second 33-strain panel. The number of patterns and the D values were obtained. After comparison, protocols *Spe*I (switch times 1-20s) and *Xba*I (switch times 1.5-29s) showed the highest discriminatory power with two respective enzymes. These two protocols were also used in our further studies of PFGE with 106 isolates, which also had much higher discriminatory power than the standardized *Xba*I-PFGE and *Bln*I-PFGE protocols for the subtyping of *Salmonella* serotypes. The bands yielded with *Spe*I digestion using the optimal electrophoresis parameters determined in this study distributed evenly. Therefore, for the PFGE typing of *S.* Paratyphi A, we recommend *Spe*I protocol as the first choice and *Xba*I protocol is the second choice, for the further subtyping if higher discriminatory power is needed for the subtyping of *S.* Paratyphi A isolates.

IV. Discussion:

Laboratory data may provide more details for the surveillance and outbreak detection in the control of diarrheal disease and other infectious diseases. PulseNet, a molecular subtyping network in the diarrheal disease surveillance, has shown important roles in the outbreak detection, early warning in disease prevention, and source tracking in outbreak investigations, and has become essential component in the food safety constructions. We also started to establish PulseNet China in the bacterial diseases surveillance. In this project we plan to select some pilot regions to establish the laboratory based diarrheal disease surveillance system. Previously in China we have no such system. Establishing the laboratory subtyping capacities in provincial

CDCs is essential, so within the framestructure of planned PulseNet China and the procedure of network laboratory certification, we first performed molecular subtyping techniques transfer. And, training and quality assurance is also needed in the maintenance of such network, for its effective performance. The training enhanced the capability of software analysis and promoted the standardization of data processing for participants.

The central laboratory has responsibility in the development of such laboratory surveillance network, and take on the technical development and promotion. In this project the central laboratory in China CDC will develop molecular typing methods, especially for the pathogens which have no standardized protocols. During our surveillance of *S.* Paratyphi A, limited patterns were obtained when the strains from multiple years and multiple provinces were analyzed with the standard protocol used for *Salmonella* serotypes. In addition, *S.* Paratyphi A infection may have a longer (more than one week) preclinical period than other foodborne infections, which may be a disadvantage during outbreak investigations, because this makes it more difficult to determine exposure factors and to perform source tracing. Much more sensitive molecular subtyping method is needed. In this study we tried to assess some new protocols for the subtyping of *S.* Paratyphi A isolates based on the standardized *Xba*I-PFGE and *Bln*I-PFGE protocols for the subtyping of *Salmonella*. New protocols showed higher discriminatory power. Practically, we think after *S.* Paratyphi A is identified, the protocols optimized in this study may be used as the supplementary analysis when more subtyping information is needed in the epidemiological

investigation, such as the difficultly interpretative inconsistency between the primary PFGE result and epidemiological information and more accurate case definition. In addition, the optimized protocols will be more useful in the clonal structure analysis of the *S.* Paratyphi A strains collected from different regions and years.

In the first year of project, we mainly focused on the capacity building of the provincial CDC laboratories for their molecular subtyping of enteropathgens in some provinces. In the following years, the pilot surveillance system based on isolation and PFGE in the local laboratories and hospitals will be practically performed.

2. Preparedness for Bioterrorism and Emerging Infectious Diseases

Title: Molecular Epidemiology of Severe Febrile and Thrombocytopenia Syndrome Virus (SFTSV), A New Bunyavirus, in China

Name of researcher: De-Xin Li,

Affiliation: National Institute for Viral Disease Control and Prevention, Beijing, China CDC

Summary:

To identify the character of SFTS virus transmission and distribution in China, the potential role of domestic animals in transmission cycles of SFTS virus was investigated. Sera from domestic animals were collected and tested for viral infection in 2011. Viral antibodies were detected from 64.9% of sheep (n=1013), 52.5% of cattle (n=842), 28.7% of dogs (n=359), 1.3% of pigs (n=839) and 44.6% of chicken (n=527). Small proportion of studied animals was found carrying low levels of viral RNA with a short detectable viremia. Viral isolates were obtained from the sera of sheep, dog and human patients, genetic analysis revealed a pairwise distance of S segment less than 4.6%. In the cohort of 38 sheep grazing at two sites, viral infection from July to November rise from 0 to 76.5% (n=17) and 100% (n=21). These data demonstrated an intense epizoonics and endemic of SFTS virus in the studied area.

