT0CT0ATAACCCAATT0AAT0AATTCAATT0AAT0AA
P Translate P Consensus	AARGGGTITGCCACTATGGATAAACCCAATAGCGGGCGCCATATCCGCCGCCTATACTGCCGCTTCAGAMATACCGTACGAGCAGTGATGCAGCACTGATGCAGCAATGCCGAGCGATTGCCCAGTCTTTCCCCAGTCTTTAGGATCGTAT
NC_016822.1(1>4988504) Untitled Seg 22#8.seg(1>253 Position: 152	$\stackrel{\rightarrow}{\rightarrow} \begin{array}{c} ABGOBTITECCACTATEGACACACTGATAACCCBATAACCCBCTCACTATECCOCCBCCTATACCCCCCBCTCAGACATAACCCBACAGACATGATETABCACACTGATETABCAGCACTGATGACAGCACTGATGACGACATGACGCCTTTTCCCCACTCTTTAGGACCACTGATGATGACGACACTGATGATGACGACACTGATGATGACGACACTGATGATGACGACACTGATGATGACGACACTGATGATGACGACACTGATGATGACGACACTGATGATGACGACACTGATGATGACGACACTGATGATGACGACACTGATGATGACGACACTGATGATGACGACACTGATGATGACGACACTGATGATGACGACACTGATGATGACGACACTGATGATGACGACGACTGATGATGATGATGATGATGATGATGATGATGATGATGAT$
D Translate D Consensus	3176470 3176480 3176500 3176500 3176510 3176520 3176530 3176540 3176550 3176550 3176570 3176580 3176590 3176690 3176610
NC_016822.1(1>4988504) Untitled Seq 25#9.seq(1>256 Position: 257	→ ТСТВАСТИСТИАТСАВОТИСТОВОВОН БОСТОВСТВОСТОВСТВОСТАТИТОВААТОССТИТОВСАСАВСОВААТСАВАТТАВАТАВАТОВААТОВААТОВ
Longer Colores	3176500 3176500 3176600 3176610 3176620 3176630 317660 3176650 3176650 3176650 3176650 3176650 3176650 3176670 3176710 31

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 \rightarrow T), 3176641 (T \rightarrow A), and 3176662 (A \rightarrow G). Furthermore, except strain nos. 4–1 and 9, all strains showed point mutations in site 3176482 (A \rightarrow T), and strain nos. 6, 7, 8–2, 12, and 13–2 showed point mutations in site 3176611 (A \rightarrow T). Additionally, strain nos. 7 and 9 showed a base A deletion 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 \rightarrow C), 3177019 (T \rightarrow C), 3177037 (T \rightarrow C), 3177076 (T \rightarrow C), and 3177082 (A \rightarrow G). Except for strain nos. 4–1, the remaining strains showed point mutations in 3177171 (G \rightarrow 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 \rightarrow T) and 3176100 (A \rightarrow G). In addition, strain nos. 4–1 and 9 showed point mutations in site 3176156 (G \rightarrow T), whereas the rest of the strains showed point mutations in sites 3176063 (G \rightarrow A), 3176120 (C \rightarrow T), 317138 (A \rightarrow T), 3176147 (G \rightarrow A), 3176149 (T \rightarrow A), 317150 (A \rightarrow G), 3176183 (T \rightarrow G), 3176186 (T \rightarrow C), 3176192 (A \rightarrow T), 31769195 (A \rightarrow G), and 3176201 (C \rightarrow 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:

