Original Article

Serological Investigation into the Infected Genotypes of Patients with Japanese Encephalitis in the Coastal Provinces of China*



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Abstract

Objective Genotypes (G) 1, 3, and 5 of the Japanese encephalitis virus (JEV) have been isolated in China, but the dominant genotype circulating in Chinese coastal areas remains unknown. We searched for G5 JEV-infected cases and attempted to elucidate which JEV genotype was most closely related to human Japanese encephalitis (JE) in the coastal provinces of China.

Methods In this study, we collected serum specimens from patients with JE in three coastal provinces of China (Guangdong, Zhejiang, and Shandong) from 2018 to 2020 and conducted JEV cross-neutralization tests against G1, G3, and G5.

Results Acute serum specimens from clinically reported JE cases were obtained for laboratory confirmation from hospitals in Shandong (92 patients), Zhejiang (192 patients), and Guangdong (77 patients), China, from 2018 to 2020. Seventy of the 361 serum specimens were laboratory-confirmed to be infected with JEV. Two cases were confirmed to be infected with G1 JEV, 32 with G3 JEV, and two with G5 JEV.

Conclusion G3 was the primary infection genotype among JE cases with a definite infection genotype, and the infection caused by G5 JEV was confirmed serologically in China.

Key words: Japanese encephalitis virus; Serological investigation; Plaque reduction neutralization test; Cross-neutralization test; Genotype

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INTRODUCTION

apanese encephalitis (JE) is a mosquitoborne zoonotic disease caused by the Japanese encephalitis virus (JEV)^[1]. JEV is the major cause of viral encephalitis in the Asia-Pacific region^[2,3]. More than three billion individuals living in 24 countries in this region are at risk of JEV infection. The estimated annual number of JE cases was 67,900^[3,4]. Although most JE cases are asymptomatic, the fatality rate among patients with encephalitis is as high as 20%–30%, and

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approximately 30%–50% of survivors have long-term neurological sequelae^[2,5]. Encephalitis primarily affects children. Most adults in endemic countries develop natural immunity after childhood infections. However, individuals of any age can be affected^[6].

JEV belongs to the genus *Orthoflavivirus*, the family *Flaviviridae*, and the JEV serological complex. The JEV genome spans approximately 11,000 nucleotides and contains a single open reading frame that encodes three structural proteins (C, PrM/M, and E) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). The viral genome is flanked by 5' and 3' untranslated regions^[1,7,8]. Moreover, it is divided into five genotypes (G1–G5) based on the E gene and the whole genome^[9,10]. G1, G3, and G5 JEVs have been isolated in China^[11,12].

JE is a disease that can be prevented *via* vaccination. Vaccination has significantly reduced the incidence of JE and JE-related deaths in endemic areas^[13]. All JE vaccines currently used are derived from G3 JEV^[14], which provides protection against G1 and G3 JEV infections but does not provide adequate protection against G5 JEV infections^[15-17]. In 2015, a case of JE caused by G5 JEV was reported in a South Korean patient with a history of JE vaccination^[18]. In China, a G5 JEV strain was isolated from *Culex tritaeniorhynchus* collected in Nyingchi, Tibet^[19], which confirmed the presence of G5 JEV in China. This suggested that attention should be given to the existence of this genotype in JEV cases in China.

The primary diagnostic criteria for JE are viral isolation, serological evidence, and molecular biology evidence^[20,21]. However, JEV has only one serotype; therefore, current enzyme-linked immunosorbent assay (ELISA) methods cannot distinguish the viral genotype when detecting JEV infections. Furthermore, the detection of JEV nucleic acids in cerebrospinal fluid and serum specimens is complicated. Therefore, it is difficult to distinguish between the JEV genotypes in most cases. Crossneutralization experiments have been used to distinguish flaviviral infections^[22], and serological investigations have aimed to determine the JEV genotype^[23,24]. In this study, we compared neutralizing antibody (nAb) titers produced by serum specimens from the same patient against G1, G3, and G5 JEV strains. A 4-fold difference in the nAb titer enabled the identification of the JEV genotype causing the infection.

