groups between 15 and 49 years old, the detection rate was relatively high, with DEC detected more frequently.

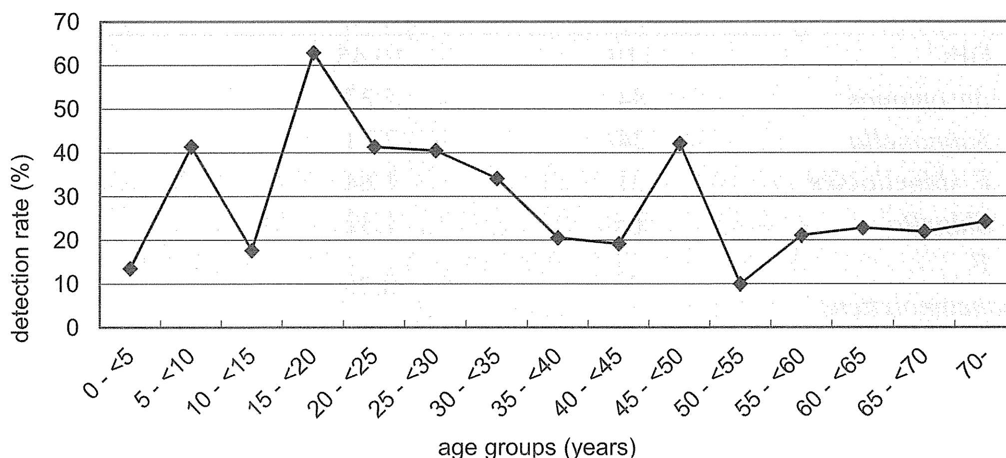


Figure 2. The detection rate of target bacteria in age groups.

The detection rate was 22.8% for male and 23.7% for female. There was no obvious gender difference in the detection rates of DEC, *Aeromonas*, and *Salmonella.* The detection rate of *P. shigelloides* in male was twice higher than that in female, while *Shigella* in female was 7 times higher than that in male.

The detection rate varied with occupation, the highest was 36.2% for students and the lowest was 13.9% for children.

4) PFGE of the isolates

All strains were analyzed by PFGE. For the 119 DEC, 84 *Aeromonas*, 24 *Salmonella* and 21 *P. shigelloides* isolates, the numbers of clusters with the same patterns were 9, 9, 4 and 1 respectively (Fig. 3-6). No *Shigella* isolates had the same pattern.

P. shigelloides and *Aeromonas* are not the strict diarrheal pathogens, it may raise the question whether the common exposure exist since the strains with the same PFGE patterns occurred in the patients.