I.Purpose:

SFTS Bunyvirus (SFTSV) is a newly identified phlebovirus in the family of Bunyaviridae, which causes human severe fever with thrombocytopenia syndrome (SFTS), with a case fatality rate of 10-12 % (1). The distribution of SFTS is sporadic and widespread, which circulates in central and north-east China. Although *Haemaphysalis longjicornis* was found to be a competent vector for SFTSV infection, the natural reservoirs host and transmission cycles for the virus still remain unclear. As an emerging zoonotic disease, our understanding of the transmission cycle(s) remains very incomplete. So far, we can only speculate the importance of variables that maintain transmission and stimulate epizootics or epidemics, which may include the density of competent vector ticks, particularly *Haemaphysalis longjicornis* and the relative abundance of vertebrates that serve as both hosts to ticks and as possible reservoirs of SFTS virus, however, both have not yet been systematically studied within a particular geographic region.

To establish and identify an arbovirus transmission cycle, except vectors competence, the susceptibility of the vertebrate host for the virus is essential, which should produce a high-level viremia to allow other vectors to become infected, and the vector and vertebrate host should be available in sufficient numbers at the same time and at the same place (2-3). Ticks which bite humans usually parasitize domestic animals by preference, the infection rate of animal hosts, levels of viremia and maintenance play a key role in transmission and spreading of the virus in transmission cycle of human infection (2).

Systematic investigations of SFTSV seroprevalence and infection rate in

vertebrates, in particular in domestic animals from SFTS endemic areas, are necessary to identify the pathogens sources, reservoirs hosts and the transmission routes to human, therefore to support the evidence-based disease control and prevention strategy. Through China CDC surveillance system, here we reported an investigation of SFTS virus infections and seroprevalence in domestic animals of sheep, cattle, dog, pig and chicken, from two counties Laizhou and Penglai of Shandong provinces, as well as the genetic analysis of SFTSV strains isolated from the animals and the local SFTS patients.

II.Methods:

Study design and sample collection

To assess the prevalence of SFTS virus infection in animal hosts at endemic areas, a cross-sectional and cohort study were designed, sheep, cattle, dogs, pig and chicken were used as sentinel animals and screened for presence of SFTS viral specific RNA and antibodies. Animals were sampled at 6 villages from each county to reflect the most severe areas of SFTS virus transmission according to reported SFTS cases in 2010. For each animal, the following data were collected: place of origin (owner's address), sampling date, age, breed, gender and carrying ticks. To facilitate the field work, collaborations were established with local CDC, village medical doctors, and local veterinary hospitals.

The blood samples from all of above animals were collected once a month from April to November 2011 at Laizhou and Penglai counties in Shandong province, all sampled animals in each collection month were marked with a unique labels. The

collected animal sera were separated into aliquots locally, and screened for viral RNAs and SFTSV N protein specific antibodies. The sampled animals with antibodies positive were out of the study group in next month while new candidates would enter the sampling lists. The sampled animals with viral RNA positive were selected for further sampling every 2 days.

In order to define the infection rate and viremia of SFTS virus infection in animal host, a cohort was established using 38 lambs (age≤1 month) without viral infection proofs in the screening tests, 17 from Laizhou County and 21 from Penglai County, which were bred on grazing at the sampled villages. The animals in the cohort were sampled every 10 days from 20 June to 30 November. In addition, sera sample from human patients reported during the period of this study from the sampled villages were also collected for SFTS viral specific RNA and antibodies detection.

Double antigen sandwich ELISA

N specific total antibodies detection was performed using double antigen sandwich ELISA, in brief, the SFTS viral N protein was used as antigen for antibodies capturing and N protein labeled with horseradish peroxidase (HRP) was used to detect the SFTS viral specific antibodies.

