Report 2014

STUDY GENERAL TITLE

Laboratory-based collaboration net work of infectious diseases in Asia

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1. Epidemiology of HFMD and genetic characterization of HEV71and CVA16

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Whole genome sequencing of 50 EV-A71 strains isolated in mainland of China

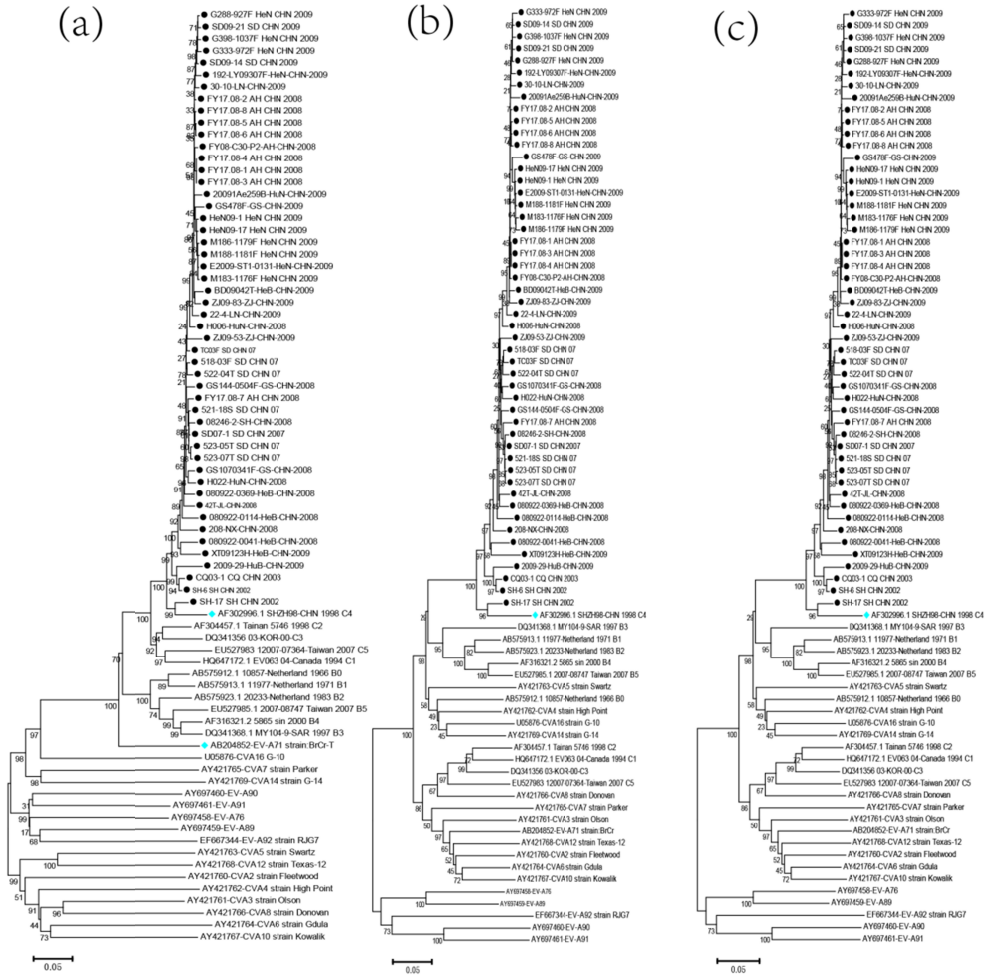
50 EV-A71 strains isolated in mainland of China were sequenced. All EV-A71 strains have 7404-7406bp in length, and nucleotide insertions or deletions were found in some of the EV-A71 strains. But in coding region, all 50 EV-A71 strains have the same length, 6579bp, which coding 2193 amino acids.

All 50 EV-A71 strains share 92.7-99.8% nucleotide similarities in VP1 region, while share 89.2%-99.7%nucleotide similarities in the whole genome. Compared with EV-A71 prototype strain (BrCr strain), they share 82.3%-84.0% nucleotide similaritiesin VP1 region, and share 81.3%-82.9%, 77.7%-79.1%, 77.2%-78.2%nucleotide similarities in P1 region, P2 region, P3 region, respectively.

In order to find the evolutionary relationship among 50 EV-A71 strains, other EV-A71 strains downloaded from GenBank database, and other enterovirus species A, 203 whole genomic sequences of EV-A71 strains from mainland of China and 123 whole genomic sequences of EV-A71 strains from other countries and regions (including Viet Nam, Russia, Thailand, Canada, Malaysia, Singapore, Taiwan, and Hongkong), downloaded in GenBank database, and Among them, 30 whole genomic sequences of EV-A71 strains were selected based on their nucleotide variation, and phylogenetic trees were constructed based on their whole genomic sequences, P1 region sequences, P2 region sequences, P3 region sequences, respectively.

Seen from the phylogenetic trees based on whole genomic sequences, all EV-A71 from mainland of China could be divided into two branches, which are C4a evolutionary branch and C4b evolutionary branch; EV-A71 isolated after 2007

belonged to C4a evolutionary branch, and EV-A71 isolated before 2003 belonged to C4b evolutionary branch.Seen from the phylogenetic trees based on P1 region sequences and P2 region sequences, all 50 EV-A71 strains have the highest nucleotide similarity with C4 genotype EV-A71 strains; while seen from the phylogenetic trees based on P3 region sequences, only 47 EV-A71 strains have the highest nucleotide similarity with C4 genotype EV-A71 strains, two strains (XT09123H-HeB-CHN-2009 and 080922-0114-HeB-CHN-2008) are still stay in the same branch with C4 genotype but have large genetic distance, one strain (2009-29-HuB-CHN-2009) has the highest nucleotide similarity with B3 genotype EV-A71 strains, which indicates recombinationoccurred in P3 region sequence (Figure).