Clusters	PFGE patterns	Strain codes

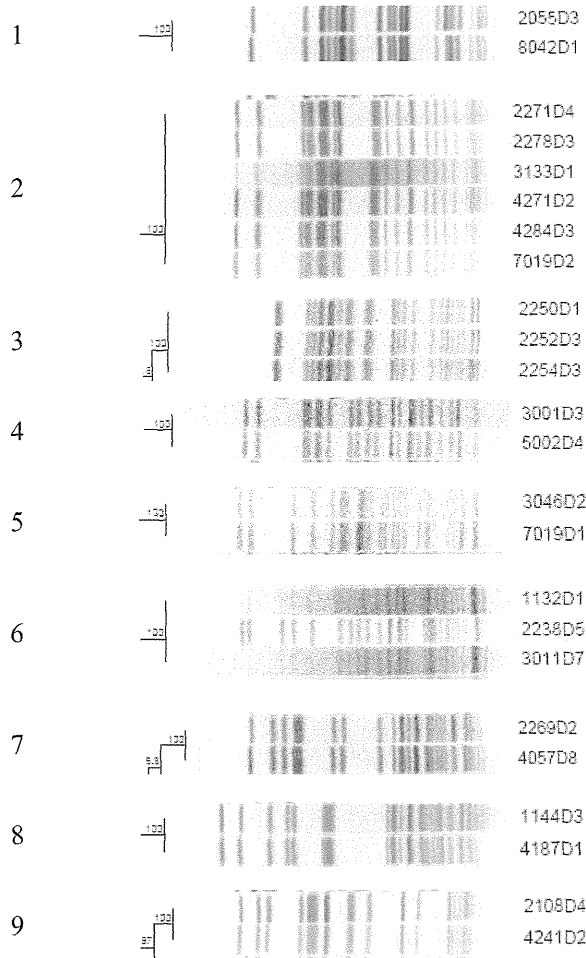
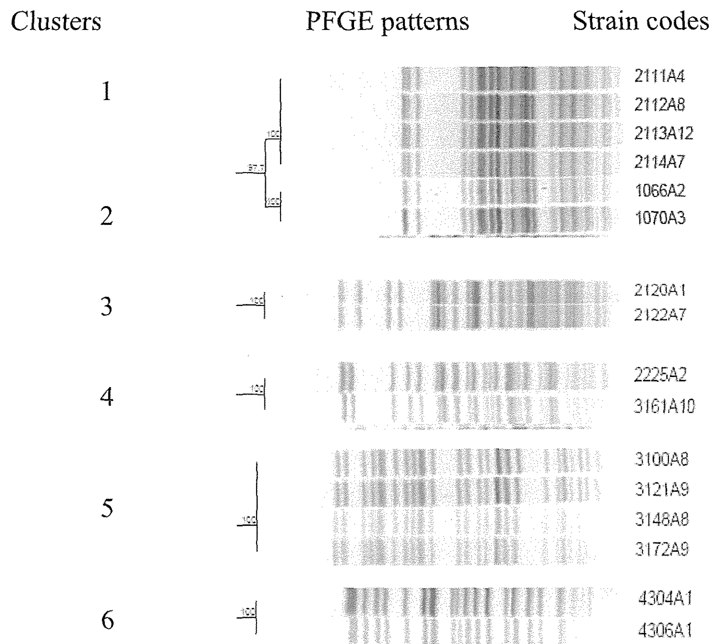


Figure 3. PFGE cluster patterns of the DEC strains.



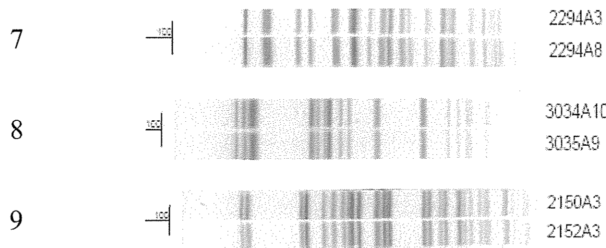


Figure 4. PFGE cluster patterns of the *Aeromonas* strains.