 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

Position: 205	1000 50100
3	3176970 3176980 3176990 3177000 3177010 3177020 3177030 3177040 3177050 3177060 3177070 3177080 3177090 3177100 31771
Translate D Consensus	
NC_016822.1(1>4988504) → 4-1_TSS20160119-028-1532.seg(1>204)← Position: 3177159	
	317/030 317/040 317/050 317/060 317/070 317/060 317/100 317/110 317/120 317/130 317/140 317/150 317/140 317/150
Translate Consensus	GGAAACTCTYGTTCCCGGTTCGAGCATAATGCAGGCGACCCGATCCCACYGGAATRTGCGTGCGGATCCCGGTTTGTCGATCAGCACGAAAGCGCCGTCCAGTACGTCGATTTGACCGTACTGGAGGAGAACATCGGAAGGC
NC_016822.1(1>4988504)	BSAAACTCTIGTICCC0GTICGASCATAATGCAGOCGACCCATCCGAGCATATGCGTGGGBATCCCCGGTITIGTCGGACGAGCGGGCCGTCCAGTACGTCGGTTGGACGATGGAGGGC GGAAACTCTCGTTCCC0GTICGASCATAATGCAGCGACCGATCCCACGGAGTGCGGGGGCCCGGTITIGTCGGTCAGCACGAAAGGGCCGTCCAGTACGTCGATTGACCGTACGGAGGAGGACGA GGAAACTCTCGTTCCC0GTICGASCATAATGCAGGCGACCGATCCCACGGAGTGCGGGGGCCCGGTITIGTCGGTCAGCACGAAAGGGCCGTCCAGTACGTCGGTTGGAGGAGGC 9840.5040
3	31/87/0 31/8760 31/970 31/100 31/100 31/020 31/030 31/040 31/060 31/070 31/060 31/070 31/100 31/100
▶ Translate ▶ Consensus	COSCITCACCGACCCAGACCAGYAGYGTYCCCACCGTGGCGGCCAGATGSACYGCCGCGGGGGAACTCTYGTTCCCGGTTCGAGCATAATGCAGGACCGATCCCACYGGAATBTGCGTGCGGATCCCGGTTTTGTCGATCAGC
NC_016822.1(1>4988504) → 6_TSS20160119-028-1532.seq(1>204)+ Position: 3177158	COGCT CACCGACCGAACCGGCAGTCCCCACCGTGGCGGCCGAATGGACGGCGGCGGGGGAAACTCTTGTTCCCGGTTCGAGCATATCCAGGCGACCGATCCCACTGGAATATCCGGGCGGCGGCGGGCG
3	3177030 3177040 3177050 3177060 3177070 3177080 3177090 3177100 3177120 3177130 3177140 3177150 3177160 31771
▶ Translate ▶ Consensus	IGGGAAACTCTYGITCCCGGTTCGAGCATAATGCAGGCGACCCGATCCCACYGGAATRIGCGTGCGGAICCCGGTTTGTCGATCAGCACCGAAAGCGCCGTTCGAGCGATGCGATGCGGAGGAAGaTCCAGGAAGaTCCAGGAAGGCCG
NC_016822.1(1>4988504) → 6_TSS20160119-028-1532.seg(1>204)← Position: 59	1995AAACICTISTICCC601TC6A5CATAAT9CA9C6ACC6AT0CCACT69AATAIT9C059C596ATCCC59TIT9IC6ATCASCAC6AAA5C50C5TCCA5TAC55C6ATT36ACC5TACT69A96AA6ATCAT56AA5A5C 1995AACTCTC5TICCC601TC0A5CATAAT9CA9C6ACC6AT0CCAC560AAT9IGC55GC5G6GATCCC56TIT15IC6ATCASCAC6AAA5C50C5TCCA5TAC55CA 499E.5042b
3	3176970 3176980 3176990 3177080 3177010 3177020 3177030 3177040 3177050 3177060 3177070 3177080 3177090 3177100 3177110
Translate Consensus	CCGCTTCACCGACCCAGACCAGCGTGTCCCACCGTGGCGGCCAGATGGACCCGCGTGGGAAACTCTYGTTCCCGGTTCGAGCATAATGCAGGCGACCGATCCCACYGGAATBTGCGTGCGGATCCCGGTTTTGTCGATCAGCA
