



Persistent Asymptomatic Human Infections by *Salmonella enterica* Serovar Newport in China

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ABSTRACT *Salmonella enterica* serovar Newport (*S. Newport*) infections are gradually on the rise in China from the last decade. For humans' infections, *S. Newport* has been ranked among the top five serovars responsible for persistent infections, globally. A total of 290 *S. Newport* strains with their relevant clinical metadata were analyzed, and the strains were subjected to whole-genome sequence analysis. Among these, 62.4% ($n = 181$) were from diarrheic patients and 28.9% ($n = 84$) were from asymptomatic individuals (including adults and youngsters) while 8.6% ($n = 25$) were from cases of persistent diarrhea in infants (28%, $n = 7$) and toddlers (72%, $n = 18$). The association between the sequence types (STs) and the variations in the clinical presentation was statistically significant ($P = 0.0432$), with ST46 causing diarrhea or representing asymptomatic patients and ST31 or ST68 causing persistent diarrhea. Genomic analysis revealed that the highest proportion of the isolates (98.5%, $n = 279$), primarily from patients with or without diarrhea rather than from asymptomatic individuals, carried antimicrobial resistance determinants corresponding to the aminoglycosides and beta-lactams, highlighting the need for cautionary usage of antimicrobials in such patients. These findings also suggest that cases of nontyphoidal *Salmonella* infection with symptoms of acute diarrhea or persistent diarrhea caused by *S. Newport* should be handled with caution, due to the high chance of development of an antimicrobial resistance phenotype that might lead to therapeutic failures. Together, *S. Newport* ST31 and ST46, which have the highest frequency of carriage of multidrug resistance, are potentially responsible for antimicrobial-resistant diarrhea/persistent diarrhea in infants and children, while adult humans are more likely to be (asymptomatic) carriers of the *S. Newport* strains.


IMPORTANCE Human infections caused by *Salmonella* Newport generally lead to gastrointestinal diseases. These infections are normally self-limiting; however, in certain cases, broad-spectrum antimicrobials are prescribed for the treatment. The Chinese National Foodborne Disease Surveillance Network has reported a gradual increase in the incidence of multidrug-resistant *S. Newport* infections in humans. After careful evaluation of the dynamic relationship among the clinical findings, the age group, and the genomic sequence data, it was found that young patients represented the major group with persistent diarrhea, whereas adults were either asymp-

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 #SalmonellaNewport

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tomatic or diarrheic. Furthermore, all these strains contained multiple acquired antimicrobial resistance determinants, which limited the use of antimicrobials for human patients of all age groups. This analysis of the laboratory-confirmed cases, coupled with genetic analysis of the corresponding pathogen, revealed that antimicrobial treatment of persistent infections by *S. Newport* in infants and toddlers, and in asymptomatic or diarrheic adults, may not be successful. If the antimicrobials must be prescribed at all, they must be used with caution because of the presence of multiple acquired antimicrobial resistance determinants in such strains.

KEYWORDS age, *Salmonella* Newport, antimicrobial resistance, human symptoms

Salmonella is one of the leading causes of diarrhea among all age groups, in particular, for children (<5 years of age) in low-income and middle-income countries (1). Typhoidal *Salmonella* infections can be acute or chronic, and the asymptomatic carrier state has also been reported. For example, nontyphoidal *Salmonella* (NTS) infections typically cause acute self-limiting gastroenteritis in humans; in certain cases, however, such infections can cause invasive febrile bacteremia with fatal outcome. In some cases, a successful treatment could result in recovery from the symptoms but without clearance of the pathogens from the infected individual. Asymptomatic or chronic carrier is defined as a persistent infection, representing a phenomenon that is poorly understood. While cases of chronic carriers of typhoidal salmonellosis are reported frequently (2, 3), those for nontyphoidal salmonellosis are scarce and largely underestimated. In China, the incidence of infections of humans by *Salmonella enterica* serovar Newport has been gradually on the rise (4–7), and such infections are among those commonly reported from multiple different sources (8, 9).