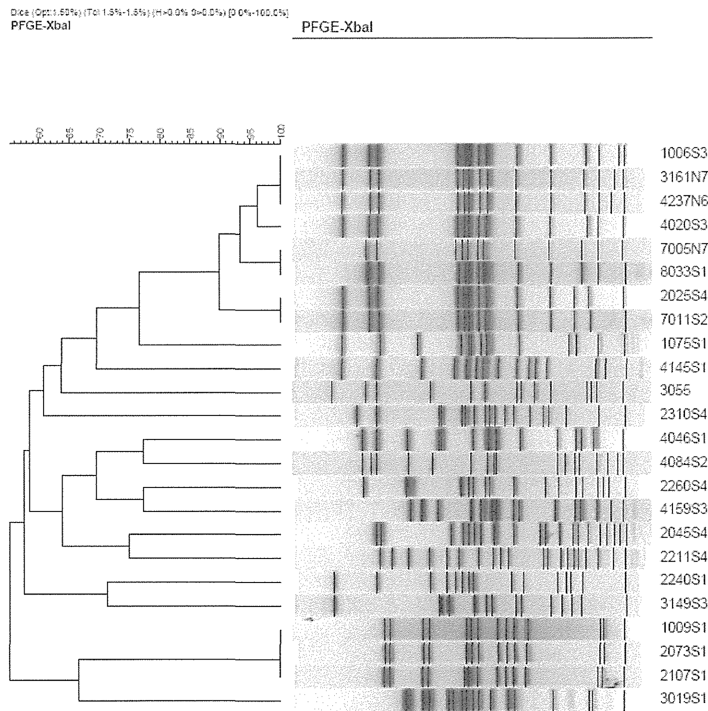


Figure 5. PFGE patterns of the *Salmonella* isolates. Four patterns were shown by more than one isolate.

PFGE patterns Strain codes

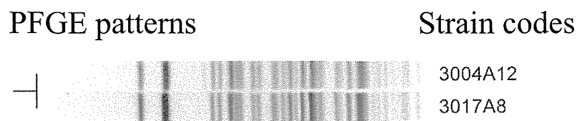


Figure 6. PFGE cluster pattern of the *P. shigelloides* isolates

IV. Discussion:

Laboratory data may provide more details for the surveillance and outbreak detection in the control of diarrheal disease. PulseNet China, the molecular subtyping network within the international network, is working on the laboratory surveillance of

the infectious diseases including diarrheal diseases. Since lots of pathogens may cause diarrhea, a high efficient identification of etiology of the diarrheal patients is needed. The important but difficult step in the laboratory surveillance is to obtain the pathogen from the diarrheal patients. Normally the isolation of bacterial pathogens is conducted in the laboratories of the hospitals and city level CDCs in China. Based on the support of this and other projects, we performed a one-year survey of bacterial pathogens from the diarrheal patients in Yuxi city CDC laboratory. One purpose of the survey is to form the procedure and protocol on the isolation of multiple bacterial species related to diarrhea.

From the survey data in Yuxi city, the diarrheal pathogens mostly often isolated from the diarrheal patients is DEC, followed by *Aeromonas* and *Salmonella.* The situation was quite different with some reports. For example, in the study in Beijing from April 2010 to December 2011 [1], *Shigella*, *V. parahaemolyticus* and *Salmonella* were the common pathogens, the DEC ranked the forth, whereas in Yuxi study the DEC was the most common and *V. parahaemolyticus* was rare. A survey in Henan province in the whole year of 2007 showed the similar rank with Yuxi study, DEC, *Shigella* and *Salmonella* were the top three bacterial pathogens [2]. The social and economic conditions, the component of food, and the sanitary conditions of food consumption might be the reasons for the difference of the pathogens.

The detection rate varied with season. In Dec and Jan, the number of diarrheal cases in the sentinel hospitals reached the highest level while the detection rate fell to the lowest level. Diarrheal diseases in the winter may be caused by reasons other than bacterial infections.

Within this study, *Aeromonas* and *Plesiomonas* are also included in the target bacteria list in the isolation. It's interesting the positive rates were so high within the patient samples. Normally it is difficult to identify the role of these bacteria in the development of human diarrhea. In this study we tried to find the possible evidence based on the PFGE subtyping, which means, if a cluster with the same PFGE pattern were found in some patients, the outbreak possibility may arise, since different patients have the same *Aeromonas* or *Plesiomonas* strains in their stool. The problem

in this study is that the epidemiological investigations were not conducted immediately, although some cases had the same patterns of these bacteria, no epidemiological data could suggest or support the common exposure of these patients.

PFGE was also performed, and the cluster characters of these bacterial isolates were obtained. Some clusters with the same patterns were found, since there's no PFGE instruments in Yuxi CDC laboratory, the isolates were transferred to Yunan provincial CDC and national CDC laboratories to do PFGE, the time was too long to get the results. Once the epidemiological investigation team joins the study, the isolates should be transferred to the provincial CDC laboratory against time to do the subtyping.

