Report 2013

STUDY GENERAL TITLE

Laboratory-based collaboration net work of infectious diseases in Asia

PI general from CCDC: Xiao-Ping Dong, PhD & MD

Prof., Director of Division of Science and Technology, China CDC; Deputy Director of State Key Laboratory for Infectious Disease Control and Prevention, China CDC; Director, Dept. of Prion disease, National Institute for Viral Disease Control and Prevention, China CDC

1. Epidemiology of HFMD and genetic characterization of HEV71and CVA16

Study faculty: Institute for Viral Disease Control and Prevention, China CDC, Changbai road 155, Changping Qu, Beijing 102206, China

Name and official title of PI: Wenbo Xu, Prof., Assistant Director of National Institute for Viral Disease Control and Prevention in China CDC

1. Epidemiology

1.1. Profile of the outbreak in 2013

Totally, 1,828,377 cases were reported throughout year 2013, including 252 fatal cases. The number of reporting cases in year 2013 decreased compared with that in 2012 (2,170,248), and increased compared with that in 2011 (1,620,430). However, the number of fatal cases decreased dramatically, which is 509 in 2011, and 563 in 2012.

1.2. Age distribution

Cases were reported among all of the age groups, ranging from 0 to 85 years old. Young children less than 6 years old are still the majority suffering from HFMD, which account for 95% of the reporting cases. Young children especially 1-year-old group indicated highest incidence and fatality.

1.3. Spatial distribution

In 2013, there were cases reported from all over China. The top 6 provinces of reporting cases are Guangdong, Guangxi, Zhejiang, Hunan, Jiangsu, and Anhui, reporting cases of which account for 53% of national reporting cases. By fatal cases, Sichuan, Anhui, Hebei, Chongqing, Hunan, and Yunnan were the top 6 provinces. By the incidence, Hainan was still the top 1, followed by Guangxi, Guangdong, Fujian, Zhejiang, and Shanghai provinces.

1.4. Week distribution (Figure 1)

HFMD cases were reported throughout the year in 2013. The case number began to increase from week 8, and reached the peak in week 22. The first, also

the largest, round epidemics were lasting from week 10-34, followed by the second round epidemics in autumn, from week 13-33. The amount of cases and fatalities reported during the first round epidemic account for 61% and 72% of the cases in 2013 respectively. The second epidemic peak occurred from week 36-41, including 13% reporting cases and 7% fatalities.

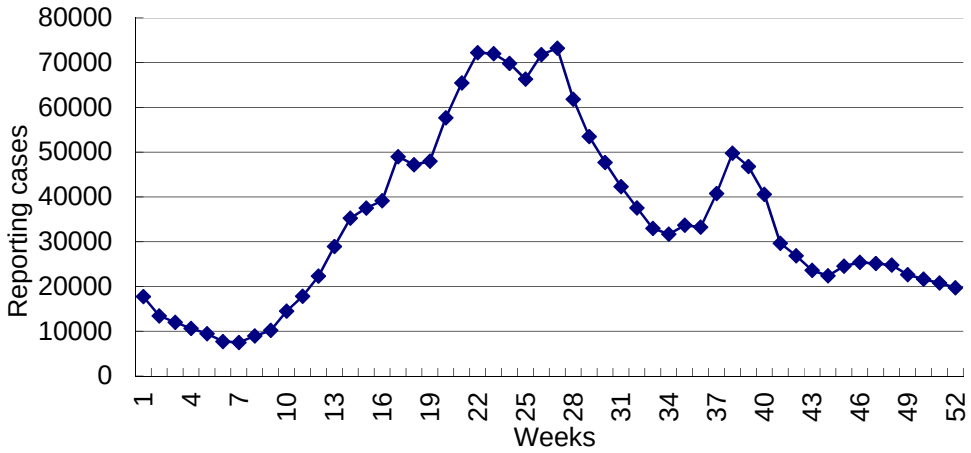


Figure 1. The reporting cases by week from Jan 1, 2013-Dec, 31, 2013

2. Pathogenic surveillance

2.1. Pathogenic spectrum (Figure 2)

In 2013, totally 87,454 cases, 4.8% of the reporting clinical cases, were confirmed by lab. The lab result indicated that other EV and EV71became the most major pathogen of HFMD in China, account for 48% and 37% of the positive respectively. EV71 is the dominant serotype during week 12-22. From week 22-49, other EV became the majority instead of EV71.

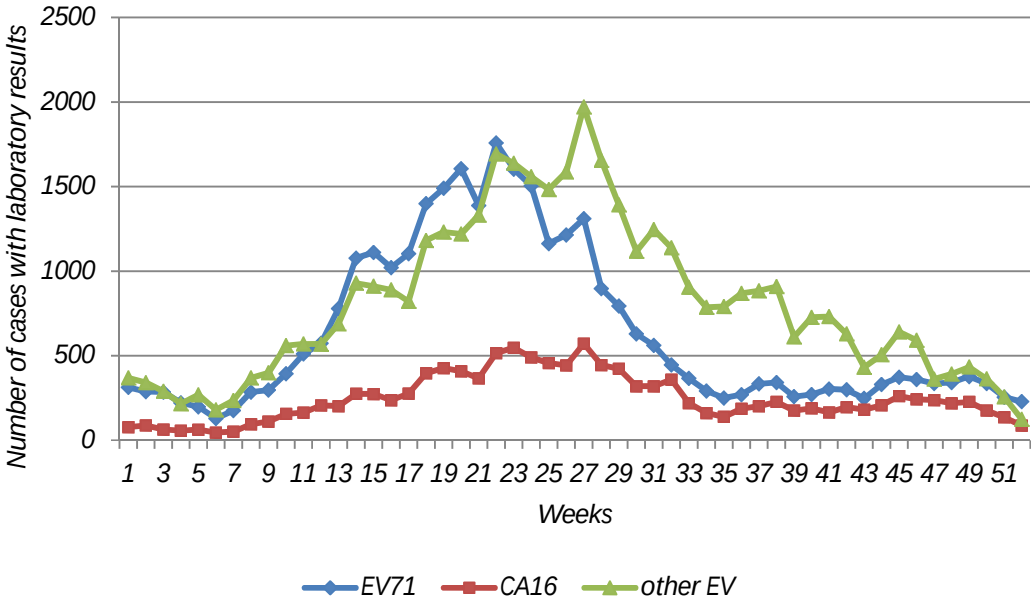


Figure 2. Pathogenic results in year 2013

2.2. Molecular epidemiology of EV71 and CA16

Phylogenetic trees of the EV71 and CA16 isolates in 2013 were reconstructed respectively, based on VP1 encoding region (891bp), with Kimura 2-parameter nucleotide substitution model. All the EV71 isolates in 2013 clustered together, showing the nearest phylogenetic relationship with subgenotype C4a, which was predominant in mainland China since 2004. For CA16, clade B1a and B1b still co-circulated in China in 2013. No predominance was found in specific provinces for both B1a and B1b.