By using *S. Newport* data from the Chinese National Foodborne Disease Surveillance Network (active surveillance network), we too observed an increasing incidence of persistent and asymptomatic infections in human in China. One Chinese study reported that for the years 2005 to 2011, *S. Newport* was among the top 10 serovars responsible for human clinical cases (10). An Israeli study showed that the persistence index of *S. Newport* in human infections was 1.3 and that *S. Newport* was ranked among the top five serovars (11). The human cases of *S. Newport* and its persistence are generally of low concern in Chinese medical practice due to its nonsignificant clinical outcome in most of the cases. The attitude of the Chinese medical practitioners is probably a consequence of the fact that many enteric bacteria manifest similar clinical symptoms and that the usage of broad-spectrum antimicrobials substantially helps to reduce the severity of the symptoms. Here, we analyzed the clinical and genomic data on *S. Newport* isolates and evaluated the bacterial genetic factors associated with clinical presentations.

RESULTS

The *S. Newport* strains of human origin, along with the relevant metadata collected from various age groups in China over a period of 18 years (1991 to 2018), were analyzed. The stool samples were collected from human subjects presenting themselves at the health care facilities at 13 provinces, while the Shanghai region contributed the largest number of isolates used in our analysis.

Among the total 290 strains, the highest proportion, 70% ($n = 203$), were isolated from adults whereas the lowest proportion, 2% ($n = 6$), were from children. Concerning the clinical symptoms, 62.4% ($n = 181$) were from diarrheic subjects whereas 28.9% ($n = 84$) were from asymptomatic individuals and 8.6% ($n = 25$) from individuals with cases of persistent diarrhea. It was a peculiar feature that *S. Newport* was isolated from cases of persistent diarrhea only in infants (28%, $n = 7$) and toddlers (72%, $n = 18$). Adult and other age groups reported only diarrhea or were apparently healthy or asymptomatic (Fig. 1).

The genomic sequence was analyzed for the sequence type (ST) and the acquired antimicrobial resistance genes in 282 strains. Among these 282 strains, 31 different

	<1	1-5	5-10	11-19	>19
Asym.				9.5	90.5
Diar.	9.4	14.4	3.3	2.8	70.2
P. Diar.	72.0	28.0			

FIG 1 Prevalence by age, symptoms, and clinical findings. Data represent percentages of *S. Newport* (values of individual cells) isolated from different clinical presentations (left side) for various age categories (patient age on top). Blank cells represent the absence of data. The age categories (x axis, in years, top) are classified as infant (<1 year of age), toddler (1 to 5 years of age), child (6 to 10 years of age), adolescent (11 to 19 years of age), and adult (>19 years of age). The symptoms (y axis) are abbreviated as follows: asymptomatic, Asym.; diarrhea, Diar.; persistent diarrhea, P. Diar.

types of known STs along with 6 as-yet-unidentified STs were recorded. The top five STs were ST46 ($n = 122$), ST31 ($n = 54$), ST2364 ($n = 21$), ST166 ($n = 19$), and ST45 ($n = 17$). The six unidentified STs belonged to the strains isolated from stool samples of female adult and children, showing symptoms of diarrhea at Shanghai (2017, 2011, 2010, and 2009, $n = 1$ each), Shenzhen (2009, $n = 1$), and Jiangsu (2010, $n = 1$). ST46 was responsible for nine cases of persistent diarrhea in infants and toddlers and ST31 for seven cases. The analysis also showed that ST46 was isolated mostly from the cases of diarrhea or asymptomatic patients whereas ST31 and ST68 were isolated from the cases of persistent diarrhea. The contribution of ST to the outcome of different clinical presentations was analyzed in two-way analysis of variance (ANOVA). The analysis showed that the ST was a statistically significant ($P = 0.0432$) contributor to variation in clinical presentation.

The genomic sequence was also scanned for other relevant features such as the number of antimicrobial resistance determinants (ARDs) and the number of mutations. Genomic analysis revealed that the highest proportion of the isolates (98.5%, $n = 279$) carried multiple ARDs corresponding to the aminoglycosides and beta-lactams whereas the lowest proportion of the isolates had carriage of ARDs corresponding to colistin (3.5%, $n = 10$). The genotypic resistance was plotted against the clinical presentation (Fig. 2). This showed that the isolates from patients with symptoms of diarrhea or from