A protocol for bacterial pathogen isolation was optimized, including the real-time PCR detection for DEC strains. The protocol will be more satisfactory if a new technique is introduced. To distinguish DEC, several target genes need to be detected, and at least 5 clones should be screened on the selection culture plate. The work is tedious and laborious. To improve the detection efficiency, a nucleic acid based assay with high sensitivity and high throughput is in urgent need. Since DNA has been extracted from some fecal and swab samples, we will evaluate the application of high throughput assays in the detection of DEC.

As a collaboration project, 30 non-O1/non-O139 *V. cholerae* strains from the environmental samples and diarrheal patients, and 10 *V. fluvialis* strains form diarrheal patients have been transferred to NIID laboratory for the serotype identification and further studies, to promote the study on the uncommon diarrheal pathogens.

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

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Name and official title of PI: Dexin Li, MD, Prof., Director of National Institute for Viral Disease Control and Prevention, China CDC

Summary:

To investigate severe fever with thrombocytopenia syndrome virus (SFTSV) in ticks in the endemic region of China, a total of 3,145 ticks were collected from animals (71.6%), and grazing fields (28.4%) in an endemic region of Shandong province, China, in 2011. Of these, the majority was *H. longicornis* (96.9%), the other species were *R. microplus* (2.6%), *H. campanulata* (0.3%), and *D. sinicus* (0.2%), which included both adults (59.4%), and the immature ticks (40.6%). All ticks were assessed for SFTSV RNA, 4% of *H. longicornis* and 4.8% of the adult *R. microplus* were tested SFTSV RNA positive. Viral RNA was identified from all developmental stage of *H. longicornis* including adult, nymphal, larval, and eggs. Sequences analysis revealed that tick-derived sequences shared high degree of homology with human-, and animal-derived viral isolates. These findings may extend the understanding of SFTSV natural maintenance and transmission.

I. Purpose:

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

II. Methods:

Tick collection and processing.

Ticks were collected at Yantai region, a SFTSV endemic region of Shandong

province (119°34′–121°57′E, 36°16′–38°23′N) (Figure 1), where SFTS human cases had been reported since 2010, SFTSV infection among domesticated animals had been studied in 2011 [9].