2.3. Genomic characteristics

To clarify the genetic characteristics and the epidemic patterns of CVA16 in mainland China, comprehensive bioinformatics analyses were performed by using 35 CVA16 whole genome sequences from 1998 to 2011, 593 complete CVA16 VP1 sequences from 1981 to 2011, and prototype strains of human enterovirus species A (EV-A). Analysis based on complete VP1 sequences revealed that clade B1a and B1b were prevalent strains and have been co-circulating in many Asian countries since 2000, especially in mainland China for at least 13 years. While the prevalence of clade B1c (totally 20 strains) was much limited, only found in Malaysia from 2005 to 2007 and in France in 2010.

Genotype B2 only caused epidemic in Japan and Malaysia from 1981 to 2000. Both clade B1a and B1b were potential recombinant viruses containing sequences from other EV-A donors in the 5'-untranslated region and P2, P3 non-structural protein encoding regions.

1. Epidemiology of HFMD and genetic characterization of HEV71and CVA16

Study faculty: Institute for Viral Disease Control and Prevention, China CDC, Changbai road 155, Changping Qu, Beijing 102206, China

Name and official title of PI: Wenbo Xu, Prof., Assistant Director of National Institute for Viral Disease Control and Prevention in China CDC

3. Epidemiology

3.1. Profile of the outbreak in 2012

The nationwide epidemics of HFMD were continuing in China in 2012. Totally, 2,170,248 cases were reported throughout the whole year, including 20,949 severe cases, and 563 fatal cases (Table 1). The case number of all three types increased compared with that in 2011. However, the fatality rate were decreasing year by years, as showed in table 1.

Table 1. Summary of the outbreak during 2011-2012.

Items	2011	2012	2011 vs 2010	2012 vs 2011
Reporting cases	1,620,430	2,170,248	-9%	+34%
Severe cases	18,717	20,949	-33%	+12%
Fatal cases	509	563	-44%	+11%

3.2. Age distribution

Cases were reported among all of the age groups, ranging from 0 to 85 years old, but ~94% of the cases were young children less than 5 years old, which is consistent with the previous data in China. Furthermore, the amount of both reporting cases and fatal cases were indicated to be the largest in 1-year-old group, accounting for ~30% and ~47% of the all reporting cases and the fatal cases respectively. Young children especially 1-year-old group was showed to have higher risk for HFMD.

3.3. Spatial distribution

In 2012, the southeast of mainland China was still the most severe area for HFMD epidemic. By both the reporting cases and fatal cases, Guangdong, Guangxi, Hunan, Zhejiang, and Jiangsu were the top 5 provinces. However, by the incidence, Hainan was the top 1, followed by Guangxi, Guangdong, Hunan, and Zhejiang provinces.

3.4. Temporal distribution (figure 2)

HFMD cases were reported throughout the year in 2012. The case number began to increase from week ~10, and reached the peak in week ~21. The first, also the largest, round epidemics were lasting from week 10-34, followed by the second round epidemics in autumn, from week 35-50. The amount of cases and fatalities reported during the first round epidemic account for ~50% and 53% of the cases in 2012 respectively.

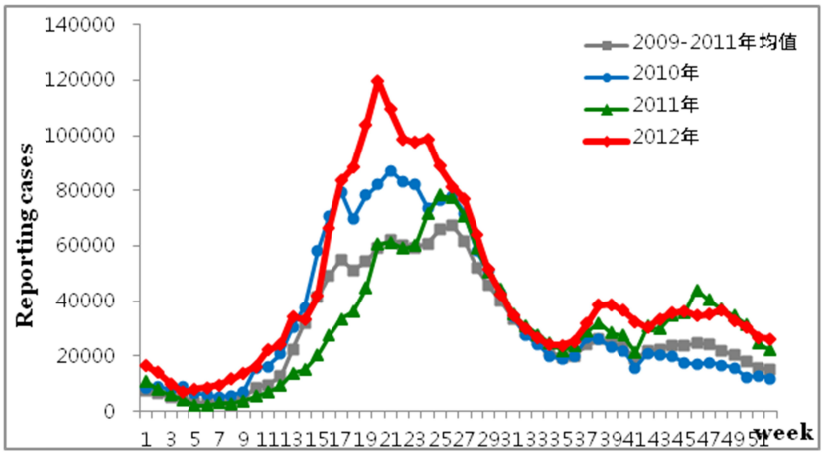


Figure 2. The reporting cases by week from Jan 1, 2010-Dec, 31, 2012

4. Pathogenic surveillance

4.1. Pathogenic spectrum

In 2012, totally 105,380 cases, 4.9% of the clinically reporting cases, were confirmed by lab. The lab result indicated that EV71 and CA16 still were the most major pathogen of HFMD in China, account for 48.9% and 30.6% of all of the positive respectively. Still there were 20.5% of the confirmed cases associated with other enteroviruses.

4.2. Molecular epidemiology of EV71 and CA16

Phylogenetic trees of the EV71 and CA16 isolates in 2012 were reconstructed respectively, based on VP1 encoding region (891bp), with Kimura 2-parameter nucleotide substitution model. All the EV71 isolates in 2012 clustered together, showing the nearest phylogenetic relationship with subgenotype C4a, which was predominant in mainland China since 1998. Different with the unique genotype of

EV71 circulating in China, there were 2 subgenotype of CA16, B1a and B1b, co-circulating in China in the recent years. No significant predominance were found in specific provinces for both B1a and B1b.