Shandong, Zhejiang, and Guangdong provinces were selected for serological investigations. Crossneutralization tests were performed on laboratoryconfirmed JE cases using G1, G3, and G5 JEV strains to determine whether G5 JE cases had appeared in China. We aimed to identify the major genotypes of JEV that cause human diseases in selected coastal areas of China.

MATERIALS AND METHODS

Serum Specimen Collection and Ethics Consideration

Serum specimens were collected from patients diagnosed with JE in the Guangdong, Zhejiang, and Shandong provinces of China from 2018 to 2020. This was a retrospective study, and epidemiological information was obtained from the JE Reporting Information Management System. Serum specimens were obtained as part of routine diagnostic testing, and the names and other identifying information of the patients were not included in the data analysis. Therefore, the Ethical Review Committee of the Chinese Center for Disease Control and Prevention exempted this study from review by the Institutional Review Board.

Viruses and Cells

The JEV strains used in this study were maintained at the Department of Arbovirus, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention (IVDC, China CDC). G1 JEV is the NX1889 strain (GenBank accession no.: MT134112.1), G3 JEV is the P3 strain (GenBank accession no.: U47032.1), and G5 JEV is the XZ0934 strain (GenBank accession no.: JF915894.1). The titers of the three JEV strains were measured^[16], and the viral suspensions were stored at -80 °C. BHK-21 cells were aseptically cultured in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) at 37 °C in an atmosphere of 5% CO₂.

Immunoglobulin (Ig) M Antibody Detection

The serum specimens were subjected to anti-JEV IgM antibody detection. A JEV IgM Capture ELISA kit (Shanghai B & C Enterprise Development Co., Ltd., Shanghai, China) was used to detect JEV IgM antibodies. Detection was performed according to the manufacturer's instructions.

JEV Genome Analysis

Nucleic acids were extracted from all serum specimens using a QIAamp Viral RNA Mini Kit (QIAGEN, Dusseldorf, Germany) and subjected to a reverse transcription one-step polymerase chain reaction (RT-PCR). The RNA (5 μ L) was added to the reaction volume (20 μ L) according to the instructions of the TaqPath-IDTM 1-Step Multiplex Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). Universal primers and probes for JEV have been used to detect G1, G3, and G5 JEV^[25]. Specimens with *Ct* values of < 35.00 were considered positive.

Detection of Neutralizing Antibodies (nAbs)

A 90% plaque reduction neutralization test (PRNT₉₀) was used to measure the titers of nAbs against G1, G3, and G5 JEV. Dispensed BHK-21 cells were seeded in six-well plates and cultured until a monolayer was formed. Serum specimens were inactivated at 56 °C for 30 min and diluted 2-fold from 1:5 to 1:10,240. Each dilution was mixed with an equal volume of virus suspension at a titer of 200 plaque-forming units (PFU)/100 µL. After incubation for 1 h at 37 °C, the mixture was used to inoculate BHK-21 cells in six-well plates. In addition, the virus was added (100, 10, and 1 PFU) to the cells in sixwell plates as a reference. All specimens and references were incubated at 37 °C for 1 h. The liquid in each well was discarded, and the cells were overlaid with methylcellulose-MEM containing 2% FBS and 1% PS, followed by incubation at 37 °C for 5 d. The methylcellulose-MEM in each well was discarded, and the cells were stained with crystal violet. Next, the number of plaques in each well was determined. The titers of nAbs were determined as the maximum dilution level that resulted in a 90% reduction in plaque number compared to the reference.

Statistical Analysis

The value of PRNT₉₀ \geq 1:10 was defined as a positive result. The ratios of the nAb titers for the various viral strains were calculated. If one nAb titer was 4-fold different from the other two or only if this nAb was positive in the same serum specimen, we determined that the patient was infected with the JEV genotype. Statistical analyses were performed using the R software (Version 3.6.3). Proportions were compared using a χ^2 test with a test level of a = 0.05.