Real-Time, conventional RT-PCR

RNA was extracted from sera sample using QIAamp Viral RNA Kit (Qiagen, Germany) according to manufacture instruction. SFTS viral RNA was detected using quantitative, real-time one-step RT-PCR according to previously published method (5). Two-step conventional RT-PCR was performed for real-time PCR positive samples by

using SuperScript III First strand cDNA synthesis kit (Invitrogen) to generate cDNA and Fast Start Taq DNA Polymerase High Fidelity (Roche) for the PCR. Viral S segment gene was amplified, analyzed by agarose gel electrophoresis and sequenced as described (6).

Virus isolation

Virus isolation was attempted by inoculation of Vero cells using 10-fold diluted sera. Virus identification was made by an indirect immunofluorescent test on Vero cells, using polyclonal and monoclonal antibodies. The identity of virus isolates was confirmed by RT-PCR and nucleic acid sequencing. Phylogenetic and molecular analysis of the obtained sequences to previously published STSF virus sequences in GenBank were performed with MEGA 5 (7).

III.Results:

A total of 1013 sheep, 842 cattle, 359 dogs, 839 pigs and 527 chicken were bled between April and November 2011 from Laizhou and Penglai counties, Shangdong province, China. quantitative Real-time PCR examination of all sera sample indicated a low detection rate of viral RNA in the sera of studied animals; Investigation result showed that 5% (51/1013) of sheep, 3.7% (31/842) of cattle, 2.8% (10/359) of dogs, 3% (25/839) of pigs and 2.1% (11/527) of chicken were viral RNA positive after detection (Figure 1), while the viral copies in all of above positive samples were at lower level, less than 10^3 RNA copies per milliter(data not shown). The overall seroprevalence of SFTSV from the two counties was 64.9% (657/1013) in sheep, 52.5% (442/842) in cattle, 28.7% (103/359) in dogs, 1.3% (11/839) in pigs and

44.6% (235/527) chicken (Figure 1). The prevalence of SFTSV virus infection and seroconversion from the two counties were not significantly different, and their geography location was not far.

SFTS virus infection in domestic animals was analyzed over time (Figure 1).The percentage of viral RNA positive samples was fluctuated slightly from April to September, declined apparently in October and no viral RNA was detected from mammals since then (Figure 1). The percentage of antibodies positive sheep, varied from 5.9% of lambs (≤1 months) (collected in July, n=51) to 74% (collected in November, n=77) from Laizhou county; and varied from 46% (collected in April, n=52) to 100% (collected in November, n=82) from Penglai county (Figure 1). Sera prevalence in cattle was varied from 33.3% (n=48) to 63.7% (n=80) in Laizhou county, and from 58.1% (n=43) to 78.7% (n=80) in Penglai county in the period from April to November inclusive (Figure 1). The seraprevalence in dog was relative stable among the sample collected from different month, varied from 31.4% (n=35) to 38.9% (n=54) in Laizhou county, and from 29.6% (n=44) to 31.7% (n=41) in Penglai county (Figure 1). The prevalence of antibodies in dog sera was lower than in sheep and cattle sera, which might due to captive breeding and living styles of dogs. The positive rate of SFTSV viral antibodies in pig was apparently low, only samples from April, May and July were detected in Penglai, no antibodies were detected from samples collected in Laizhou county (n=427) (Figure 1). This might be partly due to farm breeding and short lifelong of the sampled pigs. Also no apparent monthly

fluctuations of antibodies prevalence in Chicken sera were detected, varied from 47.5% (n=40) to 57.5% (n=40) in Laizhou county, and from 32% (n=25) to 41.9% (n=43) in Penglai county (Figure 1).