Phylogenetic trees of EV-A71 strains. (a): P1 region; (b): P2 region; (c):P3 region。

2. Laboratory based surveillance and outbreak detection for multiple foodborne diseases

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Summary:
We estimated preliminarily a multiple PCR method which is developed for the detection of genes of the common bacterial pathogens causing diarrhea, by using the outpatients' samples collected in the hospitals. Another cooperation work was the transfer and identification of non-o1/non-O139 *Vibrio cholerae, V. fluvialis* and some other suspected strains, and their major virulence-related genes conducted in NIID.

I. Purpose:

To estimate an GeXP principle based multiple PCR detection for diarrheal bacterial pathogens, by using a clinical sample collection from diarrheal outpatients. Another work is non-O1/non-O139 *V. cholerae* strains from the environmental samples and diarrheal patients, and the *V. fluvialis* and the suspected strains form diarrheal patients were transferred to NIID for the serotype identification and further studies.

II. Results:

1) The preliminary estimation of the multiplex PCR method in diarrheal detection caused by bacterial pathogens.

A total of 243 clinical diarrheal samples were collected from May 2013 to April 2014 in Wuxi Center for Disease Control and Prevention. Total nucleic acids of these samples were extracted and examined with a 16-plex PCR assay we developed based

on the GeXP technique, in parallel with the comparison methods (including a multiplex PCR assay targeting diarrheagenic *E. coli*, and other real-time PCR and conventional PCR detecting single gene of the pathogens listed in Table 1). The discordant results between the 16-plex assay and the comparison methods were resolved by a third single-plex PCR and sequencing. The sensitivities and specificities of the two methods were listed in the Table 1. In all, 43 samples (17.7%) were positive with at least one target gene, and 6 samples were positive in 2 or more target genes (two were *sth*/*elt*+, one *eaeA*/EIEC+, one *eaeA*/EAEC+, one *st*h/EAEC+, and one *sth*/*elt*/*eaeA*+). Compared with conventional culture methods, the detection of nucleic acids will show more positive samples. This is a preliminary estimation of this 16-plex PCR method for detection of the common bacterial pathogens which may cause diarrhea.

Table 1. Comparison of 16-plex PCR method with the routinely used PCR methods in the detection of diarrheal outpatient samples.

Target genes or pathogens (numbers of positive samples)	16-plex PCR		comparison methods	
	sensitivity	specificity	sensitivity	specificity
Genes for diarrheagenic *E. coli* and *Shigella*				
sth (16)	15/16	98.7%	11/16	100.0%
elt (9)	5/9	100%	7/9	100%
eaeA (7)	7/7	99.6%	4/7	99.6%
aggR (5)	4/5	100.0%	4/5	100.0%
virA (3)	3/3	100.0%	3/3	100.0%
stx1 (3)	3/3	100.0%	1/3	100.0%
stx2 (0)	-	-	-	-
bfpA (0)	-	-	-	-
stp(0)	-	-	-	-
Other pathogens				
Salmonella (4)	1/4	100.0%	4/4	100.0%
C. jejuni (2)	2/2	100.0%	2/2	100.0%
C. coli (0)	-	-	-	-
V. cholearae (2)	0/2	100.0%	2/2	100.0%
V. parahaemolyticus (2)	0/2	100.0%	2/2	100.0%
Y. enterocolitica (0)	-	-	-	-
Total positive: 50				

2) Identification of non-O1/non-O139 *Vibrio cholerae*, *V. fluvialis* and Isolation of the bacteria from the samples

In 2013, 30 suspected non-O1/non-O139 *V. cholerae* strains from the environmental samples and diarrheal patients in the previous surveillance and 10 suspected *V. fluvialis* strains from diarrheal patients were transferred to NIID for the serotype identification. 25 of these strains were indentified as non-O1/non-O139 *V. cholerae* (including 21 serotypes), 10 strains as *V. fluvialis*, two strains as *A. sobria*, two strains as *A. hydrophila*, and one strain as *V. parahaemolytic* respectively. Some toxigenic genes including *ctx*, nag-*st*, *trh* and T3SS gene were also identified.

In this year, 20 suspected non-O1/non-O139 *V. cholerae* strains and 20 suspected *V. fluvialis* strains will be transferred to NIID for the serotype identification again, and the relevant documents are under preparation.

III. Discussion:

We estimated the GeXP technique based multiple PCR method for the target gene detection of the common diarrheal bacterial pathogens. The results showed a good prospect for the application of this method, though the amount of the samples was not so huge and some pathogens were not found. But improvement is also needed. We found that *V. cholerae* and *V. parahaemolyticus* were negative in 16-plex PCR method but were detected with the commonly used PCR, it may suggest that the primers in the 16-plex PCR method for the detection of these two vibrios are needed to be optimized. We develop this multiplex PCR method for the purpose of the application of PCR based screening for the diarrheal bacterial pathogens. PCR screening and followed by the culture based on the indication of target bacteria suggested by PCR, will improve the efficiency of bacterial diarrheal pathogen surveillance.

We also perform the cooperation on the Vibrio identification and virulence related genes detection. We have identified some serotypes of non-O1/non-O139 *V.*

cholerae strains, and got the virulence gene profiles of these strains. More suspected *Vibrio* strains will be transferred to NIID for the further identification.

3. Molecular Epidemiology of Severe Febrile and Thrombocytopenia Syndrome Virus (SFTSV), a New Bunyavirus, in China

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Summary:

A total of 3,145 ticks of the species *Haemaphysalis longicornis* (*H. longicornis*; 3048, 96.9%), *Rhipicephalus microplus* (*R. microplus*; 82, 2.6%), *Haemaphysalis campanulata* (*H. campanulata*; 9, 0.3%), and *Dermacentor sinicus* (*D. sinicus*; 5, 0.2%) were collected from animals and vegetation at Yantai in Shandong province. Both adult and immature ticks were obtained, and all ticks collected from vegetation were unfed. Eggs were obtained from 22 blood-fed female ticksthrough maintenance at room temperature after collection. Severe fever with thrombocytopenia syndrome virus (SFTSV) viral RNA was identified in *H. longicornis* and *R. microplus* with a prevalence of 4.75 per 100 ticks (95% CI: 3.87-5.63) for ticks collected from animals and 2.24 per 100 ticks (95% CI: 1.27-3.21) for ticks collected from vegetation. The possibility that SFTSV transmission may occur by both the transstadial and transovarial routes was suggested by the fact that viral RNA was detected in *H. longicornis* at all developmental stages. Tick-derived sequences shared over 95.6% identity with human- and animal-derived isolates. This study provides evidence that implicates ticks not only as vectors but also as reservoirs of SFTSV.