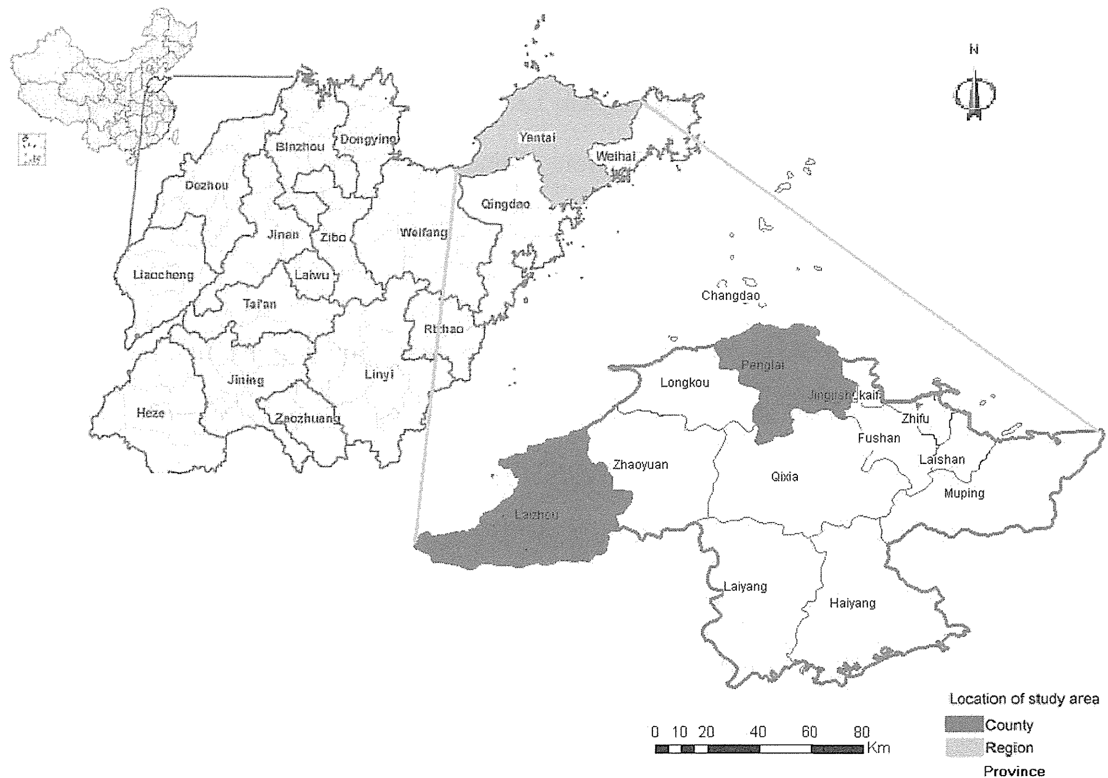

Figure 1 *Map of Yantai region indicating location of sampling areas.*

Maps show the location of Yantai region in Shandong Province, China, where an epidemiological investigation of SFTSV infection in domesticated animals had ever been performed in the study area in 2011. Map at bottom shows the sampling areas at Yantai region (red shading), map in the middle indicates Yantai region in Shandong Province, map at left and up shows Shandong Province in China.

Tick collection methods included dragging and collection from animals. Dragging was performed at the area near farmland and land used for grazing. Collection of adult ticks from livestock (cattle, sheep and dog) was done with the

permission of the owners, and care was taken to minimize discomfort for the animals. At the time of collection, ticks were placed in collecting tubes, and labels identifying the date, location, and host (or collection site) were added to the vials, and kept alive at room temperature for one week for depletion of blood and collection of eggs from adult female ticks, then store at -80□. Ticks were transported to National Institute for Viral Diseases Control and Prevention, where they were identified and separated into pools based on species, host, collection site, life stage. Ticks were grouped into pools adult ticks was one, five to eight for nymphs, and 10 for larvae, eggs were separated according to the adult female mother ticks.

RNA extraction and virus detection.

Ticks were surface sterilized with sequential washes with DMEM medium containing antibiotics for 2 times. Tick pools were homogenized in chilled 500 μl DMEM medium by TissueLyser (QIAGEN, Germany). The tick homogenates were poured into 1.5 mL microtubes and centrifuged at 4°C and 10,000 RPM for 1 min with an Eppendorf centrifuge 5415R. A 140 μL aliquot of the clarified supernatant was transferred to an identically labeled 1.5 mL microfuge tube for RNA extraction using RNeasy mini kit (QIAGEN, Germany) according to the manufacturer's protocol. Quantitative real-time RT-PCR (qRT-PCR) was performed by adding 5 μL of each extracted RNA using a CFDA approved qRT-PCR kit (DaAn Gene, Guangzhou, China) following the manufacturer's instruction with primer and probe as published before[12]. The detection limit for this kit is 10 copies/μl, the result was considered positive if a sample had a *Ct* value below the cut-off. Viral RNA copy numbers were determined from amplification of a standard curve of positive control RNA. The infection in ticks was recorded as the minimum infection rate (MIR; MIR = [No. of positive pools/Total no. of ticks tested] × 100%).

Sequencing and phylogenetic analyses.

To generate nucleotide sequence data for phylogenetic analyses, RT-PCR amplification of the S segment of SFTSV was conducted on positive pools using primers as described previously[1]. All amplicons were sequenced using ABI BigDye Terminator V3.1 ready reaction cycle sequencing mix (Applied Biosystems, Carlsbad, CA) at Genewiz service company (Genewiz, Beijing, China) Nucleotide sequences were assembled using SeqMan software (DNASTAR) and visual inspection. Alignments were conducted using ClustalW (MEGA 5). Phylogenetic analyses were then conducted on these tick-derived whole S segment along with those of human- and animal-derived sequence using the neighbor-joining method with 1,000 replicates for bootstrap testing with MEGA 5 software[13].