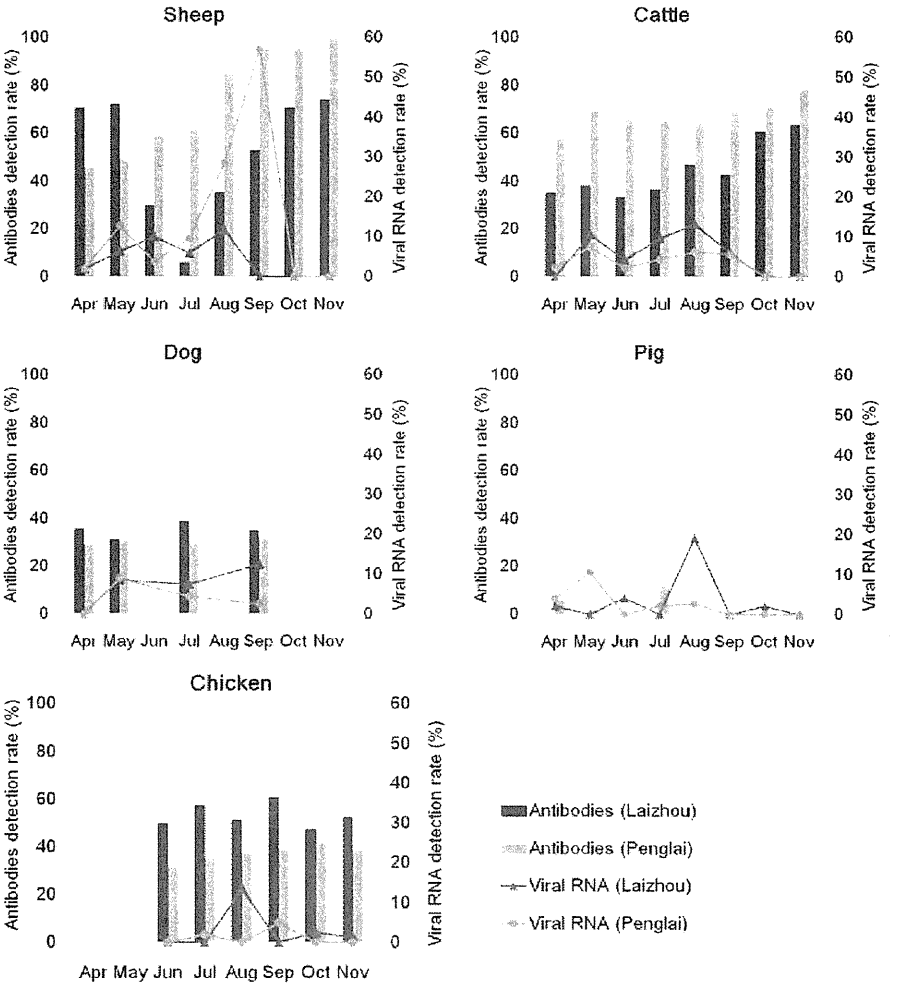

Figure 1. Monthly detection of SFTSV viral RNA and antibodies in domestic animals from two counties. The viral RNAs were detected in domestic animals of sheep, cattle, dog, pig and chicken in Laizhou (Dark line) and Penglai (Light line) from April to November , 2011; and related sera SFTSV antibodies were detected in above animals from Laizhou (Dark histogram) and Penglai (Light histogram).

The screening tests revealed a high prevalence of viral antibodies in the sera of

studied animals. To study the time course of viremia and antibodies, a cohort study of sheep was designed, the sera samples from the sheep were collected and screened at June, 2011, and a cohort of 38 lambs (age≤1 month) with negative SFTSV antibodies was established and tracked. The lambs were bred by grazing at Laizhou (n=17) and Penglai county (n=21). In this cohort, sera samples were collected from June to Novemebr at an interval of around 10 days. Totally 608 sera samples were collected, and the positive rate of antibody rise from 0 to 76.5% (n=17) at Laizhou county, and to 100% (n=21) at Penglai county. These suggested that intense transmission of SFTS virus apparently occurred in the investigation areas, which led to a high infection rate of SFTS virus. Of this cohort sheep, 17 sheep were detected to be viral RNA positive at viral RNA copy level various from less 100 copies /ml toabout 1000 copies/ml in different months, higher viral RNA positive detection rate of the17 lams appeared in August and September(Figure 2B). Among these viral RNA positive sheep, 7 were detected before or at the same time as seroconversion, 10 were detected after seroconversion, in addition, viral RNA was appeared positive twice for 6 sheep over a period of 20 to 50 days, which might suggest repeatedly infection over a short period since no viral detectable between the two detection (Figure 2B). The results indicate that sheep may develope only a short viremia with low viral titers or no detectable viremia after SFTS virus infection.