RESULTS

Specimen Collection

In this study, acute serum specimens from clinically reported JE cases were obtained for laboratory confirmation from hospitals in Shandong (92 cases), Zhejiang (192 cases), and Guangdong (77 cases) between 2018 and 2020. These specimens were stored at -80 °C and transported on dry ice to the Department of Arbovirus, National Institute for Viral Disease Control and Prevention, China CDC, for JEV serological tests and nucleic acid detection.

IgM Antibody and JEV Nucleotide Detection

All 361 specimens were negative for JEV nucleotides, and only 70 serum specimens were positive for JEV IgM antibodies.

Basic Characteristics of the 70 Participants

Seventy laboratory-confirmed JE cases from three coastal provinces of China between 2018 and 2020 were included. The highest number of cases was reported in 2018. The sex ratio of 48 males to 22 females was 2.18:1. Their mean age was 29.63 ± 25.17 years. Thirty-three individuals were 0-14 years old, 11 were 15–40 years old, and 26 were > 40 years old. Farmers, students, and children accounted for the largest proportion of JE cases. A considerable proportion of cases (24/70, 34.28%) were classified as severe. In addition, approximately one-third (21/70) of the patients received the JE vaccination. The remaining two-thirds (49/70) were unvaccinated against JE or had unknown vaccination status (Table 1). Individuals aged > 40 years accounted for the largest proportion of cases in Shandong province, whereas those aged 0-14 years dominated Zhejiang and Guangdong provinces (Figure 1).

nAbs Against JEV

Of the 36 specimens with identifiable infection genotypes, two cases of G1 (5.6%), 32 cases of G3 (88.8%), and two cases of G5 (5.6%) JEV infections were identified using the cross-neutralization test. Thirty-two patients in Shandong, Zhejiang, and Guangdong provinces were confirmed to be infected with G3 JEV. One case in Zhejiang and Guangdong provinces was G1 JEV. One case from Shandong and Zhejiang provinces was confirmed to be infected with G5 JEV (Table 2). Seven specimens tested negative for the G1, G3, and G5 JEV nAbs. The JEV genotype in the other 27 cases could not be inferred using a cross-neutralization test. Nine specimens were positive for three nAbs, 17 were positive for G1 and G3 JEV nAbs but negative for G5 JEV nAbs, and one specimen was positive for G3 and G5 JEV nAbs but negative for G1 JEV nAbs (Table 2).

Clinical Features

To explore the relationship between vaccination

status and the clinical classification of the 70 cases, the clinical classification of the 21 cases with a vaccination history was primarily mild (33.3%, 7/21) or moderate (38.1%, 8/21). The clinical classification of the 49 patients with no or unknown vaccination history was primarily mild (24.5%, 12/49) or severe (42.9%, 21/49). No statistically significant differences were observed between the two groups (Table 3). The incidence of symptoms such as acute onset, fever, depression, and dysfunction of consciousness was similar in the different vaccination status groups (all > 50%). The incidence of dizziness, nausea, irritability, convulsions, small and localized twitches, and recurrent or continuous twitches was similar (10%–50%). Abdominal pain, diarrhea, and circulatory failure were less common (< 10%). No statistically significant differences were observed between vaccination status and clinical symptoms (Table 4).