I. Purpose:

To investigate the infection of severe fever with thrombocytopenia syndrome virus (SFTSV) in ticks in the endemic region of China

II.Methods:

*Tick collection.*Ticks were collected from both animals and vegetation in the Yantai region, an SFTSV-endemic region of Shandong province (119°34′–121°57′E,

36°16′–38°23′N; Figure 1). SFTS human cases have been reported in this region since 2010.

Ticks were collected from sheep, cattle, dogs, chickens, and small wild animals in this area. The animals' bodies were examined for ticks. If fewer than ten ticks were found on one animal, then all ticks were collected; if more than ten were found, then ten ticks from each species at each developmental stages were chosen respectively and removed from each animal. The ticks were collected using forceps and placed in perforated tubes containing a moistened piece of filter paper. Labels with unique numbers were placed on each tube to identify the date, location of sample collection and the host from which the ticks had been removed. Engorged ticks were maintained individually, while other ticks from the same host were maintained in one tube until species determination. All ticks were maintained at room temperature (20 – 25°C) for one week, after which the engorged female ticks were allowed to oviposit. After oviposition, females were placed individually in 2-mLcryovials, and the cryovials were labeled and stored at -80°C. Eggs from each female that oviposited were immediately placed in a 2-mLcryovial labeled with the same identifying number as that used for the engorged female tick that had laid the eggs, followed by an extension of "egg," and stored at -80°C.

Ticks from vegetation were sampled by "the woolen flannel cloth dragging method" as described by Mejlon [12]. The size of the woolen flannel cloth was 1 m×1 m. One end of the cloth was attached to a bamboo cane and a string. The method was implemented by dragging the cloth slowly through vegetation for one hour in the morning. At 30 - 50 m intervals, the cloth was inspected, and the attached ticks were removed and collected as described above. The tubes were labeled with a unique number to identify the date, location, and collection site.

Ticks collected by both methods were transported to the Chinese National Institute for Viral Diseases Control and Prevention, where they were identified and pooled into groups by site, collection type, host, species, and stage. Adult ticks were placed individually in 2-mL cryovials, while nymphs(5-8 per cryovia) and larvae (10 per cryovia) were pooled and stored at -80°C.

RNA extraction and viral RNA detection. Individual and pooled tick specimens were surface sterilized with sequential washes with DMEM medium containing antibiotics and then homogenized in 500 μL of chilled DMEM medium using a tissue homogenizer (TissueLyser, QIAGEN, Hilden, Germany). The tick homogenates were transferred into 1.5-mL microcentrifuge tubes and centrifuged at 4°C and 9279 × g for 1 min (Eppendorf, Germany). The clarified supernatants were transferred in 150-μL aliquots to labeled 1.5-mL microcentrifuge tubes for RNA extraction using an RNeasy mini kit (QIAGEN, Germany) according to the manufacturer's instructions. Real-time quantitative reverse transcription PCR (real-time qRT-PCR) was performed using 5 μL of each aliquot of extracted RNA and a CFDA-approved qRT-PCR kit (DaAn Gene, Guangzhou, China), following the manufacturer's instructions; primer sets and probes targeted the L, M and S segments of SFTSV, as previously described[13]. The result was considered positive if a sample had a *Ct* value below the cutoff for the assay. All real-time qRT-PCR-positive samples were confirmed using qRT-PCR targeting the 3 segments of the viral genome. Viral RNA copy numbers were determined by comparison with a standard curve generated by the amplification of positive-control RNA. Two-step conventional RT-PCR was performed for real-time PCR-positive samples using the SuperScript □ First Strand cDNA Synthesis kit (Invitrogen) to generate cDNA. FastStart High Fidelity Taq DNA Polymerase (Roche) and primers were used for PCR amplification of the viral genome S segment, as previously described[1].

Virus isolation. Tick homogenates that tested positive for viral RNA were tested for the presence of viable virus using Vero cells cultured in six-well plates using a modified version of a previously published protocol[1]. Briefly, 100 μL of each viral RNA-positive sample was inoculated onto a monolayer of Vero cells. Wells were inspected daily and were tested for viral RNA and N-protein antigen on days 6-8 post-infection. If the tests were negative, the culture supernatant was used to inoculate Vero cells for another two passages.

Sequencing and phylogenetic analysis. RT-PCR amplicons of the S segment of SFTSV were sequenced by the Genewiz service company (Genewiz, Beijing, China)

using the ABI BigDye Terminator V3.1 Ready Reaction Cycle Sequencing Mix (Applied Biosystems, Carlsbad, CA). Nucleotide sequences were assembled using SeqMan software (DNASTAR) and visual inspection. Alignments were conducted using ClustalW (MEGA 5). Phylogenetic analyses of whole tick, animal, and human derived S segments of SFTSV were conducted with MEGA 5 software using the neighbor-joining method with 1,000 replicates for bootstrap testing[14].

Statistics. The prevalence of SFTSV infection in ticks is presented as the Maximum Likelihood Estimate (MLE) per 100 ticks, with a 95% confidence interval. The MLE was calculated and analyzed using SPSS (v16) software. Viral RNA (vRNA) copy numbers in different species and developmental stages of ticks collected from either animals or plants were analyzed using the Kruskal–Wallis test performed using GraphPad Prism software (ver. 5.0; GraphPad Software Inc., La Jolla, CA). Values of $P < 0.05$ were considered statistically significant.