To investigate dynamic profile of SFTSV natural infections in animal hosts, the dog we mentioned above with higher serum SFTSV viral RNA copies was quarantined and monitored with two days interval at day 8 after fist detection (Day 0),

followed up till to day 22, and again at day 90. The result showed that the viral RNA was positive at day 8 but already declined, the viremia in the dog remained at least 10 days, and was undetectable at day 12. IgG antibodies titer in the sera of the dog had reached higher level (1: 2560) at day 8 and maintained stable at high level till to day 90. These led us to suppose that the viremia of this dog was about 2-3 weeks, although for most sheep, cattle and dogs which seemed developing only a shorter viremia with lower viral titers after SFTS virus natural infection.

Further, the SFTS viruses were isolated from a sheep, a cattle and a dog and the genomic sequences were analyzed. To determine evolutionary relationships of SFTV viral genes between animal isolates and human isolates, we compared the viral RNA S gene nucleic acid sequences of above three domestic animals isolated SFTSV with the sequences of 10 SFTS patients sera, collected in 2011, in the same investigation areas of Penglai or Laizhou county . The phylogenetic analysis indicated that the sequences amplified from cattle (SDLZCattle05/2011) and dog (SDPLDog01/2011) were substantially close with the sequences from two SFTS patients (SDPLP01/2011, SDLZP01/2011) and previously published virus strains; while the sheep derived sequence (SDLZSheep01/2011) was also close with the sequences from the local SFTS patients sera (SDLZP02/2011, SDLZP03/2011, SDLZP04/2011, SDLZP05/2011, SDLZP06/2011, SDLZP08/2011 and SDLZP09/2011) as Figure 2 showed . All of above SFTSV S segment nucleic acid sequences from either animals and humans in 2011 or 2010 were generally clustered together (tool bar distance = 0.005), The pairwise distance between the isolates from domestic animals and human

patients was less than 4.6%, indicated all the sequences shared 95% genetic homology and the close evolutional relationship among SFTS viruses circulating in the endemic areas; from either domestic animals or local SFTS patients in both Laizhou or Penglai county.

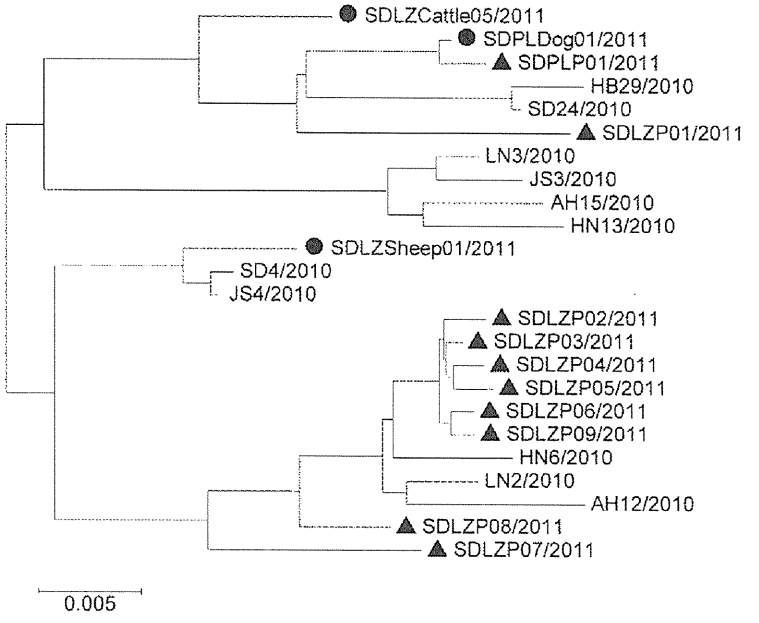

Figure 2 Phylogenetic analysis S segment of SFTS virus genome obtained from animal hosts and human patients. The whole sequences of SFTS viral genome S segment obtained in this study are indicated by black round dots (from animal hosts) and black triangle (from human patients). Sequences were labeled with viral strain name and isolation year. Neighbor-Joining tree was conducted using MEGA5, the evolutionary distances were computed using the Maximum Composite Likelihood method, A total of 1744 positions was analyzed in the final dataset.