4.3. Genomic characteristics

16 complete genome sequences of EV71 were determined, isolated from HFMD patients during the large scale outbreak and non-outbreak years since 1998 in China. These 16 full length genome sequences were aligned with another 104 genome sequences of EV71 from China mainland, available in GenBank, covering the time period of 1998-2011. Our comprehensive recombination analysis showed the evidence of genome recombination of subgenotype C4 (including C4a and C4b) sequences between structural genes from genotype C EV71 and non-structural genes from the prototype strains of CAV16, 14 and 4, but the evidence of intratypic recombination between C4 strains and B subgenotype was not enough strong (Figure 3). This intertypic recombination of C4 viruses were first identified in 1998 and became the predominant endemic viruses circulating in China mainland for at least 14 years.

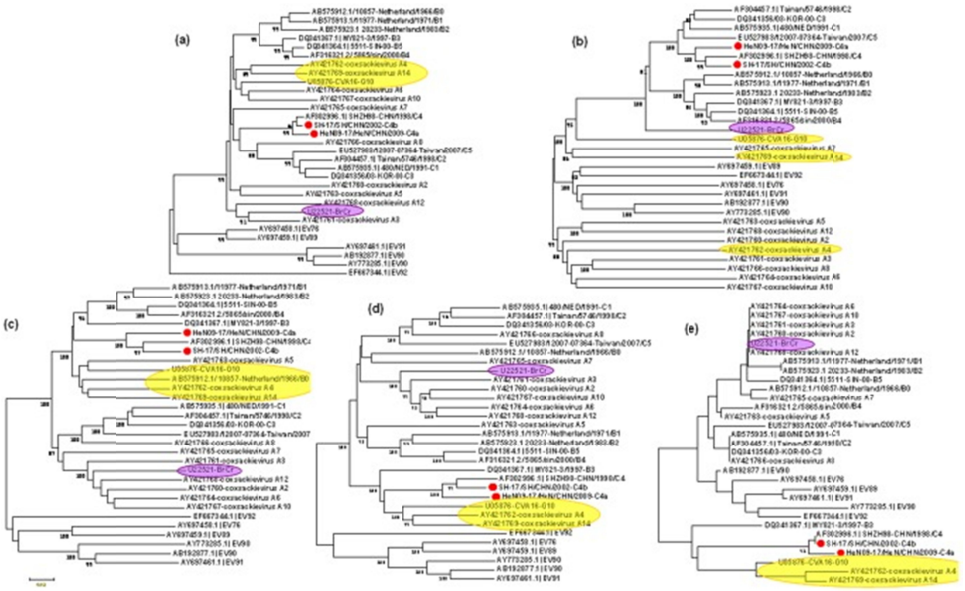


Figure 3. **Rooted phylogenetic trees showing the relationships amongst HEV-A isolates using the different genomic regions.** The neighbour-joining trees were constructed from alignment of the 5' UTR (a), P1 (b), P2 (c), P3 (d) and 3′UTR (e)

genomic region, respectively

8 complete genome sequences of CA16 from China were determined. Comprehensive phylogenetic and recombination analyses were performed on these 8 complete genome sequences and another 20 CA16 whole genome sequences from China in GenBank, during 1998 to 2011. Analysis indicated that CA16 B1a and B1b strains were potential multiple-recombinant viruses containing other HEV-A donor sequences in the 5'-untranslated region and P2, P3 non-structural protein encoding regions. The donor squences were hard to determined.

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

Study faculty: Institute for Communicable Disease Control and Prevention, China CDC, Changbai road 155, Changping Qu, Beijing 102206, China

Name and official title of PI: Biao Kan, PhD, Prof., Deputy Director of National Institute for Communicable Disease Control and Prevention, China CDC

Summary:

A survey of etiology from the diarrheal patients was conducted in the sentinel city, Yuxi of Yunnan province. The stool samples were obtained from the hospitals and isolation of multiple target bacteria pathogens was conducted in Yuxi city CDC laboratory. Molecular subtyping of the isolates was also performed. Based on the one-year survey, the distribution of bacterial pathogens was obtained, diarrheagenic *Escherichia coli* (DEC), *Aeromonas* and *Salmonella* strains were listed in the top three bacteria species. PFGE of the isolates showed complex patterns, but some clusters with the same patterns were also found. A protocol of multiple bacteria isolation from the diarrheal sample was developed generated, which can be provided to the national laboratory surveillance network as the technical support in the foodborne disease survey. In another part of work, non-O1/non-O139 *V. cholerae* strains from the environmental samples and diarrheal patients in the previous surveillance, and the *V. fluvialis* strains form diarrheal patients were transferred to NIID for the serotype identification and further studies.

I. Purpose:

To establish the multiple foodborne pathogen detection protocol and molecular subtyping surveillance which can be used in the city and provincial health institutions, to strengthen the ability of cluster and outbreak detection through laboratory based surveillance. The distribution of the bacterial pathogens will be surveyed in the outpatients with the acute diarrhea in the sentinel city. In addition, an optimized

protocol for the multiple bacterial pathogen isolation from stool sample and a multiplex real-time PCRs detection protocol will be established for the surveillance of the common foodborne pathogens, to recommend to the network of PulseNet China. This year the study will focus on the laboratory based detection of the bacterial pathogens from the diarrheal cases, and molecular subtyping of the isolates, to find the possible cluster of the isolates. Yuxi city, in Yunnan province, is selected as the sentinel site. The study includes two parts: (1) Optimization of a protocol for the isolation of multiple bacterial pathogens from diarrheal cases in the city-level laboratory. The etiology of acute diarrhea using a sentinel hospital-based surveillance will also be analyzed. (2) Molecular subtyping (pulsed-field gel electrophoresis, PFGE) of the isolates in provincial CDC laboratory, to find the pattern cluster.