III.Results:

Tick collection. A total of 2,251 ticks of the species *Haemaphysalis longicornis* (*H. longicornis*; 2,154, 95.7%), *Rhipicephalus microplus* (*R. microplus*; 83, 3.7%), *Haemaphysalis campanulata* (*H. campanulata*; 9, 0.4%), or Dermacentor sinicus (*D. sinicus*; 5, 0.2%) were collected from animal hosts. *H. longicornis*was the dominant species; ticks of this species at all stages of development were collected (1,342 adults, 652 nymphs, and 138 larvae), and eggs were obtained from 22 *H. longicornis* adults during maintenance at room temperature for 1 week after detachment (Table 1). Both adult (n =50) and nymph (n = 33) *R. microplus* were collected, while only adult *H. campanulata* (n =9) and *D. sinicus* (n = 5) were collected. Most of the ticks were collected from sheep (1,718, 76.3%), cattle (254, 11.3%), dogs (194, 8.6%), chickens (61, 2.7%) and hedgehogs (24, 1.1%). A total of 894 *H. longicornis* ticks were collected by dragging a cloth through vegetation (461 adults, 415nymphs, and 18 larvae; Table 1). All ticks collected from vegetation were unfed.

In total, 3,145 ticks from 4 species were collected in the study region. The ticks were identified and grouped into 2,044 pools by site, collection type, host, species, and stage in preparation for viral RNA detection assays.

Table 1 Number of ticks collected by origin, species, and stage

Origin	Species	Adult	Nymph	Larvae	Eggs*	Total
Animals		1406	685	138	22	2251
Sheep	*H. longicornis*	1038	545	102	22	1707
	R. microplus	9	2	0	0	11
Cattle	*H. longicornis*	199	16	0	0	215
	R. microplus	11	28	0	0	39
Dog	*H. longicornis*	101	60	0	0	161
	R. microplus	30	3	0	0	33
Chicken	*H. longicornis*	0	25	36	0	61
Hedgehog	*H. longicornis*	4	6	0	0	10
	H. campanulata	9	0	0	0	9
	D. sinicus	5	0	0	0	5
Vegetation	*H. longicornis*	461	415	18	0	894
Total		1867	1100	156	22	3145

*Eggs were laid by blood-fed female ticks collected from animals and maintained at room temperature for one week after collection. Samples are grouped according to the adult female ticks that laid eggs.

Detection of SFTSV RNA in ticks. Of the four species collected, only *H. longicornis* (larvae, nymphs, and adults) and *R. microplus* (adults) were positive for SFTSV (Tables 2). In addition, 2/22 of the egg masses oviposited by blood-fed *H. longicornis* females were positive for SFTSV.

Ticks collected from animals were divided into 1,533 pooled groups and tested; SFTSV RNA was detected in 107 of the pooled samples. The frequencies of infection in ticks were recorded as the MLEs per 100 ticks, with 95% confidence intervals. The MLE of the prevalence of infection in ticks collected from animals was 4.75 per 100 ticks (95% CI:3.87-5.63; Table 2). No significant difference in prevalence was observed among adults, nymphs, and larvae ($P>0.1$). Of these positive pools, 64.5% (69/107) were adults, 29.9% (32/107) were nymphs, and 3.7% (4/107) were larvae (Table 2). A total of 3/22 adult female *H. longicornis* that oviposited were positive for

Table 2 SFTS virus infection in ticks by origin, species, and stage.

Origin	Adult (Positive /pools/ticks)	Infection rate (95% CI)*	Nymph (Positive /pools/ticks)	Infection rate (95% CI)	Larvae (Positive /pools/ticks)	Infection rate (95% CI)
Animals	69/1,406/1,406	4.91 (3.78-6.04)	32/91/685	4.67 (3.09-6.26)	4/14/138	2.90 (0.06-5.73)
H. longicornis	65/1,342/1,342	4.84 (3.69-5.99)	32/85/1067	3.00 (1.97-4.02)	4/14/138	2.90 (0.06-5.73)
Sheep	56/1,038/1,038	5.39 (4.02-6.77)	29/70/545	5.32 (3.43-7.21)	4/10/102	3.92 (0.09-7.75)
Cattle	4/199/199	2.01 (0.04-3.98)	0/3/16/	0	-	-
Dog	5/101/101	4.95 (0.65-9.25)	3/8/60	5.00 (0-10.68)	-	-
Chicken	-	-	0/3/25/	0	0/4/36	-
Hedgehog	0/4/4/	0	0/1/6/	0	-	-
R. microplus	4/50/50	8.00(0.21-15.79)	0/33	0	-	-
Sheep	0/9/9/	0	0/1/2/	0	-	-
Cattle	1/11/11/	-	0/4/28/	0	-	-
Dog	3/30/30	-	0/1/3/	0	-	-
H. campanulata	-	-	-	-	-	-
Hedgehog	0/9/9/	0	-	-	-	-
D. sinicus	-	-	-	-	-	-
Hedgehog	0/5/5/	0				
Vegetation	6/461/461	1.30 (0.26-2.34)	13/48/415	3.13 (1.45-4.82)	0/2/18/	0
H. longicornis	6/461/461	1.30 (0.26-2.34)	13/48/415	3.13 (1.45-4.82)	0/2/18/	0

*Infection rate expressed as Maximum Likelihood Estimate/100 ticks. The ranges in parentheses are 95% confidence intervals representing the upper and lower limits.

SFTSV RNA, and a total of 2/3 egg masses laid by SFTSV-positive females were also positive. We identified 96.3% (103/107) of the SFTSV-positive ticks as *H. longicornis*, while the remaining 3.7% (4/107) were adult *R. microplus* (Table 2). No significant difference in the prevalence of SFTSV in *H. longicornis* was found between ticks collected from sheep and ticks collected from dogs (P>0.05), but the prevalence in sheep and dogs were both higher than the MLE of the prevalence in ticks collected from cattle (P<0.05).