NC_016822.1(1>4988504) → 9_TSS20160119-028-1532.seq(1>204)↔ Position: 147	CONCINCIANCEARCARCARCARCARTRICCARCOFFECERCECARATERACTERCEDEDEDEGERAACTITUTICCONFICEARCATAATECARGEDACOATCCCLIGBATATECGERECONFILTETERATCARC CONTINACCBACCARCARCARCARTRICCARCOFFECERCECARATERACTERCECONFILEMENTAL CONFILEMENTAL CONFIL
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9_TSS20160119-028-1532.seq(1>204)← Position: 61	Istogeal control and control a
	3176970 3176980 3177080 3177000 3177100 3177030 3177040 3177050 3177060 3177070 3177080 3177090 3177100 31771
Translate Consensus	CCGCTTCACC6ACCCAGYAGYGTYCCCACCGTGGCGGCCCAGATG%ACYGCCGCGTGGGAAACTCTYGTTCCCGGTTCGAGCATAATGCAGGCGACCCACYGGAATAYGCGGGCGGCGGGCGGGCGACCCGGTTTGTCGATCAC
NC_016822.1(1>4988504) → 22_TS520160119-028-1532.seg(1>205)← Position: 3177159	CCGCTTCACCBACCCABACCAG' KATGETICCCACCBTBEGGECCBATGBACTGACCCCACTGBGAAACTCTTGTTCCACCBATTCBACCATABTCCABCCBACCBATCCCACTGBAATTGCGTBCCBBATGCGTGCGBATCAGCGTTTTTGTCGATCABC CCGCTTCACCBACCCABACCAGTAGTGTCCCACCBTBGGGGCCABATGBACTGCCTCGTTCCCGGTTCGBACCTABTCGABCCBATCGGTGCGBATGGTGCGGGCCGBTCGGGTTTTGTCGATCABC 4900.501000000000000000000000000000000000
	31/1000 31/1000 31/1000 31/1010 31/1000 31/1000 31/1000 31/100 31/100 31/100 31/100 31/100 31/100 31/100 31/100
P Translate P Consensus	GRAACTCT/OTTCCCSGTTCSASCATAATGCAGGGSCGATCCCACYGGAATRISCSTGCGGATCCCCGTTTTGTCGATCASCACGAAAGCSCCGTCCAGTACGCGATTISACCGTACTGGAGAAGACCATGGAAGACCATGGAAGACCATGGAAGACCATGGAGAAGACCATGGACGACGACGAGAAGACCATGGAAGACCATGGAAGACCATGGAAGACCATGGAAGACCATGGAAGACCATGGAAGACCATGGAAGACCATGGAAGACCATGGAAGACCATGGAAGACCATGGAAGACCATGGAAGACCATGGAAGACCATGGAAGACCATGGAAGACGACGACGACGACGACGACGACGACGACGACGAC
NC_016822.1(1>4988504) 22_TSS20160119-028-1532.seq(1>205) Position: 62	99AACTCTT9TTCCC69TTCGA9CATAAT9CA99CGACCGATCCCAC199AATAT9C0T9C9GATCC09TTT9TCGATCA9CACGAAAGC9CCGTCCASTAC9TCGATT9ACC9TAC199AGGAAGHCAT19GAAT9C9 98AAACTCT09TTCCC69TTCGA9CATAAT9CA99C9ACCGATCCCAC199AAT4T9C05T9C09ATT19CCGATCA9CACGAAAGC9CCGTCCASTAC9TCGATT9ACC9TAC199AGGAAGA-CAT69AA519C2 99AACTCT09TTCC09TTCGA9CATAAT9CA99C9ACCGATCCCAC199AAT475C05TAC190AGTAC109AT19ACC9TAC199AA79C3 99BAACTCT09TTCGA9CATAAT9CA9C9ACCGATCCCAC199AAT475C05GATCCC9TT19TCGATCA9CACGAAAGC9CCGTCCASTAC9TCGATT9ACC9TAC199AGAAGA-CAT69AA519C2 99AACTCT09TTCC09TTCGA9CATAAT9CA9CCGATCCCAC199AAT475C05GATCCG9TT19TCGATCA9CACGAAAGC9CCGTCCASTAC9TCGATT9ACC9TAC199AGAAGA-CAT69AA519C2 99BAACTCT09TTCC09TTCGA9CATAAT9CA9CCGATCCCAC199AAT475C05GATCCG9TT19TCGATCA9CACGAAAGC9CCGTCCASTAC9TCGATT9ACC9TAC199AGAAGAA-CAT69AA519C2 99BAACTCT09TTCGA9CATAAT9CA9C0ACCGATCCCAC199AAT475C05GATCCG9TT19TCGATCA9CACGAAAGC9CCGTCCASTAC9TCGATT9ACC9TAC199AGAAGAA-CAT69AA519C2 