DISCUSSION

In China, JE is the most common form of viral encephalitis^[26]. Clinically reported cases of JE are

Marin alara	Data'il alaas	Shan	dong (31 c	ases)	Zhej	iang (24 ca	ises)	Guangdong (15 cases)		
iviain class	Detail class	0–14	15–40	> 40	0-14	15–40	> 40	0-14	15–40	> 40
Candar	Male	3	2	15#	13 ^{*#}	5	2	6	0	2
Gender	Female	2	2	7	2	2	0	7*	0	0
	Farmer	0	1	20	0	1	1	0	0	2
	Student	3	0	0	8	1	0	8	0	0
Occupation	Scattered children	2	0	0	7	0	0	5	0	0
Occupation	Worker	0	0	1	0	4	1	0	0	0
	Job-waiting people	0	1	1	0	0	0	0	0	0
	Unknown	0	2	0	0	1	0	0	0	0
	Mild	2	1	3	3	2	1	6	0	1
	Moderate	2	1	5	4	3	0	4	0	0
Clinic classification	Severity	1	2	10	7	1	1	2	0	1
	Extreme Severity	0	0	2	0	1	0	1	0	0
	Unclassified	0	0	2	1	0	0	0	0	0
	Vaccinated	2	0	0	10	0	0	9	0	0
JE vaccination status	Unvaccinated	2	1	6	2	1	1	4	0	1
	Unknown	1	3	16	3	6	1	0	0	1

Table 1. Summary of basic characteristics of the laboratory-confirmed case of JE, 2018–2020

Note. ^{*}Indicates the G1 JEV infection case is included in this group. [#]Indicates the G5 JEV infection case is included in this group. JE, Japanese encephalitis; JEV, Japanese encephalitis virus.



Figure 1. Demographic characteristics of the JE case for our survey. The pyramid shows the number of laboratory-confirmed JE cases and the age and gender of participants from each province. A total of 70 laboratory-confirmed cases of JE are observed, with more males than females. The number of cases from Shandong, Zhejiang, and Guangdong Provinces was 31, 24, and 15, respectively. The majority of cases in Shandong province were > 40 years old, whereas the majority of cases in Zhejiang Province and Guangdong Province were 0–14 years old.

usually confirmed using ELISA methods for detecting JEV IgM antibodies in specimens. However, this method cannot distinguish between genotypes of infected JEV. Furthermore, because it is difficult to

detect viral nucleic acids in case specimens, the infected JEV genotype cannot be identified using molecular biology methods. In this study, the nAb titers of G1, G3, and G5 JEV strains against the same

Table 2. Results of PRNT ₉₀ and the information of	JE cases with definite JEV infection genotype result
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						Tit	ers of nA	Abs			
No.	Province ^a	Year	Age	Gender⁵	Days after onset ^c		(1:-)		Genotype ^d	Clinic classification	Vaccination status ^e
						G1	G3	G5			
1	ZJ	2019	14	М	-	320	20	< 10	G1	Severe	UK
2	GD	2019	8	F	4	10	< 10	< 10	G1	Extremely severe	V
3	SD	2018	18	М	4	40	320	40	G3	Mild	UK
4	SD	2018	19	F	14	10	2,560	80	G3	Severe	UK
5	SD	2018	53	М	5	20	320	10	G3	Extremely severe	UK
6	SD	2018	57	М	-	160	5,120	< 10	G3	Unclassified	UK
7	SD	2018	60	М	20	20	80	< 10	G3	Unclassified	UK
8	SD	2018	67	М	13	10	40	< 10	G3	Moderate	No
9	SD	2018	67	F	21	< 10	160	< 10	G3	Severe	No
10	SD	2018	73	М	31	< 10	40	< 10	G3	Severe	UK
11	SD	2018	74	М	7	< 10	40	< 10	G3	Mild	No
12	SD	2019	2 m ^f	М	7	< 10	20	< 10	G3	Mild	No
13	SD	2019	55	F	-	< 10	20	< 10	G3	Unclassified	UK
14	SD	2020	34	М	10	320	1,280	320	G3	Moderate	No
15	SD	2020	49	М	7	40	2,560	< 10	G3	Severe	UK
16	SD	2020	54	F	3	< 10	20	< 10	G3	Unclassified	No
17	SD	2020	54	М	5	160	640	< 10	G3	Moderate	UK
18	SD	2020	55	F	11	< 10	10	< 10	G3	Mild	UK
19	SD	2020	60	М	0	< 10	20	< 10	G3	Unclassified	UK
20	SD	2020	63	М	7	< 10	10	< 10	G3	Severe	UK
21	ZJ	2018	1	М	6	10	80	10	G3	Mild	V
22	ZJ	2018	10	М	-	20	640	10	G3	Severe	UK
23	ZJ	2018	15	М	-	320	1,280	10	G3	Moderate	UK
24	ZJ	2019	14	М	-	80	320	< 10	G3	Severe	V
25	ZJ	2019	15	М	11	10	40	< 10	G3	Mild	UK
26	ZJ	2019	55	М	17	40	320	< 10	G3	Unclassified	No
27	ZJ	2020	1	М	16	10	160	< 10	G3	Mild	V
28	ZJ	2020	30	F	11	10	40	< 10	G3	Mild	No
29	ZJ	2020	31	М	7	20	320	< 10	G3	Moderate	UK
30	GD	2018	3	F	18	< 10	10	< 10	G3	Unclassified	No
31	GD	2018	71	М	25	< 10	10	< 10	G3	Unclassified	UK
32	GD	2020	10 m ^f	М	2	< 10	10	< 10	G3	Moderate	V
33	GD	2020	8	М	6	40	1,280	10	G3	Moderate	V
34	GD	2020	66	М	4	< 10	10	< 10	G3	Mild	No