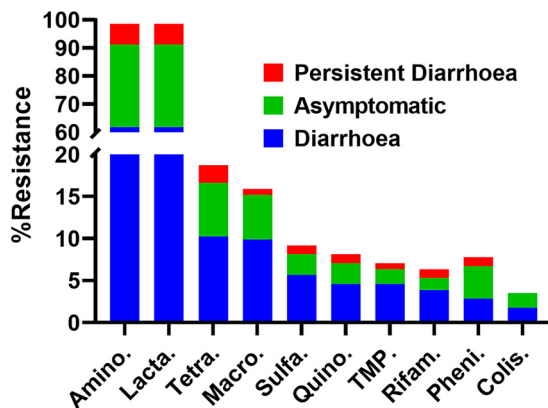


FIG 2 Antimicrobial resistance and clinical findings. Data represent percentages of carriage of acquired resistance genetic determinants (genes) by the *S. Newport* strains isolated from various clinical presentations. The x axis gives the classes of antimicrobials as follows: Amino., aminoglycosides; Lacta., beta-lactams; Tetra., tetracyclines; Macro., macrolides; Sulfa., sulfonamides; Quino., quinolones; TMP, trimethoprim; Rifam., rifampin; Pheni., phenicol; Colis., colistin.

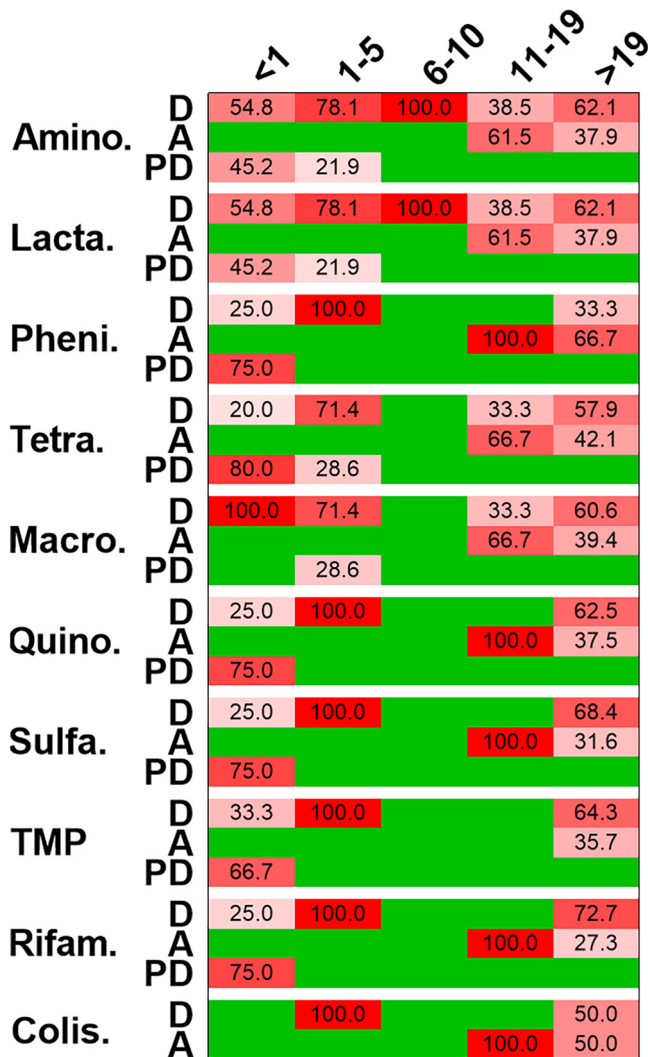


FIG 3 Antimicrobial resistance, age, and clinical findings. The heat map shows the percentages of isolates that were genotypically resistant for the given category of drugs (as described in the Fig. 2 legend) and the clinical symptoms (D, diarrhea; A, asymptomatic; PD, persistent diarrhea) as indicated on the right. The top gives the classifications of age (as described in the Fig. 1 legend). The shades of red show the various percentages of carriage of acquired resistance determinants, while green indicates that the isolates did not contain any such determinants, based on genomic analysis. Values in the cells indicate percentages of carriage of acquired antimicrobial resistance determinants.

asymptomatic patients had the highest rate of carriage of strains with ARDs.

Our next aim was to evaluate the relationship between the clinical presentation and the degree of carriage of ARDs in these isolates for various age categories of the patients. The analysis showed that most of the isolates from young patients (<5 years of age) and adults (>19) generally had a higher carriage rate of the ARDs (Fig. 3).