III.Results:

A total of 3,145 ticks were collected at Laizhou and Penglai Counties of Yantai region, Shandong Province, China (Figure 1). Of these, 70.8% of ticks were recovered feeding on cattle, sheep and dogs, and 29.2% were collected during dragging, *H. longicornis* were the dominant species (96.9%). Of the ticks collected, 59.4% were adult ticks of *H. longicornis* (n = 1803), *R. microplus* (n = 50), *H. campanulata* (n =9), and *D. sinicus* (n = 5), and the remaining 40.6% were immature forms of either *H. longicornis* (n = 1,245) or *R. microplus* (n = 33). 86.1% of immature forms were at stage of nymphae (n = 1100), 12.2% were larval (n = 156), and 22 pools of eggs were harvested from 22 female adult ticks of *H. longicornis* recovered from sheep.

Total RNA were extracted from 2044 tick pools for real-time RT-PCR detection. 126 pools of ticks was detected SFTSV RNA positive, of which 107 pools were out of 2,251 ticks recovered from animals and 19 pools were from 894 field ticks. 97% of

the SFTSV positive ticks belonged to *H. longicornis*, 122 pools of 3,048 *H. longicornis* were detected positive, the remaining SFTSV RNA positive 4 pools were tested from 83 *R. microplus* pools (Table 1). The MIR was 4% and 4.8% respectively.

Out of the 126 SFTSV positive tick pools, positive pools of *H. longicornis* (n = 122), and *R. microplus* (n = 4), were identified from sheep (n = 91), cattle (n = 5), dog (n = 11), and dragging (n = 19, Table 2). The minimum infection rate (MIR) of SFTSV in *H. longicornis* collected from sheep and dog is higher than from cattle and dragging (Table1, table 2). SFTSV RNA was detected from both adult ticks and the pools of immature ticks of *H. longicornis*, of which 71 were from 1803 adult ticks, 45 pools from 1067 nymphs and 4 pools from 156 larvae (Table 1). Of the 22 female adult *H. longicornis* that laid eggs, 3 were detected SFTSV RNA positive, while 2 pools of eggs were detected positive (Table 1). Virus isolation was attempted for all viral RNA positive tick samples, no viable virus was obtained, however, we have ever obtained an isolates from one of 140 *H. longicornis* tick pools (1/140) collected at this area in 2010.

Table 1. Results of real-time RT-PCR for detection of SFTS virus RNA in different developmental stage of ticks collected from Yantai of Shandong province, China

Tick species	Developmental stage	No. positive/no. examined ticks	Minimum Infection Rate (%)
H. longicornis		122/3048	4
	Adult	71/1803	3.94
	Nymphal	45/1067	4.22
	Larval	4/156	2.56
	Eggs	2/22	9.09
R. microplus		4/88	4.82
	Adult	4/50	8
	Nymphae	0/33	0
	Larval	-	-
	Eggs	-	-
H.	Adult	0/9	0

campanulata			
D. sinicus	Adult	0/5	0

Table 2. Results of real-time RT-PCR for detection of SFTS virus RNA in ticks collected from Yantai of Shandong province, China

Tick species	Source	No. positive/no. examined	Minimum Infection Rate (%)
H. longicornis	Sheep	91/1707	5.33 (
	Cattle	4/215	1.86
	Dog	8/161	4.97
	Chicken	0/61	0
	Hedgehog	0/10	0
	Dragging	19/894	2.13
R. microplus	Sheep	0/11	0
	Cattle	1/39	2.56
	Dog	3/33	9.09
H. campanulata	Hedgehog	0/9	0
D. sinicus	Hedgehog	0/5	0