7	2	1

											Continued
						Tite	ers of n	Abs			
No.	Province ^a	Year	Age	Gender⁵	Days after onset ^c		(1:-)		Genotype ^d	Clinic classification	Vaccination status ^e
						G1	G3	G5			514145
35	SD	2020	58	М	8	40	160	640	G5	Severe	UK
36	ZJ	2019	2	М	8	40	40	320	G5	Moderate	V
37	SD	2020	14	F	7	< 10	< 10	< 10	-	Mild	No
38	SD	2020	61	F	5	< 10	< 10	< 10	-	Moderate	UK
39	SD	2020	65	М	13	< 10	< 10	< 10	-	Severe	UK
40	SD	2020	70	М	5	< 10	< 10	< 10	-	Severe	UK
41	ZJ	2020	1	М	4	< 10	< 10	< 10	-	Moderate	No
42	GD	2018	11	F	10	< 10	< 10	< 10	-	Mild	V
43	GD	2019	5	F	22	< 10	< 10	< 10	-	Mild	V
44	SD	2018	14	М	7	10	20	< 10	-	Severe	UK
45	SD	2018	32	F	27	10	20	< 10	-	Severe	UK
46	SD	2018	53	F	13	20	20	< 10	-	Severe	UK
47	SD	2018	62	F	10	20	20	< 10	-	Moderate	No
48	SD	2018	64	М	3	10	20	< 10	-	Severe	No
49	SD	2019	9	F	5	10	20	< 10	-	Unclassified	V
50	SD	2020	7	М	5	20	40	< 10	-	Moderate	V
51	ZJ	2018	10	М	-	80	80	< 10	-	Severe	V
52	ZJ	2018	49	М	13	10	10	< 10	-	Severe	UK
53	ZJ	2019	14	М	5	20	20	< 10	-	Mild	V
54	GD	2018	1	М	1	20	40	< 10	-	Moderate	No
55	GD	2018	8	F	9	20	20	< 10	-	Mild	V
56	GD	2018	14	F	18	20	10	< 10	-	Severe	V
57	GD	2019	8	М	35	20	40	< 10	-	Mild	V
58	GD	2019	10	М	26	80	160	< 10	-	Mild	No
59	GD	2019	14	F	-	20	10	< 10	-	Severe	No
60	GD	2020	3	М	1	10	20	< 10	-	Moderate	V
61	ZJ	2018	26	М	6	< 10	20	20	-	Extremely severe	UK
62	SD	2018	53	М	5	20	40	10	-	Severe	UK
63	ZJ	2018	1	F	8	40	80	20	-	Severe	No
64	ZJ	2018	6	М	-	160	320	160	-	Moderate	V
65	ZJ	2018	11	М	8	160	320	10	-	Unclassified	V
66	ZJ	2018	12	М	9	10	20	10	-	Severe	V
67	ZJ	2018	18	М	5	20	20	20	-	Unclassified	UK
68	ZJ	2018	22	F	3	40	80	20	-	Severe	UK
69	ZJ	2019	4	F	18	10	10	10	-	Severe	UK
70	ZJ	2019	8	М	-	160	160	20	-	Unclassified	V