II. Methods:

Design of the project. The study focused on the pathogen isolation from the diarrheal patients in the health institution in the city level, which is an important step to connect the outpatients and molecular subtyping based surveillance in the network such as PulseNet. For this purpose, work plan of the project was made in Yuxi city, a district in Yunnan province. Four sentinel hospitals, including one children hospital, were selected. During the study, other three hospitals including one children hospital also joined and submitted some samples of the diarrheal outpatients. The samples were collected from the hospitals and transferred (in C-B transport media) to the laboratory in Yuxi Center for Disease Control and Prevention. A group from the Yunnan provincial CDC joined to coordinate the project in the field and worked in the laboratory simultaneously. The study conducted for one year, from Jun 2012 to May 2013.

Detection of bacteria from the outpatient samples. The fecal or swab samples of the outpatients with diarrhea were collected for the detection. The following bacteria were included as the targets for the isolation: *Salmonella*, *Shigella*, *diarrheagenic Escherichia coli* (DEC), *Vibrio*, *Plesiomonas shigelloides* and *Aeromonas*. The isolation methods for the different pathogens were integrated into a protocol,

including the enrichment or plating directly (for the isolation of *Shigella* and DEC), the suspected colony selection. The biochemical tests were used for the primary identification of the strains. For the DEC, the toxigenic related genes were detected with PCR for its identification. The biotypes and serotypes were determined with the corresponding methods and typing sera.

Pulsed-field gel electrophoresis (PFGE). These strains were analyzed by PFGE according to the protocols from PulseNet International in the laboratories of Yunnan CDC and China CDC.

III. Results:

1) Samples collected in the hospitals.

From Jun 1, 2012 to May 31, 2013, totally 1139 samples of diarrheal outpatients were collected, including feces and swabs. The samples were collected and cultured weekly for the isolation of the target bacteria. All ages of patients were covered. The samples were preserved in the C-B transport media and transferred to the CDC laboratory. The main symptoms of the patients were also recorded, most patients (53.2%) had 3-5 times of diarrhea per day, 42.3% had 6-10 times per day, and 4.5% had more than 10 times diarrhea per day. Other symptoms were also recorded.

Table 1. The collected samples and the isolation rates.

Hospital	Number of samples	Number of positive samples*	Positive rate (%)
Beicheng Central Hospital	303	77	25.4
Chunhe Hospital	191	48	25.1
Dayingjie Hospital	303	72	23.8
Liqi Hospital	23	8	34.8
Beiyuan Hospital	13	4	30.8
Yuxi Children Hospital	217	19	8.8
Yuxi Women and Children Health Care Hospital	89	9	10.1
Sum	1139	237	20.8

* The samples from which the target bacteria were obtained.

2) Isolation of the bacteria from the samples

In the 1139 samples, the target bacteria were recovered from 237 patients (20.8% positive rate), including 24 possible mixed infections, since more than one target bacteria species were obtained (Table 2). Mixed infection often occurred among DEC, *Aeromonas*, *P. shigelloides* and *Salmonella*, and didn't find *Shigella* and *Vibrio* involved (Table 3). Within all the 265 isolates, the distribution of bacteria from high frequency to low frequency is DEC (obtained from 10.45% patients), *Aeromonas* (7.37% in total), *Salmonella*, *P. shigelloides*, *Shigella*, and *Vibrio* (Table 4).

Table 2. Bacterial isolation in the samples of diarrheal patients.

Recovered bacterium	Number of samples	Recovery rate (%)
DEC	96	
Aeromonas	65	
Salmonella	21	
P. shigelloides	14	
Shigella	15	
V. paraheamolyticus	1	
V. fluvialis	1	
Mixed bacteria*	24	2.1
Sum	237	20.8

* More than one target bacteria were obtained from one sample.

Table 3. Mixed infection in samples.

Recovered bacterium	Number of samples
DEC and DEC	6
DEC and *A.hydrophila*	3
DEC and *P. shigelloides*	3
DEC and *A. sobria*	2
DEC and *Salmonella*	1
A.hydrophila and *A. sobria*	3
A.hydrophila and *P. shigelloides*	2
Salmonella, *P. shigelloides* and *A. sobria*	1
Salmonella, *A.hydrophila* and *A. sobria*	1
A.hydrophila, DEC and *P. shigelloides*	1
A.hydrophila, DEC and *A. sobria*	1

Sum	24

Table 4. The bacteria isolated from the diarrheal patient samples.

Bacterium	Number of isolates	Recovery rate (%)	Constituent ratio (%)
DEC	119	10.45	44.9
Aeromonas	84	7.37	31.7
Salmonella	24	2.11	9.1
P. shigelloides	21	1.84	7.9
Shigella	15	1.32	5.7
V. paraheamolyticus	1	0.09	0.4
V. fluvialis	1	0.09	0.4
Sum	265	23.27	100

3) The distribution of detection rate

During the period from 2012 June to 2013 May, the monthly detection rate of target bacteria was between 8% and 38.9% (Fig.1). The detection rate rose rapidly in February, reached the peak in March, began to decline after July, fell to the lowest value in January. The number of diarrhea patients had two peaks during the year, one in the summer with a high detection rate, and the other in the winter with a low detection rate.

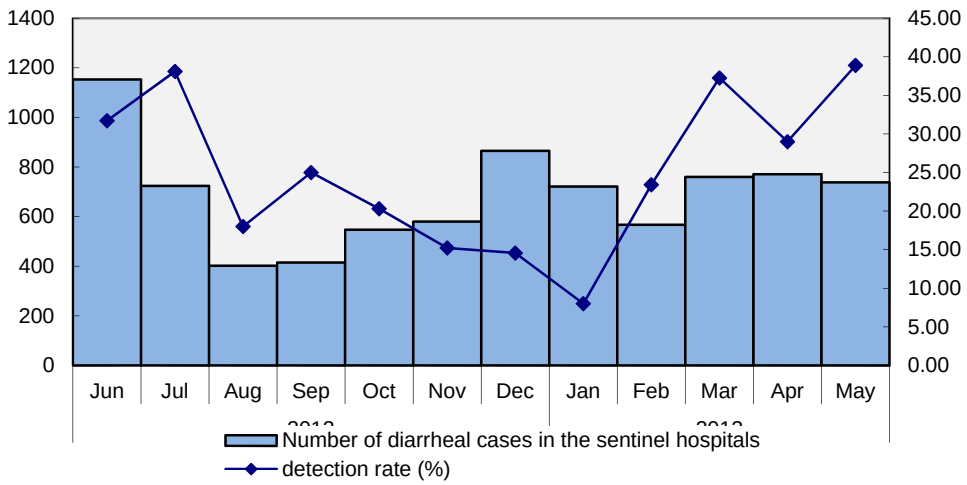


Figure 1. The monthly detection rate of target bacteria.