A total of 894 *H. longicornis* ticks were collected by dragging cloths through vegetation. These ticks were examined in 511 pools, out of which 6 of 461 adult pools and 13 of 48 nymphpools were SFTSV RNA-positive. The MLE of the prevalence was significantly higher in nymphs (3.13%) than in adults (1.3%, P<0.05; Table 2). No viral RNA was detected in 2 pools of 18 larvae. In total, the MLE of the prevalence in ticks from vegetation was 2.13 per 100 ticks (95% CI:1.18-3.07; Table 3), which was significantly lower than that in ticks from animals (P = 0.001).

The detected viral RNA concentration was approximately 3.0×10^4 copies/mL (95% CI: $2.6\text{-}3.5\times10^4$) in ticks from animals and 3.5×10^4 copies/mL (95% CI: $2.2\text{-}4.8\times10^4$) in ticks from vegetation. The difference in RNA copy number between ticks collected from animals and ticks collected from vegetation was not statistically significant (P = 0.336).The S segment of SFTSV was sequenced from ten pools composed of *H. longicornis* eggs, nymphs, and adults. Virus could not be grown from any SFTSV-positive tick samples.

Phylogenetic analysis. The S segment of SFTSV was amplified and sequenced from 11/126 of the RT-PCR-positive tick samples. Phylogenetic analyses were performed, and the S-segment sequences were compared with previously published SFTSV sequences obtained from patients, animals and ticks. The SFTSV sequences we obtained from ticks have a 95.6% to 99.9% nucleotide identity with each other and a 94.6% to 99.8% identity with sequences reported from patients and animals from both the same area and other provinces. Neighbor-joining (NJ) trees constructed with full S-segment sequences demonstrated that SFTSV from local patients, dogs and cattle clustered with some of the sequences we obtained from ticks.

DISCUSSION

SFTSV RNA was detected in *H. longicornis*at different developmental stages (in larvae, nymphs, and adults), as well as in eggs oviposited by blood-fed adult females. These results suggest that transmission of SFTSV may occur by the transstadial and transovarial routes, and demonstrate that *H. longicornis* plays a role in SFTSV maintenance and transmission.

Of the four species collected, *H. longicornis* and *R. microplus* were the two most frequently encountered ticks on both domestic animals and vegetation in the study region. The tick species and infection rates are similar to those reported from previous tick surveys from areas with SFTS cases in Henan Province and Hubei Province[15]. No statistically significant difference was observed for the rate of SFTSV infection among the different stages of *H. longicornis*, but the prevelance in ticks from animals was significantly higher than that in ticks from vegetation. Furthermore, the infection rate in nymphs was higher than in unfed adults from vegetation. This might imply that blood feeding has an impact on viral RNA detection. Surveillance of SFTSV in ticks, animals and humans provides epidemiological information about the maintenance and risks associated with epizootic transmission to domestic animals and incidental transmission to humans. All developmental stages of *H. longicornis*, as well as eggs from SFTSV-positive oviposited females, were positive for SFTSV, which demonstrated a mode for the maintenance of the virus in vector populations in the absence of feeding on viremic hosts. However, our data were only collected from the field through epidemiologic investigation, and only few egg masses were collected from SFTSV-positive oviposited females, therefore, further studies are needed to confirm in the laboratory the transmission of SFTSV in ticks by the transstadial and transovarial routes. In *R. microplus*, only adults were positive, suggesting that this species may not be effective at maintaining the virus in nature. Although *H. campanulata* and *D. sinicus* were SFTSV-negative, their role in SFTSV maintenance and transmission cannot be dismissed because few ticks of these species were collected for analysis. No viable virus was recovered from ticks in this study; however, this finding cannot be construed as evidence of absence of the virus, and it may

instead be due to the extremely low titers of the virus in field ticks, as indicated by the low RNA copy numbers in our qRT-PCR analysis. Several separate field investigations have been performed in areas of China with SFTS cases. One isolate was obtained from 1/140 *H. longicornis* ticks, collected in 2010, that possessed genetic and identical serological characteristics similar to those of isolates obtained from humans and domestic animals[4,15].

Infectious virus, a competent vector, and susceptible vertebrate hosts are necessary to establish and maintain arbovirus transmission cycles[16]. The natural vertebrate reservoirs of SFTSV have not been identified. Domestic animals (including sheep, goats, cattle, and dogs) are considered to be incidental hosts because they do not develop substantial viremia and because long viremic periods have not been observed in these animals[7, 21].This finding suggests that the role of viremic domestic animals in transporting SFTSV into new areas to re-infect ticks during a blood meal may be limited. Threshold levels of viremia are usually required for the infection of vectors with virus; however, ticks do not require substantial viremia in an infected host to take up an arbovirus[17-20]. While blood-feeding, ticks are attached to their hosts for days, which is crucial for virus transmission in ticks through a mechanism known as co-feeding. During co-feeding, viruses are transferred from one tick to another [24]. Adults and immature ticks (either larvae or nymphs) feed on the same host, which may also lead to transmission and maintenance of the arbovirus between different life cycle stages of the vector.

The risk of SFTSV to humans is related to vector abundance, the infection rate in various habitats, human exposure, tick feeding preferences, and prevention measures employed to reduce the frequency of tick bites[14, 15]. The distribution of SFTSV cases is therefore shaped by distributions of human populations and behaviors that favor human-tick contact. The high seroprevalence of antibodies to SFTSV detected in sheep (69.5%), cattle (60.4%), dogs (37.9%), and chickens (47.4%)[7] may indicate that the virus is more widespread than is evidenced by human cases. It would be beneficial to identify zoonotic hosts that may contribute to the maintenance and distribution of SFTSV.

RNA viruses usually have relatively high mutation rates, which when combined with natural selection, allow viruses to quickly adapt to changes in the host environment. We found that viral sequences from ticks, domestic animals, and local patients could be grouped into different sublineages. The lack of strict phylogenetic linkages among the viral genomic sequences detected in ticks, animals and human patients from the same geographic location may reflect the evolutionary dynamics of SFTSV, as is observed with other RNA viruses. In addition, the emergence and geographic dispersal of distinct phylogenetic lineages in the same geographic location may be enhanced by the movement of tick-infested animals, which may be introduced into new areas intentionally or through natural migratory activities.