99BAACTT19CC09TTCGA9CACGATCCCAC199AAT475C05GATCCG9TT19TCGATCA9CACGAAAGC9CCGTCCASTAC9TCGATT9ACC9TAC199AGAAGAA-CAT69AA519C2 99BAACTT19ACC5ACCGATCCCAC199AAT475C05GATCCCG9TT19TCGATCA9CACGAAACC9CCGTCCASTAC9CACGATCC6ATC6AGAAGAA-CAT69AA519C2 99BAACTT19CC09TTCGA9CC5ACCGATCCCAC199AAT475C05C03TCCC9TT19TCGATCA9CACGAACCGACCGGCCGCCCACGACCGATCCCAC199AAC
	3 3176970 3176980 3176990 3177000 3177010 3177020 3177030 3177040 3177050 3177060 3177080 3177080 3177100 3177
▶ Translate ▶ Consensus	CCGCTTCACCGACCCAGACCAGYAGYGTYCCCACCGTGGGGGCCAGATGGACCGCCGCGGGGGAAACTCTYGTTCCCGGTTCGAGCATAATGCAGGCGACCCGATCCCACYGGAATATGCAGCGGACCCACYGGAATATGCAGCGGACCCACYGGAATATGCAGCGGACCCACYGGAATATGCAGCGGACCCACYGGAATATGCAGCGGACCGGAC
NC_016822.1(1>4988504) 25-2_TSS20160119-028-1532.seg(1>204) Position: 1	COSCITCACCGACCAGACGAGCAGIGITCCCACCGIGGGGCCGGATGGCGGCGGGGGGGGAAACTCTCTTCTCCCGGTCGAGCATATGCAGGCGACCAGICGGCGGCGGGGGGGGGG
	\$177030 3177040 3177050 3177060 3177060 3177090 3177100 3177120 3177130 3177140 3177160 300000000000000000000000000000000000
▶ Translate ▶ Consensus	GAAACTCTYGTTCCC0GTTCGAGCATAATGCA0GCGACCGATCCCACY0GAATRT9CGTGCGGATCCC0GTTTIGTCGATCA0CACGAAAGCGCCGTCCAGTACGTCGATTGACCGTACTGGAGGAAGACCATGGAAGTGC
NC_016822.1(1>4988504) 25-2_TSS20160119-028-1532.seq(1>204)	GAAACICTIGTICCC66TIC6ASCATAAT9CA69CGACCGATCCCACT6GAATAT9C605GCG6ATCCC69TITIGTC6ATCAGCACGAAAGCGCC6TCCASTAC9TC6ATTGACCGTACT69A6GAAGATCAT9GAAGGACG GAAACICTG6TICCC6GTIC6ASCATAAT9CA69CGACCGATCCCACT69AATAT9CC6GCG6CCG6GATCCC69TITIGTC6ATCASCACGAAAGCGCCGTCCASTAC9TC6ATTGACCGTACT69A6GAAGATCAT9GAAGT9C

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.

Position: 61		3176040	3176050	3176060	3176070	3176080	3176090	3176100	3176110	3176120	3176130	3176140	3176150	3176160	4988.504kb 3176170
▶ Translate ▶ Consensus	TA	ACCCATCCAN	ATCCACCOGAJ	TACGTCTGT	TTTCTCCCCA	GICIGAAATI	CAAAACCCGA	TCGRTATTO	GICGCCCAGG	CCATCACCAC	ATTICCGCAJ	ACCAGCCAGTI	GGGTAATTTG	TGCCAGATO	ATCTCCCGAATA
NC_016822.1(1>4988504) 4-1_TSS20160119-028-1534.seq(1> Position: 3176237	→ TA 204)← ITA	ACCCATCCAAJ	ATCCACCGGAJ ATCCACCGGAJ	ATACGTCIGT ATACGTCIGT	ITICICCCAG	GTCTGAAATI GTCTGAAATI	CAAAACCCGA	CICGATATIO	GTCGCCCAGG GTCGCCCAGG	CCATCACCAC CCATCACCAC	ATTTCCGCAJ	ACCAGCCAGTI ACCAGCCAGTI	GGGTAATTIG GGGTAATTIG	TOCCAGATO	ATCTCCCGAATA ATCTCCCGAATA 4988.504kb
		3176100	3176110	3176120	3176130	3176140	3176150	3176160	3176170	3176180	3176190	3176200	3176210	3176220	3176230