The S segment of SFTSV were amplified and sequenced from 11 of the RT-PCR positive tick pools. Phylogenetic analysis was performed compared with local SFTS patient- and animal-derived SFTSV isolates in the study areas as well as previous published sequences of viral isolates from SFTS patients in other province of China. Phylogenetic analyses revealed that these tick-derived sequences shared 95.6% to 99.9% nucleotide identity with each other, and a high level of homology with those patient- and animal-derived isolates from both the same area and other provinces. Neighbor-joining (NJ) trees were constructed based on the full S segment sequences obtained in this study as well as representative sequences retrieved from GenBank. It demonstrated that local patient-, dog- and cattle-derived viral sequences were clustered together with some of these tick-derived sequences, but these sequences

were grouped into different sublineages regardless of hosts and geographical location.

Figure 2 *Phylogenetic tree based upon S segment sequences for representative viruses derived from humans, animals and ticks inferred by the neighbor joining method using MEGA 5. Tree was drawn to scale with branch lengths representing the number of nucleotide substitutions per site. Dots indicate the patient-derived sequences amplified in 2010; triangles indicate the sequences amplified from ticks in this study; diamonds indicate the sequences isolated from patients; squares indicate the sequences isolated from animals in 2011 from the same study area.*

V. Discussion

We tested and analyses ticks that were collected during our epidemiologic

investigation of SFTSV infection among domesticated animals from Yantai region, Shandong province, China in 2011[9]. SFTSV RNA was detected in ticks at different developmental stages from larvae to nymphs and adults as well as eggs, and the relationship of SFTSV infection among ticks, animals and human was demonstrated via phylogenetic analysis.

Monitoring directly for virus among vectors, animals and patients provides valuable information as to the link of SFTSV transmission from hosts, vectors to humans. Molecular methods (reverse transcription-PCR) were be used to detect viruses in the tick samples. SFTSV RNA was detected from *H. longicornis* and *R. microplus,* which were the two most frequently encountered ticks, while the majority was *H. longicornis*. No SFTSV RNA was detected from *H. campanulata* and *D. sinicus,* which may due to very few ticks collected in this study. The MIR of SFTSV in *H. longicornis* and *R. microplus* was 4% and 4.8%respectively, which may contribute to the transmission of the virus to humans and animals in the region. And the presence of SFTSV RNA in ticks collected from animals and by dragging, which suggested the circulation of SFTSV among ticks. Especially, SFTSV RNA was detected in all four life stages of *H. longicornis,* from eggs, larvae to nymphs and adults, which suggested that SFTSV might be transmitted transstadially (from larvae to nymphs and adults) and transovarially in *H. longicornis* [10, 11]. Although viable virus was not recovered from these tick pools in this study, we have previously obtained an isolates from one of 140 *H. longicornis* tick pools (1/140) collected at this area in 2010, which possessed a similar genetic and identical serological characters to human- and animal-derived isolates[6]. The inability to recover a viable SFTSV by using cell culture cultivation followed by reverse transcription-PCR cannot be construed as evidence of its absence, which might due to the extremely low titers of

virus in the field ticks as indicated by the low RNA copies numbers in the RT-PCR detection, or more sensitive methods for cultivation of SFTSV from field ticks samples should be used.

Like other tick-borne viral diseases, the prevalence of SFTSV in ticks should be a suitable marker for risk analysis in natural foci, but cannot be directly translated into a risk for the population, since the risk of human infection is the product of the number of infected ticks, which is a product of tick abundance and pathogen infection prevalence, and contact rate between the infected ticks and humans[14, 15]. The distribution of SFTSV cases is therefore shaped by the distribution of the human population and the behavior that favours human-tick contact. We have reported that a high seroprevalence of SFTSV infection were detected in sheep (69.5%), cattle (60.4%), dogs (37.9%), and chickens (47.4%), and human cases were continued reported since the identification of SFTSV in this study area[9]. It is rational to conclude that SFTSV is likely to be far more widespread than revealed by human cases.