The target bacteria were isolated in every age group (Fig.2). For children below 5 years old, the detection rate was low and no *P. shigelloides* was isolated. For age

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

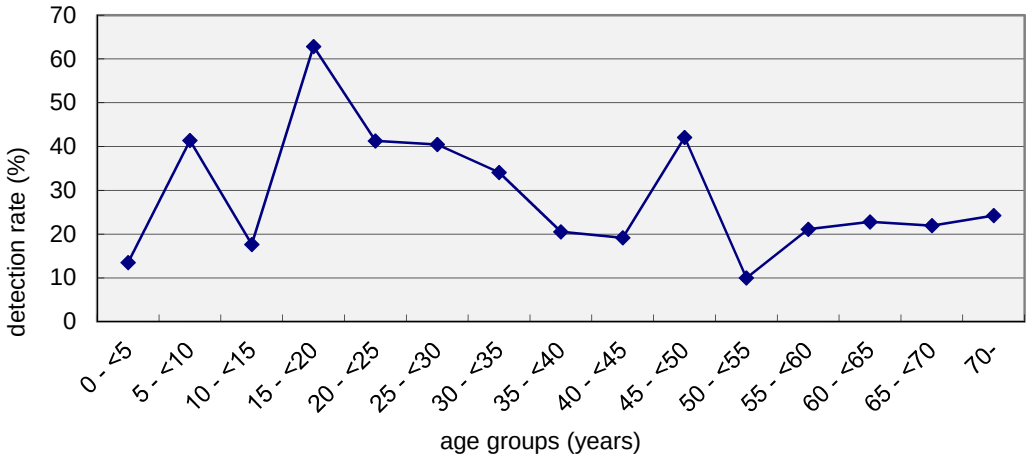


Figure 2. The detection rate of target bacteria in age groups.

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

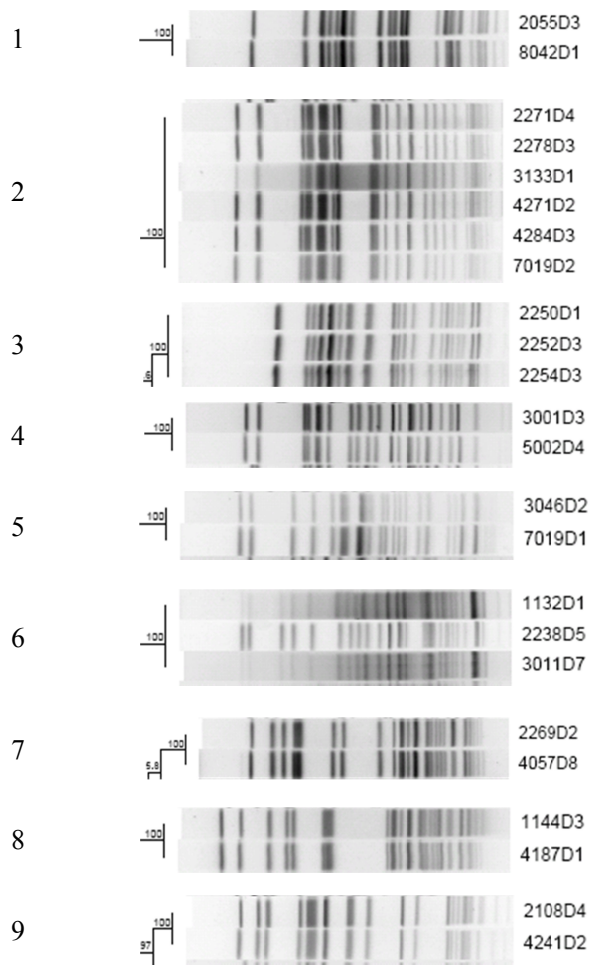
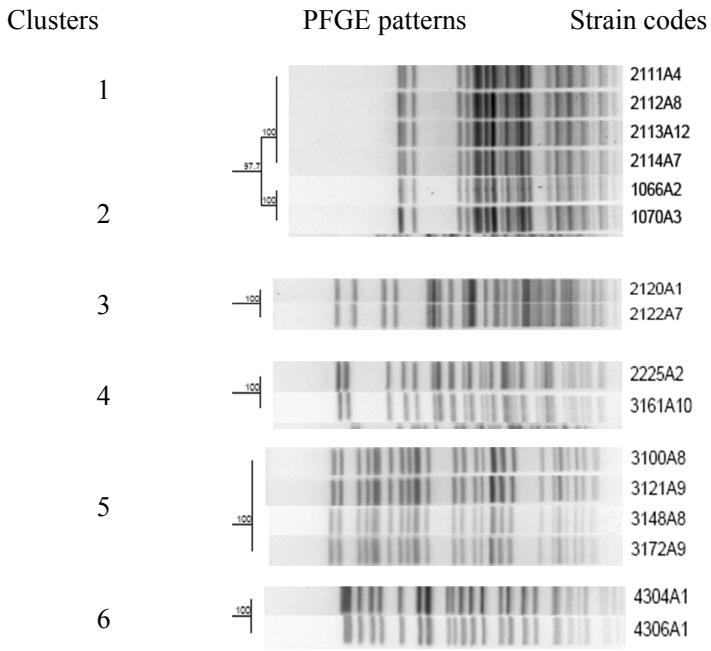


Figure 3. PFGE cluster patterns of the DEC strains.