▶ Translate ▶ Consensus	CO	BAYTCORTATT	GGTCGCCCAG	GCCATCACCA	CATTICCOCA	ACCAGCCAGT	IGGGTAATTIC	SYTECCAGAT	CATCICCCGA	ATACGTITIG.	ATGTATCACO	AACATACACA	CCGGCACGCA	CTTCCAGTA	CCAGATTGCGAR
NC_016822.1(1>4988504) 4-1_TSS20160119-028-1534.seg(1> Position: 153	→ co 204)← co	ACTOGATATT SATTCGGTATT	GGICGCCCAG GGICGCCCAG	GCCATCACCA GCCATCACCA	CATTICCGCA	ACCAGCCAGT	IGGGTAATTTO IGGGTAATTTO	SCIGCCAGAT SIIGCCAGAT	CAICICCCGA	ATACGITITG ATACGITITG	ATGTATCACC	AACATACACA	CCGGCACGCA CCGGCACGCA	CTTCCAGTA	CCAGATIGCGAG CCAGATIGCGAA 4988.504kb
C. 10 (10) (20)	3176040	3176050	3176060	3176070	3176080	3176090	3176100	3176110	3176120	3176130	3176140	3176150	3176160	3176170	3176180
▶ Translate ▶ Consensus	AACCCATCCA	AATCCACCGG	AATACGICIRI	TTTTCWCCCCC	AGGTCTGAAAT	TCAAAACCCG	ACTCGRIATI	GGTCGCCCA	GCCATYACCA	CATTICCGCA	ACCINGCCAG	TIGREMRATTI	GCTGCCAGAT	CATCICCCG	ATACGTITKGAY
NC_016822.1(1>4988504) → Untitled Seg 6#2.seg(1>202)→	AACCCATCCA	AATCCACCGG	ATACGICISI AATACGICIAI	TTTCICCCC	AGGTCTGAAAT	TCAAAACCCG	ACTCG&TATT	GGTCGCCCA	GCCATCACCA	CATTICCGCA	ACCAGCCAG	ITGGGTAAITI ITGAGAGATII	GCTGCCAGA1 GCTGCCAGA1	CATCICCCG	ATACGTTTIGAT
Position: 3176201	217500	1176100	2176110	2176120	2176110	1176140	2176160	2176160	2126120	9176190	1176100	2176200	2126210	9176220	4988.504kb
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P Translate P Consensus	TICAAAACC	CGACICGRIAT	TGGICGCCCA	GGCCATYACO	ACATITICCGC	AACCHGCCAG	TIGRGMEATI	IGCIGCCAGA	TCATCICCCG	AATACGITIK	GAYGIAICWO	CRACATA	ACCOGCACOC	ACTICCAGI	GCCAGATIGCGA
NC_016822.1(1>4988504) → Untitled Seq 6#2.seq(1>202)→ Position: 103	TICAAAACC	CGACTOGATAI	IGGICGCCCA	GGCCATCACO GGCCATTACO	ACATTICCGC	AACCAGCCAG AACCIGCCAG	IIGGGTAAIT IIGAGAGAIT	IGCIGCCAGA IGCIGCCAGA	ICAICICCCG ICAICICCCG	AATACGIIII AATACGIIIG	GAIGTAICAG GACGIAICIG	CAACATACAC	ACCOGCACOC ACCOGCACOC	ACTICCAGI	GCCAGATIGCGA GCCAGATIGCGA 4988.504kb
	3176040	3176050	3176060	3176070	3176080	3176090	3176100	3176110	3176120	3176130	3176140	3176150	3176160	3176170	3176180
Translate Consensus	ACCCATCCAL	ATCCACCGGA	ATACGICIRT	TTTC CCCCA	GGTCTGAAAT	TCAAAACCCG	ACTOGRIATIO	GTCGCCCAG	GCCATYACCA	CATTICCGCA	ACCINGCCAGI	TGRGWRATTT	GCTGCCAGAT	CATCICCCG	ATACGITIKGAY
NC_016822.1(1>4988504) →	ACCCATCCA	ATCCACCGGA	ATACGICIGT	TTTCTCCCCA	GGTCTGAAAT	TCAAAACCCG	ACTOGATATTO	GICGCCCAG	GCCATCACCA	CATTICCOCA	ACCAGCCAGT	TGOGIAATTT	GCTGCCAGAT	CATCTCCCG	ATACGTTTTGAT
Untitled Seq 7#3.seq(1>201)→ Position: 202	ACCCATCCAJ	ATCCACCGGA	ATACGTCIAT	TTICACCCCA	GGTCTGAAAT	TCAAAACCCG	ACTCGOTATIO	BETCOCCAG	GCCATTACCA	CATITICCOCA	ACCIGCCAGI	TGAGAGATTT	GCTGCCAGAT	CATCTCCCG	4988.504kb