Note. ^aSD, Shandong Province; ZJ, Zhejiang Province; GD, Guangdong Province. ^bGender: M, male; F, female. ^cDays after onset, the days from onset at serum collection. ^dGenotype, the genotype of JEV that caused JE of the patient. ^eUK, unknown; No, unvaccinated; V, vaccinated. JE, Japanese encephalitis. ^fm, month.

serum specimen were used to determine the infection genotypes of the JE cases. G1, G3, and G5 JEV infections were evident among JE cases in Shandong, Zhejiang, and Guangdong provinces from 2018 to 2020. G3 was the primary infection genotype among the JE cases with a definite infection genotype, and infection caused by G5 JEV was confirmed in China.

there have been no previous reports of G5 JEV infections^[19]. In this study, two cases of G5 JEV infection were identified in the Zhejiang and Shandong provinces in 2019 and 2020, respectively. In contrast to the age and sex characteristics of patients infected with G5 JEV reported in Korea (27 years of age and females, respectively)^[18], the patients in this study were both males (2 and 58 years of age). These findings suggest that G5 JEV can

Although G5 JEV was isolated in 2009 in China,

	Vaccinat	ion history, <i>n</i> (%)			
Clinical typing	Yes (<i>n</i> = 21)	No/Ominous (n = 49)	X ²	P value	
Mild	7 (33.3)	12 (24.5)	0.581	0.553	
Moderate	8 (38.1)	11 (22.4)	1.82	0.242	
Severe	4 (19.0)	21 (42.9)	3.63	0.091	
Extremely severe	1 (4.8)	3 (6.1)	0.051	1.000	
Unclassified	1 (4.8)	2 (4.1)	0.017	1.000	

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	Vaccinat	tion history, <i>n</i> (%)	2		
Clinical symptoms	Yes (<i>n</i> = 21)	No/Ominous (n = 49)	Ϋ́ Χ΄	P value	
Acute onset	14 (66.7)	46 (93.9)	2.694	0.131	
Fever	16 (76.2)	41 (83.7)	0.039	1.000	
< 39 °C	7 (33.3)	21 (42.9)	0.287	0.779	
39–40 °C	6 (28.6)	13 (26.5)	0.144	0.762	
> 40 °C	3 (14.3)	7 (14.3)	0.016	1.000	
Headache	8 (38.1)	27 (55.1)	1.241	0.381	
Dizziness	8 (38.1)	17 (34.7)	0.284	0.777	
Abdominal pain	0 (0.0)	3 (6.1)	1.248	0.559	
Diarrhea	2 (9.5)	4 (8.2)	0.082	1.000	
Nausea	6 (28.6)	18 (36.7)	0.219	0.778	
Vomiting	11 (52.4)	17 (34.7)	3.101	0.096	
Jet vomiting	3 (14.3)	3 (6.1)	1.541	0.332	
Depression	14 (66.7)	36 (73.5)	0.016	1.000	
Irritability	1 (4.8)	11 (22.4)	2.955	0.15	
Drowsiness	9 (42.9)	30 (61.2)	1.516	0.236	
Dysphoria	5 (23.8)	20 (40.8)	1.466	0.251	
Convulsions	3 (14.3)	11 (22.4)	0.429	0.74	
Dysfunction of consciousness	11 (52.4)	27 (55.1)	0.019	1.000	
Small localized twitches	3 (14.3)	9 (18.4)	0.082	1.000	
Recurrent or continuous twitches	4 (19.0)	9 (18.4)	0.048	1.000	
Respiratory failure	2 (9.5)	12 (24.5)	1.775	0.308	
Cirulatory failure	0 (0.0)	3 (6.1)	1.248	0.559	