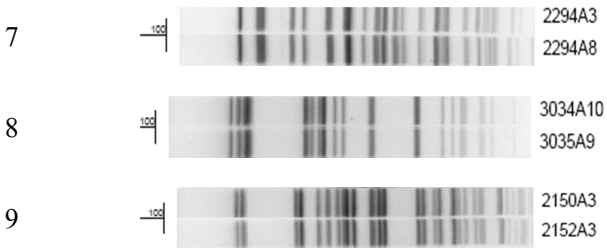


Figure 4. PFGE cluster patterns of the *Aeromonas* strains.

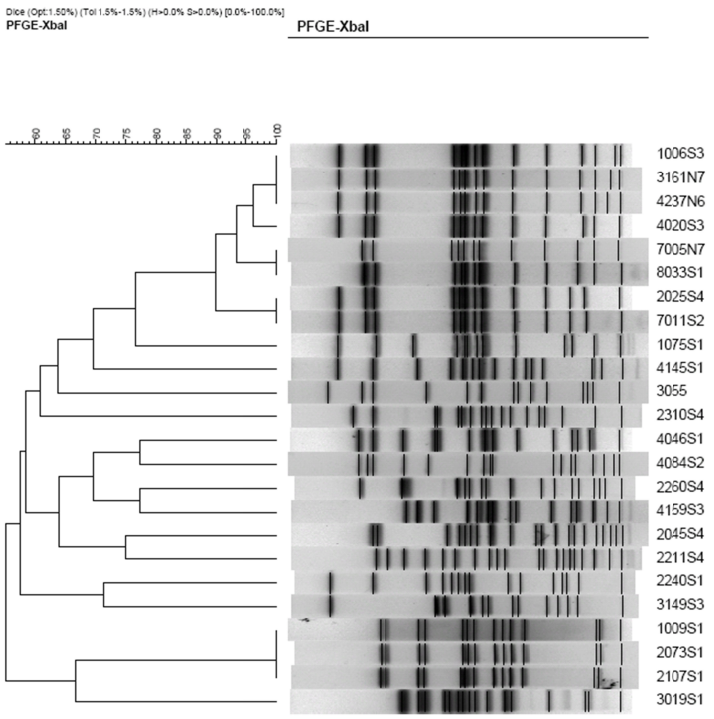


Figure 5. PFGE patterns of the *Salmonella* isolates. Four patterns were shown by more than one isolate.

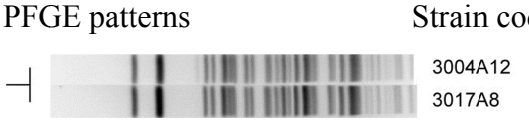


Figure 6. PFGE cluster pattern of the *P. shigelloides* isolates

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.

V. References:

1) Qu, M., Deng, Y., Zhang, X., et al. Etiology of acute diarrhea due to enteropathogenic bacteria in Beijing, China. J Infect. 65: 214-222, 2012.

2) Zhu, M., Cui, S., Lin, L., et al: Analysis of the aetiology of diarrhoea in outpatients in 2007, Henan province, China. Epidemiol Infect. 7:1-9, 2012.

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

Study faculty: Institute for Viral Disease Control and Prevention, China CDC, Changbai Road 155, Changping Qu, Beijing 102206, China

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].

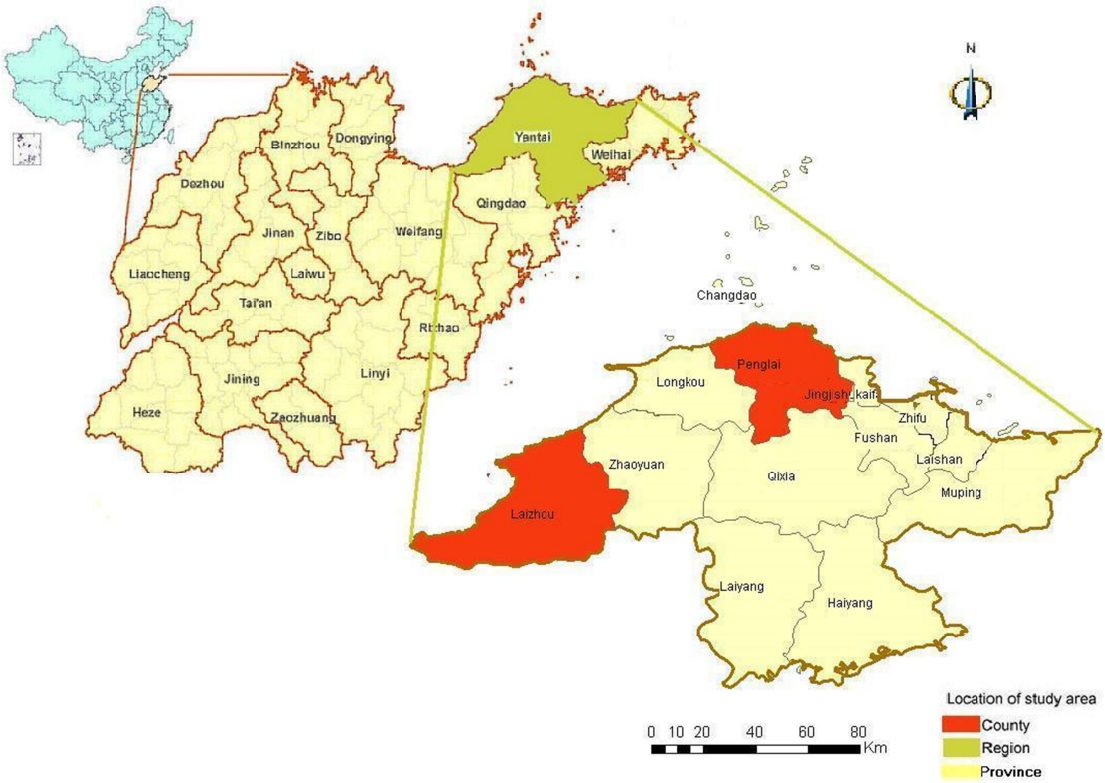


Figure 1 Map of Yantai region indicating location of sampling areas.

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.