	3176090	3176100	3176110	3176120	3176130	3176140	3176150	3176160	3176170	3176180	3176190	3176200	3176210	3176220	3176230
Firanslate Consensus	TCAAAACCCC	GACTOGRIAIT	GGTCGCCCAG	GCCATVACCI	ACATTICCGCA	ACCINGCCAGI	TGRGWRATTT	GCTGCCAGA	ICATCTCCCGJ	ATACGITIK	GAYGTATCWC	CRACATANAC	ACCOGCACOC	ACTICCAGI	GCCAGATIGCGA
NC_016822.1(1>4988504) →	TCAAAACCCC	GACICGATATI	GGICGCCCAG	GCCATCACCA	ACATTICCGCA	ACCAGCCAGT	TGGGTRATTT	GCTGCCAGA	CATCICCCG	ATACGITIT	GAIGTAICAC	CRACATACAC	ACCGGCACGC	ACTICCAGI	GCCAGATIGCGA
Position: 1	ICAAAACCCC	SACIOGOIAII	GGICGCCCAG	GCCALLACCA	ACALLICCOCA	ACCIGCCAGI	IGAGACAIII	GCIGCCAGA.	ICATCICCCGA	AIACGIIIG	AGIAICIC	COACATANAC	ACCEGEACEE	ACTICCAGI	4988.504kb
	3176040	3176050	3176060	3176070	3176080	3176090	3176100	3176110	3176120	3176130	3176140	3176150	3176160	3176170	3176180
▶ Translate ▶ Consensus	ACCCATCCA	ATCCACCGGA	ATACGTCTGT	TTTCTCCCCA	GGICIGAAAT	TCAAAACCCG	AYTCGRIATIC	GICGCCCAG	GCCATCACCA	CATTICCOCA	ACCAGCCAGI	TGGGTAATTT	GYTGCCAGAT	CATCTCCCG	ATACGTTTTGAT
NC_016822.1(1>4988504) →	ACCCATCCAR	ATCCACCGGA	ATACGICIGI	TTICTCCCCA	GGICIGAAAT	ICAAAACCCG	ACTOGATATIO	GTCGCCCAG	GCCATCACCA	CATTICCGCA	ACCAGCCAGT	IGGGIAAITI	GCTGCCAGAT	CATCICCCG	ATACGTTTTGAT
Untitled Seq 9#5.seq(1>201) ->	ACCCATCCAJ	AICCACCGGA	ATACGICIGT	TTTCTCCCCA	GGTCTGAAAT	TCAAAACCCG	ATTCGGTATIO	GICGCCCAG	GCCATCACCA	CATTICCGCA	ACCAGCCAGT	TGGGTAATTT	GTIGCCAGAT	CATCTCCCGA	ATACGTITIGAT
POBLEIGH. 202	3176090	3176100	3176110	3176120	3176130	3176140	3176150	3176160	3176170	3176180	3176190	3176200	3176210	3176220	3176230
h Translate h Conservat	TCANARCOC	Children I Harris	Luuluu	Luuluu	Lunding and a second	Lunding and	TOCOTANTTE	Lunding and the second	Luuluu	ATACOTTE	ATOTATOAC	CAACATACAC	duuluu	ACTTOCACT	accessor traces
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Untitled Seg 9#5.seg(1>201)->	TCAAAACCCC	GATICGGIATI	GGTCGCCCAG	GCCATCACCI	ACATITICCGCA	ACCAGCCAGI	TGGGTAATTT	GITGCCAGA	ICATCICCCG	ATACGITIT	SAIGTAICAC	CAACATACAC	ACCGGCACGC	ACTICCAGIA	GCCAGATTGCGA
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	3176040	3176050	3176060	3176070	3176080	3176090	3176100	3176110	3176120	3176130	3176140	3176150	3176160	3176170	3176180
▶ Translate ▶ Consensus	CCCATCCAA	ATCCACCGGA	ATACGICIRI	ITTC#CCCCA	GGTCTGAAAT	ICAAAACCCG	ACTCGRTATIC	GICGCCCAG	GCCATYACCA	CATTICCGCA	ACCWGCCAGI	IGRGWRAITI	GCTGCCAGAT	CATCTCCCG	ATACGTITKGAY
NC_016822.1(1>4988504) → Untitled Seg 12#6.seg(1>198)→	CCCATCCAA	ATCCACCGGA	ATACGICIGT	ITTCICCCCA	GGTCTGAAAT GGTCTGAAAT	ICAAAACCCGI ICAAAACCCGI	ACTEGRIATIC	GICGCCCAG GICGCCCAG	GCCATCACCA	CATTICCGCA	ACCAGCCAGI	TGAGAGATIT	GCIGCCAGAI GCIGCCAGAI	CATCICCCG	ATACGITITGAT ATACGITIGAC