Table 4. Comparison of clinical symptoms in different vaccination status

cause JE infections in all age groups. Similar to the Korean case described above, the child infected with G5 JEV in this study was vaccinated with two doses of the inactivated vaccine. These findings support the hypothesis that G3 JEV-derived JE vaccines do not provide adequate protection against G5 JEV at the individual level^[16,17]. The three individual cases mentioned above show that G5 JEV has an underestimated capacity and potential to spread among humans, and there is a risk of G5 JEV transmission in China. These results suggest that it is necessary to conduct G5 JEV nAb surveillance in healthy populations to gain a more comprehensive understanding of G5 JEV infections. G4, a relatively rare JEV genotype similar to G5, caused an outbreak in Australia in 2022. Inactivated vaccines and chimeric-attenuated live vaccines used to control outbreaks in Australia are derived from G3 JEV. To date, no relevant research has evaluated the protective effects of G3 vaccines against G4 JEV infection. Further studies focusing on the protective efficiency of JE vaccines against G4 and G5 JEV are required, and a polyvalent vaccine to prevent epidemics caused by the five JEV genotypes should be developed.

JE is a disease that can be prevented by vaccination. The vaccines currently in domestic use in China are the live attenuated JE vaccine (SA-14-14-2) and the inactivated JE vaccine (P3)^[27,28]. Shandong, Zhejiang, and Guangdong provinces included the JE vaccine in their childhood immunization programs beginning in 1986, 1986, and 2004, respectively^[29-31]. Children aged 8 and 24 months received two free doses of the live-attenuated vaccine. This has led to a decline in JE incidences^[26,32]. In 2008, China included the JE vaccine in the Expanded Program on Immunization, continuously improving JE vaccination in children and strengthening JE surveillance, making the recording and preservation of vaccination information complete and traceable^[33]. In this study, among the 21 patients with a history of JE vaccination, six completed the full vaccination schedule with two doses of the live-attenuated vaccine. However, a majority (49/70) of the patients had either not been vaccinated or had unclear vaccination histories; 25.71% (18/70) had not received the JE vaccine; and 44.29% (31/70) had unclear vaccination backgrounds. Most of those who had not been vaccinated or whose vaccination status was unclear were middle-aged and elderly individuals born during an era when the JE vaccine was not widely available. Therefore, among the objectives of this study, the vaccination information of children can be queried, but the vaccination information of adults is incomplete or inaccurate, which impacts the statistical analysis. Statistical analyses incorporating vaccination status data failed to yield statistically significant results.

In patients 0–14 years of age, the numbers of cases from Shandong, Zhejiang, and Guangdong Provinces were 16% (5/31), 62.5% (15/24), and 86.7% (13/15), respectively. For those aged > 40years, the proportions were 71% (22/31) in Shandong, 8.3% (2/24) in Zhejiang, and 13.3% (2/15) in Guangdong (Figure 1). Based on the age demographics of JE cases in these provinces, Shandong in Northern China primarily encounters adult cases, whereas in Zhejiang and Guangdong in Southern China, pediatric cases are primarily observed, which is consistent with previous research^[34,35]. As pediatric JE is increasingly mitigated by vaccination, adult cases have become more prominent. Studies have reported lower titers of nAbs against JEV in the sera of older populations in Northern China, resulting in outbreaks in adults^[36-39]. After large-scale vaccination in these older adult age groups, the incidence and severity of individual cases decreased significantly^[40]. Therefore, surveillance for adult JE should be implemented in the northern regions of China. Vaccination programs in endemic areas should maintain and strengthen regular immunization of children and supplemental vaccination of adults with unclear vaccination Collectively, "precision histories. а dynamic immunization" strategy should be employed.