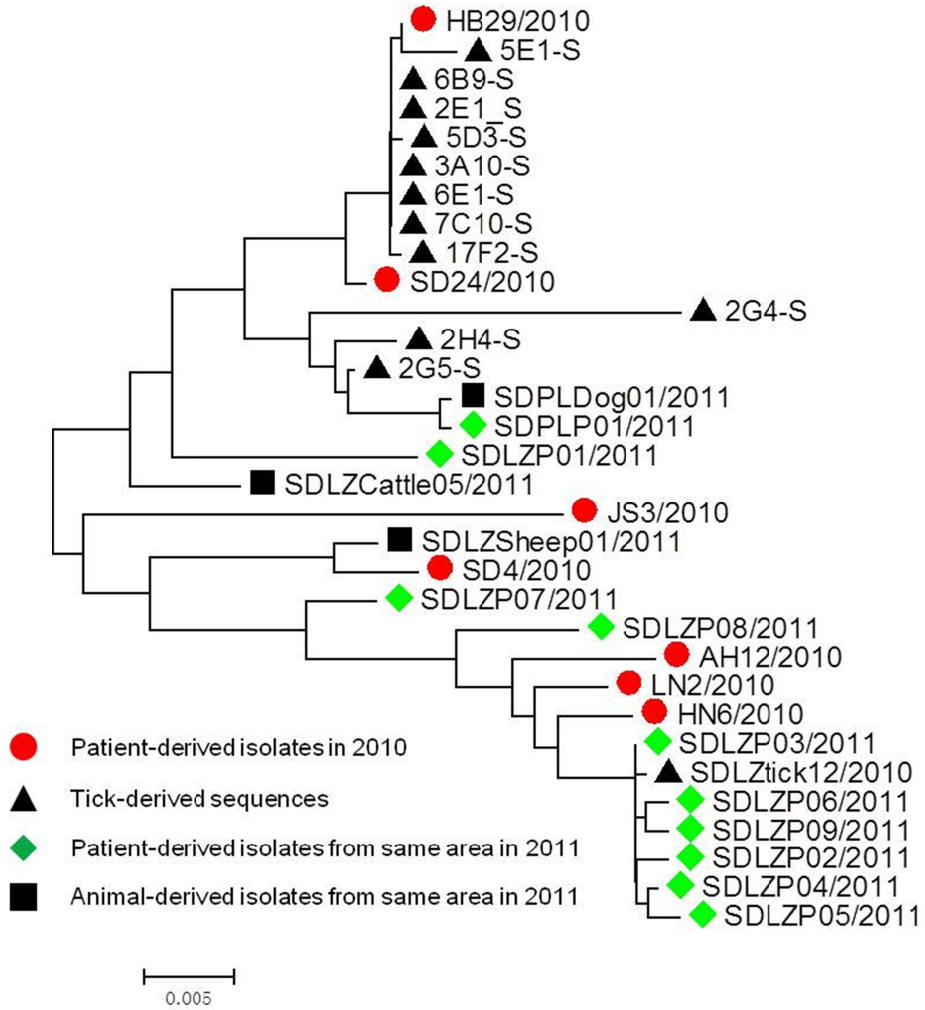


Figure 2 Phylogenetic tree based upon S segment sequences for representative viruses derived from humans, animals and ticks inferred by the neighbor joining method using MEGA 5. Tree was drawn to scale with branch lengths representing the number of nucleotide substitutions per site. Dots indicate the 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

Study faculty: Institute for Viral Disease Control and Prevention, China CDC, Changbai road 155, Changping Qu, Beijing 102206,China

Name and official title of PI: Yuelong Shu, PhD, Prof., Director of National Influenza Center, National Institute for Viral Disease Control and Prevention, China CDC.

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

Total viral RNA was extracted from all of the specimens by using a commercial kit (QIAamp Viral RNA Mini Kit, QIAGEN Inc., Valencia, CA, USA) according to manufacturer's instructions and eluted in a final volume of 40 µl of RNase-free water.

Reverse Transcriptase and PCR Amplification of Viral Nucleic Acid

Thermo RevertAid First Strand cDNA Synthesis Kit was used for cDNA Synthesis. After thawing, mix and briefly centrifuge the components of the kit. Store on ice. Add 8 µ L template RNA for each reaction then incubate 90 min at 37°C. Prepare a master mix of Seeplex RV15 ACE kit according to manufacturer's instructions. The master mix typically contains all the components required for PCR except the template cDNA. Mix the master mix thoroughly, and dispense appropriate volumes into PCR tubes. Add 5 µ L template cDNA to the individual PCR tubes. Program the thermal cycles according to manufacturer's instructions. Start the PCR program while PCR tubes are still on ice. After amplification, proceed with *Detection of Amplified Respiratory Viral* nucleic acid.

Detection of Amplified Respiratory Viral Nucleic Acid

Analyze the PCR products on the QIAcel Workstation using capillary electrophoresis. The results were determined as according to the position of the band of the PCR products.

III. Results:

We tested 202 clinical respiratory tract samples from outpatients using a multiplex PCR method which can be used for the simultaneous detection of fifteen respiratory viruses. The results showed FluA was detected in 31 patients (15.3% of total samples). Adv was find in 14 patients (6.9% of total samples). RSV B was detected in 12 patients (5.9% of total samples). 135 patients were negative for 15 respiratory viruses tested (Table 1). 8 patients presented dual viral infection (4.0%)(Table2).

Table1. Identification of the viruses in 202 respiratory tract samples from outpatients

Viruses	Number of positive samples
FluA	31
Adv	14

RSV B	12
MPV	5
HEV	5
HRV	3
RSV A	2
PIV2	1
PIV3	1
PIV4	1
Co-infection	8
Negative	135
total	**202**

Table2 Identification of the virus in 8 outpatients with co-infection

Viruses	**Number of positive samples**
RSV B+FluA	2
Adv+FluA	1
Adv+HEV	1
FluA+HEV	1
FluA+HRV	1
RSV A+HEV	1
RSV B+PIV4	1
total	**8**

IV. Discussion:

Upper respiratory tract infection (URI) represents the most common acute illness evaluated in the outpatient setting. The accurate and rapid analysis of a broad range of viral agents is critical for etiological investigations. Viruses account for most URIs. In this study, we investigated 202 clinical specimens collected from ILI cases, 33.1% samples were identified to be positive one or more viruses. However, these results may underestimate the role of virus infection, because viral loads in throat swabs, as used in this study, are usually lower than those in aspirate or lavage.