Position: 3176186															4988.504kb
	317609	90 317610	0 317611	0 317612	20 317613	0 317614	0 317615	0 317616	50 317617	0 317618	0 31761	90 31762	31762	10 31762	20 3176230
▶ Translate ▶ Consensus	TTCAAAAC	CCGACTCGRIA	ITGGICGCCC	AGGCCATYA	CACATITICCO	CAACCWGCCA	GTIGRGWRAT	TIGCIGCCA	GATCATCICCO	GAATACGTTI	REAVETATC	CCRACATAN	ACACCGGCAC	GCACTICCAG	TAGCCAGATTGC
NC_016822.1(1>4988504) → Untitled Seg 12#6.seg(1>198)→	TTCAAAAC	CCGACTCGATA	TIGGICGCCC	AGGCCATCAG	CACATITICO	CAACCAGCCA	GTTG3GTAAT	TIGCTGCCA	BATCATCICCO	GAATACGTTT	ITGATGTATC	ACCAACATAC	ACACCGGCAC ACACCGGCAC	GCACTICCAG	TAGCCAGATIGC
Position: 1															4968.504kb
	317604	0 3176050	0 3176060	317607	0 3176080	3176090	3176100	317611	0 3176120	3176130	317614	0 317615	317616	0 317617	0 3176180
▶ Translate ▶ Consensus	CCCATCO	AAATCCACCG	GAATACGICI	KTTTTC CCC	CAGGICIGAA	ATTCAAAAACC	CGACTCGRTAT	TIGGTCGCCC	AGGCCATYAC	CACATITICCG	CAACCHIGCCA	GTIGRGWRAT	IIGCIGCCAG	ATCATCICCO	GAATACGITIKG
NC_016822.1(1>4988504)		CAAATCCACCG	GAATACGICI	ATTTICACCO	CAGGICIGAA	ATTCAAAAACC	CGACTOGATAI	TIGGICGCCC TIGGICGCCC	AGGCCATCAC	CACATTICCG	CAACCAGCCA	GTTGGGTAAT	ITGCTGCCAG ITGCTGCCAG	ATCATCTCCC	GAATACGITIIG
Position: 3176113															
	3176	317	6120 31	16130	3176140	3176150	3176160	31761	3176	180 317	6190 3	1/6200	5176210	3176220	3176230
Translate Consensus	G	CCCAGGCCAT	TYACCACATI	TTCCGCAAC	CWGCCAGIT	GRGWRATTI	GCTGCCAGA	TCATCICC	CGAATACGT	TIKGAYGIA	TCWCCRAC	ATAMACACC	GGCACGCAC	TICCAGIA	GCCAGATIGCG.
NC_016822.1(1>4988504)		CCCAGGCCAT	CACCACATI	TTCCGCAAC	CAGCCAGTT	GOGIAATTI	GCTGCCAGA	TCATCICC	CGAATACGT	TTIGATGTA	TCACCAAC	ATACACACC	GGCACGCAC	TICCAGIA	GCCAGATIGCG
Position: 151	221 1 60	ULLAGGULAI	TALLALAI	LICUGURAU	CIGULAGII	GREADAILI	GUIGULAGA	AICAICICC	CGARIACGI	110GALGIA	ILICLOAD	AIAAALALL	GGLALGLAL	TICCAGIA	4988.504kb
	3176040	3176050	3176060	3176070	3176080	3176090	3176100	3176110	3176120	3176130	3176140	3176150	3176160	3176170	3176180
Translate Consensus	ACCCATCCA	AATCCACCGG	AATACGTCIR	ITTICWCCCC	AGGICIGAAA	TTCAAAACCCC	GACICGRIATI	IGGICGCCCA	GGCCATYACC	ACATTICCGC	AACCIVGCCAG	TIGRGWRATT	IGCIGCCAGA	TCATCICCCO	AATACGTTIKGA
NC_016822.1(1>4988504) →	ACCCATCCA	AATCCACCGG	AATACGICIG	IIIICICCCC	AGGICIGAAA	TTCAAAACCCO	GACICGATATI	IGGICGCCCA	GGCCATCACC	ACATTICCGC	AACCAGCCAG	TIGOGIAATI	IGCIGCCAGA	TCATCTCCCC	AATACGTTTTGA