In conclusion, our data provide important epidemiological evidence for SFTSV infection in ticks in an endemic area and suggest the possibility that transstadial transmission of SFTSV may occur among larvae, nymphs, and adults of *H. longicornis*and that vertical transovarial transmission of SFTSV may occur from infected adult female ticks to eggs and larvae. *H. longicornis*may act not only as the primary vector of SFTSV but also as a reservoir for this virus.

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4. Potential emerging respiratory infectious pathogens discovery based on national influenza surveillance network

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Summary:

Multiple viruses and bacteria could cause respiratory diseases. Though most respiratory infections were mild illness, some cases proceeded to severe pneumonia and resorted to hospitalization. Accurate pathogen identification of these severe cases is critical to determine the cause of these several pneumonia and provide target therapy.

Traditional diagnostic methods are mainly based on molecular amplification techniques such as PCR, which targeted to one or several currently known pathogens. Therefore, new viruses and variants might be missed. Next generation sequencing combined with high sensitive random amplification methods provided a powerful tool for novel virus identification and etiologicspectrum description.

In this study, we sampled nasopharyngeal swabs from 36 pneumonia cases with unknown pathogen, followed by 22 respiratory pathogens screening targetingFluA, FluB, H1N1v, RSVA, RSVB, PIV1-4, CorV-OC43, CorV-229E, CorV-NL63, CorV-HKU1,*Rhinovirus/Enterovirus*, *Adenovirus*, *human metapneumovirus*, *Boca virus I*, *chlamydia pneumonia*, *Mycoplasma bovis*, *Legionella pneumophila*, and *Bordetella pertussis*. After multiplex pathogens screening, samples negative to all the pathogens were subjected to deep sequencing.

1. Purpose:

Acute respiratoryinfections (ARI) are responsible for the most hospitalization of children below 5 years of age(1). Respiratory infections in these young children were prone to develop several case with high morbidity and mortality. Some emerging

pathogens, such as SARS and MERS virus, posed great threats to public health and also caused respiratory symptoms(2). Therefore, real-time surveillance of ARI is of great significance for potential epidemics detection.

Virus infection is the major cause of ARI. Besides virus, several bacteria, the *chlamydia pneumoniae*, *Mycoplasma bovis*, *Legionella pneumophila*, and *Bordetella pertussis*, can also lead respiratory disorders. In this study, we used the multiplex-pathogen panel to test thenasopharyngeal swabs from 36 pneumonia patients with unknown pathogen. For samples negative to all the targeted pathogens, we used deep sequencing to explore their etiological spectrums.

II Methods

Samples collection

Nasopharyngeal swabs were collected from pneumonia cases based on four criteria. First, Patients got a fever with body temperature no less than 38℃. Second, chest radiograph indicated pneumonia or acute respiratory distress syndrome. Third, patients possessed decreased or normal white blood cell counts, or with a decreased Lymphocyte count at the early stage of illness. Last, no improvement or exacerbation was observed for the patients after antibiotic treatment for 3~5 days. Base on these criteria, we got 36 samples from influenza surveillance networks in Guizhou provincefrom Feb, 2013 to Oct, 2014.

RNA Extraction

Total viral RNA was extracted from 500µl nasopharyngeal swab solution with RNeasy mini kit (QIAGEN Inc., Valencia, CA, and USA) according to the manufacturer's instructions and eluted in a final volume of 50 µl of RNease-free water.

Multiplex-pathogen identification

Extracted RNA was subjected to 22 pathogens screening with RespiFinder® SMART 22 FAST v2.0 kit (PathoFinder, Maastricht, Netherlands) targeted at FluA, FluB, H1N1v, RSVA, RSVB, PIV1-4, CorV-OC43, CorV-229E, CorV-NL63, CorV-HKU1, *Rhinovirus/Enterovirus*, *Adenovirus*, *human metapneumovirus*, *Boca virus I*, *chlamydia pneumoniae*, *Mycoplasma bovis*, *Legionella pneumophila*, *and Bordetella pertussis*. Briefly, 10ul RNA was pre-amplified with multiple primers and hybridized

with specific probes. After ligation and PCR in LightCycler® 480(Roche, Basel,Switzerland), melting curves were generated and pathogens were identified by matching the obtained Tm value to that of the specific pathogens in the kit manual.

Deep sequencing of multiplex-pathogens-free samples

In order to describe the full etiological spectrum, and identify potential new pathogens, we implemented deep sequencing and metagenomic analysis for the samples negative to all the tested viruses and bacteria. To begin with to test, the samples were filtered through a 0.22 μm filter (Ultrafree MC, Millipore). DNaseI and Benzonase was added to each aliquots, and samples were incubated for 2h at 37℃ to eliminate un-enveloped nucleic acid. RNA were extracted from treated samples and amplified with Ovation® RNA-Seq System V2 (NuGEN, San Carlos, CA, USA). The amplified products were sequenced on Ion Torrent PGM with a 200bp read length in 318V2 Ion chip.

Deep sequencing data analysis was conducted on CLC Genomics Workbench software version7.5.1. Briefly, raw reads were trimmed first to delete low-quality and short reads with length below 50bp. The trimmed reads were assembled to contigs with de novosplicing, and thenthe sequence similarity searches in the NCBI nucleotide database were performed using BLAST for highly similar sequences.

Results:

Among the 36 nasopharyngeal swabs tested for multiplex pathogens identification, 11 were*Rhinovirus/Enterovirus* positive, accounting for 30.6% of the total samples. The second most prevalent pathogens included RSVB, Flu and FluB, which were positive for three samples, respectively. Other virus, such as *Adenovirus*, *Mycoplasma pneumonia*, PIV3,PIV2,Boca virus, CorV OC43, CorV HKU1, CorV C229E, were also identified in one sample, respectively.15 samples were negative for all of the pathogens tested (Table 1).