Isolates from the children aged 6 to 10 years generally lacked these ARDs except for those corresponding to the aminoglycosides and the beta-lactams. It was alarming that the range of carriage rates of such ARDs in strains isolated from cases of persistent diarrhea in both the infants and the toddlers was quite high, measuring between 21.9% (aminoglycosides and beta-lactams) and 80% (tetracyclines). Similarly, the carriage rate of such ARDs in asymptomatic adults ranged between 27.3% (rifampin) and 66.7% (phenicols) for various classes of antimicrobial. The details of the presence or absence of the genes conferring resistance to various classes of antimicrobials are provided in Data Set S1 in the supplemental material (see Tab 1). It is notable that, contrary to expectations, there were no mutations in the genes that typically confer resistance to

fluoroquinolones. However, mutations in some genes such as *Salmonella enterica gyrA* were found that conferred resistance to fluoroquinolones. D87N was found in four strains isolated from diarrheic patients.

All these belonged to the ST31 category and carried 8 to 12 multiple ARGs corresponding to various classes of antimicrobials. Similarly, a *parC* gene that had an S80I mutation conferring resistance to fluoroquinolone and that carried 17 ARGs was seen in only one ST68 isolate. Among the ST11 isolates, *Salmonella enterica gyrA* with a mutation (D87G) conferring resistance to triclosan and carrying seven ARGs was seen only in one strain from a diarrheic patient. Similarly, *Salmonella enterica gyrA* with a mutation conferring resistance to triclosan (S83F) was seen in an array ($n = 11$) of strains isolated from adult diarrheic patients. Among these 11 isolates, 3 belonged to ST31, 6 belonged to ST46, and 2 belonged to ST33. These isolates carried 4 to 11 determinants of resistance to various classes of classes of antimicrobials.

Interestingly, among 285 strains which were detected to be positive for carriage of *bla* genes (Data set S1, Tab 2), we also found that those strains carried *bla*_{LEN} or *bla*_{OKP}, both of which are frequently found in *Klebsiella* spp. The results of scanning performed with the newly established toolkit ConFindr for detection of potential contamination showed that, in general, the chance of interspecies sequence contamination was only around approximately 0% to 0.1%, equating to a maximum of only 5 strains at the maximum level.

We calculated the multidrug resistance (MDR) genotypes on the basis of the acquired resistance determinants. In total, 85.2% ($n = 220$ of 258) of the isolates were categorized as MDR. Among these MDR isolates, 50.3% ($n = 130$) of the strains were from diarrheic subjects, 29.8% ($n = 77$) were from asymptomatic subjects, and 5% ($n = 13$) were from the persistent diarrheic subjects. For the various age categories, the MDR strains representing the highest proportion, 40.6% (13 of 32), were isolated from toddlers whereas there were no MDR strains isolated from children's samples. Among the various STs, the highest MDR frequency was in the ST31 category at 94.4% ($n = 51$ of 94) followed by ST46 at 81.1% ($n = 99$ of 122). Among the remaining STs, the MDR proportion was less than 4%.

DISCUSSION

A previous study conducted in Guangdong province between 2009 and 2016 reported that children <5 years of age were mostly affected by *Salmonella*; among those children, infants (<1 year) comprised 81.5% (1,084/1,329) of the affected population (12). In another study from Fujian, among the 163 children with *Salmonella* infection, 79 (48.5%) were ≤ 1 year of age (13). *S. Newport* is one of the most common serotypes isolated from patients with diarrhea (14). Our analysis also shows that infants and toddlers represented the second largest group (23.4%, $n = 68/290$) associated with *S. Newport* after adults (70%, $n = 203/290$). Shanghai was reported to have shown the maximum number of infant *S. Newport* cases ($n = 28/50$) among the various included regions. These data provide additional pieces of evidence for the burden of salmonellosis in infants or younger children caused by *S. Newport* in China.

Here, we show that ST46, ST31, and ST2364 are the top three STs of *S. Newport* reported from human patients with different clinical symptoms. In our earlier study (9), we reported that ST2346, usually including *S. Newport* of freshwater animal origin, is unique to China whereas ST46 and ST31 are primarily associated with reptile-associated cold-blooded animals and seafood-associated animals, respectively. Importantly, this ST46 had been reported from patients in the coastal regions of Shanghai, Fujian, and Guangxi (14).