Position: 3176187	ACCCATCCA	AATCCACCGG	AATACGICIA	TTTTCACCCC	AGGICIGAAA	TTCAAAACCCO	SACICGUIAII	IGGICGCCCA	GGCCATTACC	ACATTICCGC	AACCIGCCAG	TIGRGAGATI	IGCIGCCAGA	TCATCICCCO	4988.504kb
	3176090	3176100	3176110	3176120	3176130	3176140	3176150	3176160	3176170	3176180	3176190	3176200	3176210	3176220	3176230
▶ Translate ▶ Consensus	CAAAACCCC	BACTCORTATT	GGTCGCCCAG	GCCATYACCA	CATTICCOCA	ACCWGCCAGT	TGRGWRATTT	GCTGCCAGAT	CATCTCCCGA	ATACGITIK	AYGTATCWC	CRACATAMAC	ACCEGCACEC	ACTTCCAGTA	GCCAGATTGCGA
NC_016822.1(1>4988504) →	CAAAACCCC	BACTCGATATT	GGICGCCCAG	GCCATCACCA	CATTICCOCA	ACCAGCCAGT	TGOGTAATIT	GCTGCCAGAT	CATCICCCGA	ATACGITI	ATGTATCAC	CAACATACAC	ACCOGCACGC	ACTICCAGIA	GCCAGATTGCGA
Untitled Seq 22#8.seq(1>201) -> Position: 149	CAAAACCCC	SACICGGIAIT	GGTCGCCCAG	GCCATTACCA	CATTICCGCA	ACCIGCCAGI	TGAGAGATIT	GCTGCCAGAI	CATCICCCGA	ATACGITIGO	ACGIATCIC	COACATALAC	ACCEGCACEC	ACTICCAGIA	GCCAGATTGCGA 4988.504kb
	317604	10 317605	0 317606	0 317607	0 317608	317609	3176100	317611	0 317612	317613	317614	0 317615	317616	0 317617	0 3176180
▶ Translate ▶ Consensus	CCCATCO	CAAATCCACCG	GAATACGTCT	RTITICACCO	CAGGICIGAA	ATTCAAAACO	CGACICGRIA	ITGGTCGCCC	AGGCCATYAC	CACATTICCO	CAACCINGCCA	GTTGRGMBAT	TIGCIGCCAG	ATCATCTCC	GAATACGTITKG
NC_016822.1(1>4988504) -	CCCATCO	CAAATCCACCO	GAATACGICI	attricicco	CAGGICIGAA	ATTCAAAACC	CGACTCGATA	TIGGICGCCC	AGGCCATCAC	CACATITICCO	CAACCAGCCA	GTIGOGIAAT	TIGCIGCCAG	ATCATCTCC	GAATACGTTIIG
Untitled Seq 25-2#9.seq(1>200)-	-> CCCATCO	CAAATCCACCG	GAATACGICI	ATTTTCACCO	CAGGICIGAA	ATTCAAAACO	CGACTCGGTAT	TIGGICGCCC	AGGCCATTAC	CACATITICCG	CAACCIGCCA	GTTGAGAGAT	TIGCTGCCAG	ATCATCICCO	GAATACGTTT G
FUELCION: 201	3176090	3176100	3176110	3176120	3176130	3176140	3176150	3176160	3176170	3176180	3176190	3176200	3176210	3176220	3176230
P Translate P Consensus	A330000	GACTOGETATT	GGTCGCCC22	GCCAT VACCO	CATTICCOCT	ACCURCE	TGEGRATT	GCTGCCACA	TCATCTOCCO	ATACOTT	BAYGTATO	CRACATAWAG	ACCOGCACCO	ACTICCAGT	GCCAGATTGCCA
NC 016822.1(1>4988504) -	AAACCO	GACTOGETATT	GGTCGCCCLO	GCCATCACCI	ACATITICCOCI	ACCAGCCAGT	TONGTANTT	GCTGCCAGA	ICATCTCCCG	ATACGTTT	GATGTATCAC	CLACATACAC	ACCEGCACE	ACTICCAGT	GCCAGATTGCGA
Untitled Seg 25-2#9.seg(1>200)-	AAACCC	GACTOGGTATT	GGTCGCCCAG	GCCATTACC	ACATTICCGC	ACCIGCCAGI	TGAGAGATTT	GCTGCCAGA	TCATCTCCCG	ATACGTTTG	SACGTATCIC	CGACATAAAC	ACCOGCACOC	ACTICCAGI	GCCAGATTGCGA

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 \rightarrow T) and 3176590 (G \rightarrow 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.^[25] 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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