Table 1. Identification of the pathogens in 36 nasopharyngeal swabs sampled from patients with pneumonia.

Targetpathogen	*Number of positive samples*

	Rhinoviurs/Enterovirus	11
	Respiratory Syncytial Virus B	3
	FluA	3
	FluB	3
	Adenovirus	1
	Mycoplasma.Pneumonia	1
	PIV3	1
	PIV2	1
	Boca	1
	Coronavirus OC43	1
	Coronavirus HKU1	1
	Coronavirus C229E	1
	Negative for all the targeted pathogens	15

We then selected five samples negative for all the pathogens for deep sequencing and metageunomic analysis. For one sample collect from a baby about 1 month old, we identified 1 million reads (38.9% of the total reads) aligned to measles virus. The whole genome of this measles virus could be extracted from reads mapping. Further analysis showed genome of this measles virus is 98% identical to Chinese strain.

For the remaining four samples, we failed to identify any virus pathogens. However, we identified many bacteria usually served as normal flora in oral in these samples. For example, the *Escherichia* and the *Streptococcus* genus, were present in all the four samples. Though these bacteria are nonpathogenic in healthy people, they may cause disease in immunity-suppressed person. The accurate role of these opportunistic bacteria playsin the pneumonia samples needs further exploration.

IV. Discussion:

Acute respiratory infection accounts for the most common acute illness in hospitalized patients (3). A lot of pathogens could lead respiratory illness, which hindered the diagnosis of the cause of the illness. We used a 22 pathogens panel to screen 36 nasopharyngeal swabs from unknownpneumonia, and at least one pathogen was identified in 21 samples. The remaining 15 samples was negative for all of the targetpathogen.

Traditional virus identification methods depended on prior knowledge of the targeted pathogens to design specific primers and probes, which might miss

emerging virus or variants. Deep sequencing associated with sensitive and unbiased amplification methods shed light to novel virus discovery and full etiological spectrum description (4, 5). Using this method, we successfully identified measles virus in one sample negative to all the pathogens in the panel. Though we choose RNA-seq as the deep sequencing strategy, we identified many bacteria in the samples, which may represent rRNA of these organisms.

Several limitation should be involved into this program. Firstly, more samples should be collected to characterize the “core” pathogens presented in the samples from pneumonia with unknown pathogen. Secondly, samples from healthy person should be included in parallel study. Comparison of the organisms between patients and health persons provide key hints for which agents may contribute to the illnessdevelopment. Thirdly, metagenomics analysis targets bacteria should be conducted to the virus-free samples.

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5. Study on the epidemiological and pathogenic characteristics of inpatients of severe acute respiratory infection with Streptococcus pneumoniae in China

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Summary:

China CDC established hospital-based severe acute respiratory infection (SARI)surveillance system in central China in 2010.We found low yield of blood cultures of SP (less than 1% positive) although several efforts had been done, including boost training of SOPs. We ended Sp surveillance in Jingzhou city in 2012 and carried out another surveillance program for S.pin Kunming city, Yunnan province in 2013. However, no positive results were found. Therefore, we selected another sentinel children hospital located in Chongqing to continue Sp surveillance among Acute Lower Respiratory Infection (ALRI) patients supported partially by Japan National Institute of Infectious Disease (NIID) fund. Testing method for Sp has been changed from the whole blood culture to isolation of nasopharyngeal aspirate. From Oct 2013 to Dec 2014, total 306 case-patients were enrolled and 12% (32/268) were positive for Sp.

I. Background

Under the financial support from Japan National Institute of Infectious Disease (NIID),China CDC had been carrying outone surveillance program in hospitalized patients with severe acute respiratory infection (SARI) caused by streptococcus pneumoniae (S.p) in Jingzhou city, Hubei province from 2010 to 2012 and then in Kunming city, Yunnan province in 2013. The blood culture had always been a big issue for its low yield since the beginning in both the two sites. We have not seen

obvious improvements on this issue. Therefore, we selected the third site in Chongqing to try to isolate Sp strain from nasopharyngeal aspirate among ALRI patients.

II. Purpose:

1. To understand the prevalence of Streptococcus pneumonia (S.p) infection among inpatients cases with ALRI in sentinel hospitals.
2. To figure out the pathogenic characteristics of S.p strain isolated from nasopharyngeal aspirate of inpatients with ALRI.
3. To address the epidemiological and clinical features of inpatients with ALRI caused by S.p infection.

II. Methods:

Setting and Patients Enrollment

The Affiliated Children Hospital of Chongqing Medical University was chosen after several rounds of consideration on capacities of surveillance and laboratory testing. A national surveillance protocol including the patients' enrollment, specimens collection and laboratory testing, case report form and information reporting, and related standard operation procedures (SOPs) were developed by China CDC.

Patients admitted to the wards or intensive care unit (ICU) of departments of internal medicine, pediatrics or infectious diseases in the sentinel hospital were screened by nurses and physicians for ALRI. A patient was defined as having ALRI if they had: (1) at least one of listed manifestation of acute infection: measured fever (≥38 ˚C), abnormal white blood cell (WBC) differential, leukocytosis (a WBC count increased to over 10,000/□L) or leukopenia (a WBC count decreased to less than 4,000/μL), and chill; (2) at least one of listed respiratory tract illness: cough, sputum, shortness of breath, lung auscultation abnormality (rale or wheeze), tachypnea, and chest pain. Among the ALRI patients, those with a chest radiograph demonstrating punctate, patchy or uniform density shadow were defined as having pneumonia.

Specimen Collection and Testing

Each week or month, the first 2-5 ALRI patients were enrolled for specimen collection. After hospital admission, physicians obtained verbal consent from parent/guardians of eligible ALRI case-patients and then nasopharyngeal aspirate were collected and tested for several pathogens, including Sp.

III. Results: (if necessary, figures and tables will be included)

During the period from October, 2013 to December, 2014, we have seen that total 306 hospitalized patients meeting the ALRI case definition were enrolled after informed consent.