Evidence hints that many Chinese parents keep turtles as pets. The infants and toddlers usually interact physically and closely with such pets because docile and slow-moving small turtles are seemingly harmless for such interactions. This might explain how the infants and younger children acquired *S. Newport* and thus, in turn, exhibited persistent diarrhea, i.e., due to continuous exposure to the source (the reptilian pet) (15). Though we cannot establish the exact cause-effect relationship, the

growing numbers of publications in the literature corroborate our hypothesis (16, 17). Another possible explanation for the longer persistence in children younger than 5 years old might be found in a comparison between the less extensively developed microbiota in children and the more complex microbiota in the adult intestines or even the underdeveloped immune system of such young subjects (18). An earlier report from a study conducted in China also mentioned that *S. Newport* isolates from aquatic products shared high similarity with those from diarrheal patients in several clusters, suggesting a possible association between the isolates derived from these two types of sources (9). Other evidence also shows that seafoods such as fish and turtles, with the latter being generally underestimated players in foodborne transmission, could be among the top sources responsible for human *S. Newport* infections as well as for dissemination of acquired antimicrobial resistance genes such as *mcr* complex genes (4). The actual source (for example, foods, occupational hazards, or environmental contamination, etc.) from which the human subjects might have acquired the pathogens could not be confirmed in the absence of complete historical records in this analysis. Further analyses are being undertaken to evaluate the associations between specific adhesin alleles, host species, and antimicrobial resistance as these have shown potential for diagnosis and epidemiological studies (19).

Carriage of ARDs was found for various members of 10 classes of antimicrobials in these isolates. The carriage rate of aminoglycosides and beta-lactam resistance determinants, as well as determinants of resistance to colistin as reported in an earlier publication (20), is a serious public health concern. Among the antimicrobials, amoxicillin topped the list of the maximally used antimicrobial in human medicine in China (21). This usage might have caused massive selection pressure, thereby resulting in the occurrence of ARDs corresponding to the beta-lactams, and a similar case might be made for the aminoglycosides. In human clinical practice, where enteric pathogens generally manifest with similar symptoms, physicians prescribe the antimicrobials based on their experience rather than relying on time-consuming laboratory findings. Moreover, it is also common for people to visit more than one physician for second opinions, as a consequence of which the subject might be prescribed another type of antimicrobial. This practice rapidly gives rise to MDR strains as seen in these clinical isolates of *S. Newport*. The higher rate of carriage of MDR strains and multiple ADRs in the asymptomatic humans (who are probably carriers) might be a focal point for the spread and distribution of such strains to other human populations where herd immunity might be less than optimal. From the results, it could be seen that the strains from the infants and toddlers with diarrhea or persistent diarrhea had a high rate of MDR carriage. That is an interesting finding because infants are generally less extensively exposed to antimicrobials (clinically) than adults and yet the isolates from them showed a pattern that contrasted with that fact. *Salmonella* pathogens with a broad host range such as *Salmonella* serovar Newport can be transmitted to humans from multiple sources. There have been studies which implicated the allelic variation in multiple adhesins which have some activity in preferential host adaptation, and the use of such variations could have a potential role in source identification (22).

Carriage of *bla*_{LEN} or *bla*_{OKP} is frequently found in *Klebsiella* spp. We also checked sequences for potential interspecies contamination by using the newly established toolkit ConFindr, which has been reported to have the ability to detect cross-species contamination with high sensitivity (23). The results showed that, in general, the chance of interspecies sequence contamination is approximately 0% to 0.1%, equating to only five strains at the maximum level of contamination. Moreover, our sequence coverage was between 78 and 210, further confirming the existence of those genes in *Salmonella* Newport in China. It was well known that class A β -lactamases are usually present only in the chromosome of *Klebsiella pneumoniae* complex, *K. pneumoniae* (*bla*_{SHV}), *K. quasipneumoniae* (*bla*_{OKP}) and *K. variicola* (*bla*_{LEN}) (24), but recently, similar genes have also been reported in the *Salmonella* Heidelberg serovar of poultry origin (25), and the *bla*_{LEN} gene has also been reported in *Staphylococcus epidermidis* (26). Thus, the presence

of these peculiar AMR genes provides novel perspectives on the antimicrobial resistance of *S. Newport*, which warrants further investigations.