Serological crossover exists between flaviviruses, and serological crossover occurs between different JEV genotypes. The detection of IgM or IgG antibodies cannot distinguish the viral genotype infection, and there are no relevant studies to specifically distinguish the crossover titer. Because we only had a single serum specimens, a crossneutralization test of different JEV genotypes was used in this study to identify JE cases infected with different viral genotypes. Cross-neutralization tests revealed that among the JE cases with identifiable infection genotypes, 32 of 36 were infected with G3 JEV, and two of 36 were infected with G1 JEV. From 1949–2000, G3 was the predominant JEV genotype isolated and identified in China. Since 2000, both the G1 and G3 JEVs have been circulating in China. In recent years, G1 has become the dominant genotype in mosquitoes^[41-44]. Concurrently, G1 and G3 JEV cocirculate in the swine population. The findings of this study indicate that the natural infection rate of G3 JEV remains high among human cases in the coastal

provinces of Guangdong, Zhejiang, and Shandong. Therefore, although a shift in the JEV genotypes has occurred in mosquitoes, G3 remains the primary genotype responsible for human infections. However, the outbreaks caused by other JEV genotypes should not be ignored. In 2006, G1 JEV was first isolated from the cerebrospinal fluid of a patient with encephalitis in Guizhou province^[45]. Subsequently, more G1 JEV strains were isolated from cerebrospinal fluid specimens^[46], and multiple outbreaks caused by G1 JEV have been reported in Northern China. For example, during the 2018 JE outbreak in the Ningxia Hui Autonomous Region, both clinical and natural specimens tested positive for G1 JEV^[37], suggesting that G1 can replace G3 JEV in humans. These findings indicate the necessity for genotype monitoring in future JE cases.

Based on the cross-neutralization test, we could not deduce the causative JEV genotype in 27 cases. The timing of the production and the peak of nAbs are related to several factors. A considerable difference was observed in the titers of specific neutralizing antibodies produced after the initial exposure and re-exposure to a pathogen. Second, the time interval between onset and serum collection affected the determination of nAb titers. Previous studies have shown that on the day of onset, JEV IgG nAbs can be detected in the sera of a small number of JE cases. By the 30th day of the disease, the positivity rate of IgG nAbs reaches 100%, and the IgG antibody level peaks^[47]. In this study, the demographic and geographical patterns of the 27 cases were not observed. A follow-up investigation will increase the specimen size and continue to focus on determining the infection genotype of JE cases in other areas of the country, with the hope of drawing more comprehensive and rich conclusions.

CONCLUSION

In conclusion, this study confirmed the presence of G1, G3, and G5 JEV infections in China using serological methods. G3 was the primary infection genotype among the JE cases with a definite infection genotype. The data confirmed the insufficient protection conferred by the current JE vaccine at the individual level, and the transmission of G5 JEV in the population is important to consider. In addition, this study revealed that the majority of JE cases in northern China occurred in adults. To prevent and control JE, strengthened surveillance in adults and supplementary JE vaccination should be considered. Serological investigations in different populations are important to clarify the threat posed to population health.

AUTHOR CONTRIBUTIONS

Huanyu Wang planned the work. Weijia Zhang, Jierong Zhao, Shenghui Liu and Shihong Fu performed the experiments. Shihong Fu, Ying He, Fan Li and Qikai Yin collected the serum specimens. Weijia Zhang, Qikai Yin, Ruichen Wang and Kai Nie analyzed the data and constructed the tables and figures. Songtao Xu, Guodong Liang, Guang Yang and Huanyu Wang supervised the study. Weijia Zhang and Jierong Zhao drafted and revised the manuscript. All authors edited and commented on this manuscript. All the authors have read and agreed to the final version of the manuscript.

AVAILABILITY OF DATA AND MATERIALS

The data and materials for this study are available from the authors upon reasonable request.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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