Some limitation should be involved into this study. Firstly, the collection of samples could not cover all ages or years since we cannot exclude the possibility of outbreak samples. Furthermore, molecular investigation could miss some mutated pathogens. Additionally, to improve control and prevention for respiratory virus infection, the molecular investigation is far away of enough since novel pathogens is always a

concern in public health. Thirdly, a positive detection for a virus in throat swab by molecular techniques does not necessarily indicate respiratory disease, which is also mentioned in other studies[5].

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VI. Publication list for this work:

Hongtao Sui, Dayan Wang, Yuelong Shu.Development of Multi-Pathogen Detection Techniques for Respiratory Viruses. Chinese Journal of Virology. 2013, 9(2):238-244.

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

Study faculty: Division for Infectious Disease Control and Prevention, Chinese Center for Disease Control and Prevention, 155 Changbai road, Changping District, Beijing, 102206, China

Name and official title of PI: Hong-jie, Yu, MD, MPH, Prof., Deputy Director of Office for Disease Control and Emergency Response, China CDC

Summary:

China CDC initiated a surveillancesystemtargeting on detectingsevere acute respiratory infection (SARI)inpatientswith streptococcus pneumoniae (S.p) in Jingzhou City, Hubei province in 2010.The surveillance result showed low yield of blood cultures of SP (0.5%, 21/4,029). Therefore, we carried out another surveillance program for S.pin Kunming city, Yunnan province in 2013. However, no positive results were found. According to previous surveillance experience and awareness,the wide self-usage of antibiotics might be one important reason for such low culture positive rate, another reason should be that the standard operation procedure of blood draw, timely transfer to lab, and whole blood culture might not followed well. After pilot surveillance in two sites, we realize that whole blood culture might not yield good results at present time in China and propose to conduct more sensitive PCR method to evaluate S.p prevalence although s.p strain could not be obtained.

I. Background

Under the financial support from Japan National Institute of Infectious Disease,China CDC have been conducting a surveillance program in hospitalized patients with severe acute respiratory infection (SARI) caused by streptococcus pneumoniae (S.p)for two years in Jingzhou city, Hubei province. The surveillance activities include enrollment and investigation of inpatients matchingthe SARI case definition, collection of blood and urine, and the whole blood culture and urine antigen for

S.p.The blood culture had always been a big issue for its low yield since the beginning. We organized a boosting training course andsupported two lab staff to the national reference labs to receive more systematic training on blood culture.However, no obvious improvements have been seen. Therefore, we tried another site in Kunming city, Yunnan province to find out whether it is possible to conduct whole blood culture in surveillance program in China.

II.Purpose:

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

II.Methods:

Setting and Patients Enrollment

The Second Affiliated Hospital of Kuming Medical University was chosen after carefully considering capacities of surveillance and laboratory testing. A national surveillance protocol including patients enrollment, specimens collection and laboratory testing, case report form and information reporting, and related standard operation procedures (SOP) 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 each of these 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 ALRIpatients, 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 eligible ALRI case-patients or their parent/guardians, and then blood were collected. Collected blood samples were sent to Yunnan provincial CDC laboratory to conduct whole blood culture.

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

During the period from January, 2013 to September, 2013, we have seen that:

1. The total number of hospitalized patients within the surveillance wards was 298.
2. Epidemiological and clinical features:

1) 298CRFs had been completed.
2) All of the CRFs were from Children cases age≤14 years old and the majority (82.9%) were younger than 5 years old. 182 (61.1%) were male.
3) As for body temperature, 121(40.6%) in children cases were admitted to hospital with fever ≥38□. Cough was the most common symptom (57.4%).

3. Laboratory testing results:

All of Children cases have been collected blood samples. No samples had been cultured positive for S.p.

IV.Discussion:

It has been well known that *Streptococcus pneumoniae* (S.p) is one of the major pathogens for respiratory infection throughout the world and 5% of all children deaths were attributed to S.p. As far as we know, there is no good data for S.p prevalence in China at community level. China lacks a continuous, high quality S.p surveillance system, which is needed to characterize the burden, distribution, trends, clinical outcomes, and subtypes of S.pamong invasive S.pdisease (IPD). This is why we initiated S.p surveillance programin Jingzhou.

During two years' operation, we found that in Jingzhou it was hard to improve the yield of bacterial confirmation through blood cultures. Through field evaluation, we realized that in addition to antibiotic usage prior to and during hospitalization, other factors contributing to the low yieldincluded the timing of blood collection in the hospital, the method of blood draw, and the way blood cultures being processed.Although we had made several efforts to strengthen SOP of blood culture in Jingzhou, no big progress had been seen. So, we still decided to select another site in Yunnan province to try S.p surveillance.

As results showed that no positive were found among 298 whole blood samples, we have to reassessed our protocol. We should revise the protocol to call for collection of blood in a well-defined subset of SARI patients in whom bacterial pneumonia is more likely.According to current clinical practice and huge workload of diagnosis and treatment in China, it is hard to change to completely follow the SOPs in a short period. Furthermore, antibiotics overuse will be a long barrier for S.p detection in blood culture. Therefore, we would like to conduct more sensitive PCR test for sera or SCF.Shortage of PCR test is that we can identify its sera subtype but S.p isolate could not be available.

Total 26 S.p strains were isolated in blood culture in Jingzhou and Kunming sites. Too small number made it impossible to represent the real distribution of S.p in invasive pneumococcal diseases. Till now, no advanced laboratory testing, such as genotype, DNA sequencing, has been conducted for these isolates.We hope that we could get more funds to support it to move forward. A good platform and public health awareness in clinical departments have been gradually established. In addition, it provides experiences and lessons for hospital-based surveillance that need to be addressed in future practical implementation.