This finding suggests that cases of enteric NTS infections caused by *S. Newport* with symptoms of acute diarrhea or persistent diarrhea cannot be treated with antimicrobials, primarily because the bacteria have high carriage of AMR genes that may lead to therapeutic failures. Even for the cases of infant and toddler persistent diarrhea, there were high rates of carriage of AMR genes. It is possible that the adults who were in contact with such infants and toddlers were themselves the carriers, thus contributing to the spread of the AMR pathogen.

On the basis of the available analyses and the evidence obtained here, we can conclude with a high degree of confidence that *S. Newport* ST31 and ST46, which have the highest frequency of carriage of MDR, are potentially responsible for diarrhea/persistent diarrhea in infants and children. The adult human subjects are more likely to be the (asymptomatic) carriers of the *S. Newport* strains. This is a serious public health concern because of the fact that the presence of multiple acquired resistance genes in these isolates potentially jeopardizes the treatment regimen instituted in such cases of persistent diarrhea in infants. Collectively, these findings provide essential knowledge and updated understanding of persistent and asymptomatic infections in patients, which in turn should help efforts to provide better care in primary health care settings, which are able to influence future public health recommendations and health policy planning.

MATERIALS AND METHODS

We pooled data from the hospital records of human salmonellosis at Shanghai (~10,000 cases), Shenzhen (~4,000 cases), and Hangzhou (~3,000 cases) to analyze the historical strain collection across 13 provinces or province-level municipalities in China. From this library of approximately 17,000 strains, we were able to select only 290 *S. Newport* human strains with their relevant metadata on geographical distribution, age, gender, and major clinical presentation for the descriptive analysis. While the records showed the presence of more than 1,000 strains, there was an absence of relevant metadata for most of those isolates. Those without the related metadata were excluded from further analysis. The oldest strains in the collection dated back to 1991 ($n = 2$), while the recent ones were from 2018 ($n = 5$). The human subjects contributing the fecal samples for bacterial isolation were categorized according to age as infants (<1 year of age), toddlers (1 to 5 years of age), children (6 to 10 years of age), adolescents (11 to 19 years of age), or adults (>19 years of age). The clinical presentations were collated into three main types as individuals with diarrhea (acute, or watery), individuals with persistent diarrhea (lasting >14 days), and apparently healthy (asymptomatic) individuals based on the medical recommendation available along with the relevant metadata. The collected data were curated in Microsoft Excel and analyzed in GraphPad Prism v. 8.0.1 on a Windows platform. A descriptive analysis was performed for age, clinical presentation, and spatiotemporal variation. The interaction between the age and the clinical presentation was analyzed in one-way ANOVA, while the relationship between the top five sequence types and their clinical presentations was analyzed using two-way ANOVA at $P = 0.05$. Among these 290 isolates, 282 were subjected to whole-genome sequencing (WGS) on an Illumina HiSeq platform or BGISEQ-500. Multidrug resistance, defined as resistance to three or more classes of antimicrobials, was evaluated using the *in silico* genomic data as described previously (27). Genomic analysis was performed with an in-house script in Python. The WGS data were scanned for potential interspecies contamination by using the newly established toolkit ConFindr (23).

Data availability. The summary of the genomic analysis is given in an MS Excel spreadsheet as Data Set S1 in the supplemental material. Other data are available from the corresponding author upon request.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

DATA SET S1, XLSX file, 0.2 MB.

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We declare that there are no conflicts of interests.

Narayan Paudyal and Hang Pan analyzed the data and prepared the manuscript; Xiao Zhou, Xin Zhou, Wenqing Chai, Qingqing Wu, Shuning Li, and Fang Li recovered the frozen strains, performed the serotype reconfirmation, and prepared a strain library; Beibei Wu conducted revisions of the sequence analysis; Beibei Wu, Guimin Gu, Haoqiu Wang, Qinghua Hu, and Xuebin Xu collected the samples from humans, did the primary isolation and confirmation of the pathogen, and dispatched the frozen selected strains to Min Yue's laboratory; Yan Li provided technical input and support during the whole-genome sequencing of the isolates; Min Yue conceived the study, provided the overall management of the work, and finalized the manuscript.

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