RNA viruses usually have relatively high mutation rates, combined with natural selection, which allows viruses to quickly adapt to changes in their host environment. The progeny produced by subsequent replications are expected to be quasispecies, a large group of genotypes containing one or more mutations relative to the parent. Genetic methodology can be used to differentiate different lineages of virus found within different animal hosts. Phylogenetic analysis revealed that these tick-derived sequences shared 95.6% to 99.9% nucleotide identity with each other. Compared to those previously published sheep-, cattle- dog- and patient-derived SFTSV sequences from the same or different geographic location, all sequence were closely related to each other. It was found that three tick-derived sequences were clustered together

with several local patient- and animal-derived viral sequences, but both tick- and local patient-derived sequences could be grouped into different sublineages. The lack of strict phylogenetic linking among the viral genomic sequences detected in ticks, animals and patients from the same geographic location, it may reflect the evolution dynamics of SFTSV, which might replicate as quasispecies in the vectors and animal hosts like other RNA virus.

In summary, our study evidenced that SFTSV was circulating among ticks and the virus might be transmitted transstadially and transovarially in *H. longicornis* in this study, which may extend the understanding of SFTSV natural maintenance and transmission.

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

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Summary:

A serial of viral pathogens can cause respiratory infection, such as influenza, parainfluenza, RSV etc. Recently, more and more new pathogens associated with respiratory infection diseases have been discovered. However, it is widely believed that many pathogens related with respiratory infection remain still unidentified. Hereby, we propose to discover new respiratory infection pathogens using the methodology of multiple pathogens detection combine with virus isolation and solid high through sequencing.

In mainland China, a national wide influenza surveillance network network including 411 influenza laboratories and 556 sentinel hospitals have been established and operated smoothly. Specimens from influenza like illness patients were collected by sentinel hospitals every week. The ILI samples will be tested by multiple pathogens detection method that is able to distinguish 15 kinds of respiratory RNA and DNA pathogens with Seeplex RV15 ACE kit (Seegene, Inc. Korea). The 15 virus include FluA、FluB、RSV A、RSV B、Adv、MPV、CoV229E/NL63、CoVOC43、PIV1、PIV2、PIV3、PIV4、HRV、HEV and HBoV, with internal control(IC).

I.Purpose:

Respiratory tract infections (RTI) are among the most common infectious diseases of worldwide, causing significant morbidity and mortality. They are consequently responsible for an enormous economic burden on society in terms of visits to doctors,

treatments, hospitalizations and absences from work and school. Upper respiratory viral infections are frequent, with approximately 6-9 infections per year in children and 2-4 infections per year in adults [1]. The actual cost of upper RTI (URTI) is difficult to determine, but included are factors such as over-prescription of antibiotics or absence from work. Lower RTIs (LRTI) are less frequent than upper RTIs (URTI), but between 1 and 13% of patients with LRTI are hospitalized. The cost of these infections is higher [2].

Viral infection is the major cause of respiratory tract diseases affecting individuals of all age, and represents a major public health problem [3]. A serial of viral pathogens can cause respiratory infection, such as influenza, parainfluenza, RSV, adenovirus etc. Recently, more and more new pathogens associated with respiratory infection diseases have been discovered [4]. However, it is widely believed that many pathogens related with respiratory infection remain still unidentified. To prevent and control the respiratory infection, this study will investigated the distribution of respiratory virus, and try to find involvement of novel virus in influenza like illness in mainland China.

II.Methods:

Collection of clinical specimen

In mainland China, a national wide influenza surveillance network network including 411 influenza laboratories and 556 sentinel hospitals have been established and operated smoothly. In this study, based on Chinese influenza surveillance net-work, we collected swab samples by net-work from patient of influenza-like illness (ILI). At present, 202 swabs were taken from the networking and involved into investigation of generally respiratory viruses. The samples were collected from the sentinel hospitals of influenza surveillance network, and they are all the throat swabs. In the incidence of patients within 3 days， throat swabs were collected from the individuals who had influenza symptoms.

RNA Extraction