1. Epi curve of enrolled SARI patents

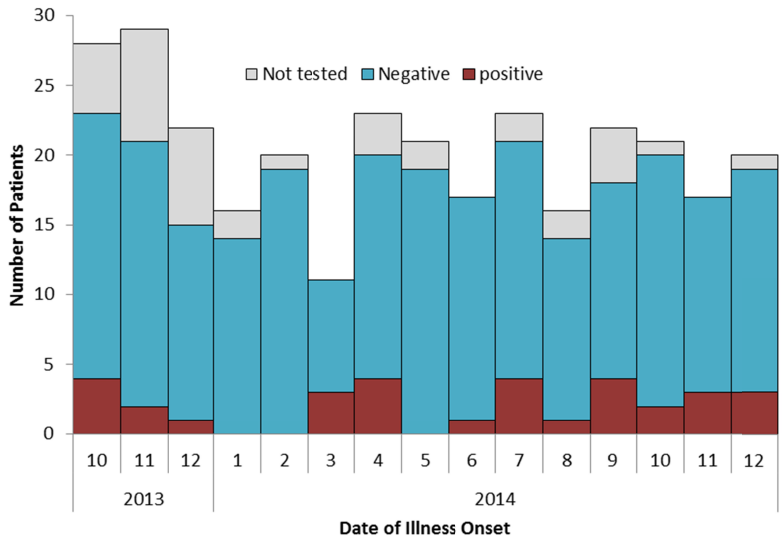

Figure 1. Epidemiological curve of enrolled ALRI inpatients by month

2. Epidemiological and clinical features:

1) Demographic characteristics

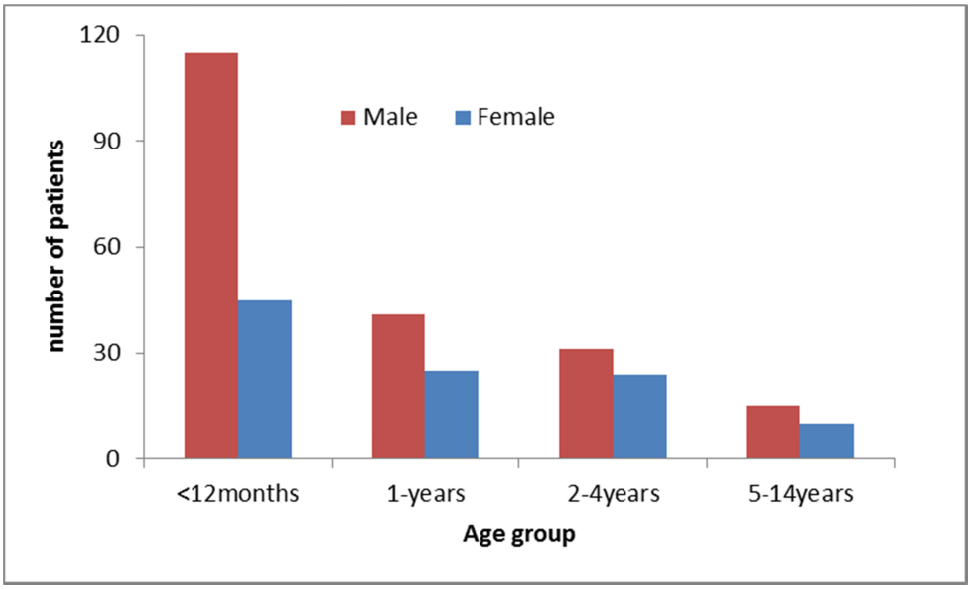

Figure 2.Age and gender distribution of enrolled ALRI inpatients

2) Clinical symptoms and Signs:

- As for body temperature, 6% (19) in children cases were admitted to hospital with fever ≥38□.
- Top four common clinical symptoms were: cough 97% (298), sputum production 78% (238), diarrhea29% (89), and breath difficulty 21% (64).
- Among 270 children cases with chest X-ray check,256 (95%) presented abnormal image.
- Only 20 (7%) children cases clearly report not to take antibiotics within one week before specimen collection.

3. Laboratory testing results:

Of those 306 enrolled children ALRI patients, nasopharyngeal aspirate have been collected from 268 (88%) cases.32(12%) samples had been cultured positive for S.p.

Table 1.Age and gender distribution of enrolled ALRI inpatients positive for Sp

Age group	Male	Female	Total
<12months	7	4	11
1-years	5	6	11
2-4years	6	3	9
5-14years	0	1	1
Total	18	14	32

IV. Discussion:

It has been estimated that 5% of all children deaths were attributed to *Streptococcus pneumoniae* (S.p). No good data for S.p prevalence in China at community level is available till now. China CDC has been trying to set up a continuous, high quality S.p surveillance system, which is needed to characterize the burden, distribution, trends, clinical outcomes, and subtypes of S.p.

Experience of Jingzhou and Kunming told us that whole blood culture for Sp would be a big challenge. On the one hand, the wide self-usage of antibiotics should be one important reason for such low culture positive rate. On the other hand, the standard operation procedure of blood draw, timely transfer to lab, and whole blood culture might not be followed well. In Chongqing sentinel hospital, we choose to culture Sp from nasopharyngeal aspirate. Advantage was to improve positive rate and disadvantage was hard to tell that ALRI was cause by Sp.

Of those 306 enrolled ALRI patients, we did not see obvious peak and found that children younger than 1 year old accounted for more than 50%. As results showed that 32samples were positive for Sp, we also did not find activity seasonality and Sp seemed to be active around the year. Also, most of those positive were mainly distributed in young children aged <2 years old. This suggests that the youngest children are at high risk for developing ALRI and being infected with Sp.

We would like to revise the protocol to call for conduct more sensitive PCR test for sera or SCF collection of blood in a well-defined subset of ALRI patients in whom bacterial pneumonia is more likely. Shortage of PCR test is that we can identify its sera subtype but S.p isolate could not be available. The real distribution of S.p in invasive pneumococcal diseases is helpful to determine whether the PCV7 or future PCV13 can works well in China.