V. Discussion

We assessed the prevalence of antibodies against SFTS virus in cross-sectional

study of sheep, cattle, dogs, pigs and chicken by means of double antigen sandwich ELISA. The seroprevalence of SFTSV specific antibodies were found in 64.9% of the sheep, 52.5% of the cattle, 28.7% of the dog, 1.3% of the pig and 44.6% of the chicken. Here we noticed that the high seroprevalence of SFTSV infections in sheep and cattle, and low rate in pig, these variation may caused by the animals living environment and behaviors, the sheep and the cattle were grazing on small hills around the villages in the two counties and easy to be bite by ticks which may carry the SFTSV ; while the dogs and pigs were in captive at famer's home, less exposure to ticks, in particular for the pig , the living behavior may not fit the tick biting and staying on the body. Here we also noticed that the high seroprevalence of SFTSV infections in chicken, a common poultry. The chicken involved in the investigation were stocking out door, living in a fenced hill, where the ticks had more chances to bite the chicken. In addition, a cohort investigation of 38 sheep, the percentage of serum antibodies positive started to rise rapidly in June, and monthly elevated till to November, when the seroprevalence reached 100% in one of the county, indicated that all sheep had ever been infected at least once. This is still likely an underestimate, as of the cohort sheep, 17 were detected to be viral RNA positive , but 10 of 17 sheep were appeared RNA positive after seroconverstion, 6 of 17 sheep appeared twice positive for viral RNA detection(Figure 2B while to the end of September, none of 17 sheep were detected for viral RNA positive. These data suggested that these sheep may have second infection and the sheep only develop a short viremia with low viral titers or no detectable viremia after SFTSV infection. In addition, in this investigation,

we tracked a dog, from the day of virus infection to the serum antibody developed, the time course described a typical SFTSV infection in the dog, and the virus maintained 12 days after the peak (as timed as day 0). No persistent viral RNA positive was found in all domestic animals in our investigation, therefore, the data we obtained so far, the sheep or the dog or others as a natural reservoir host for SFTSV transmission still can not defined although these animals may act very important role in virus transmission as a short term or more precisely to say, a temporary host.

To establish and maintain an arbovirus transmission cycle, infective virus, competent vector and susceptibility of the vertebrate host are essential (2). A minimum level of host viremia is necessary to infect vectors (8), the developed viremias of SFTS virus in the studied animals might not be high enough to infect mosquitoes and thus cannot serve for the establishment and maintenance of a transmission cycle, which might indicate that mosquitos not competent for SFTS virus. Not like mosquitoes, tick-borne viral transmision do not depend on a sufficient viremia of the infected host, due to its long-time attaching to the host to take up virus and tick saliva could assist viral transmission (9). In this investigation, the amount of ticks we trapped from the domestic animals were consistence with the sero-prevalence percentage in the animals. In addition, a mechanism of tick known as co-feeding facilitated pathogens are transferred from one tick to another[10]. These supported that ticks are competent vector for SFTS virus transmission. It is not clear if other domestic or wild animals could replicate SFTS virus titres high enough to serve as hosts for maintenance of transmission cycles. Humans infected with SFTS virus

strains could develop high titres and may therefore play a role as maintenance and amplifying hosts (11).

Phylogenetic analysis indicated that the sequences amplified from sheep, cattle and dog clustered with patient derived viral sequences, but no exclusive correlation of virus stains and geographic location could be observed, and strains from geographically distant areas can be found in one cluster, which might indicates the widespread geographic dispersal of SFTS virus in China. The tight clustering of different strains over a long distance suggests a rather recent spreading. However, not much is known about the possible ways how SFTS virus disperses over long distances. The high prevalence of antibodies in the sera of chicken, may present a hint that birds might play a potential role for the migration of SFTS virus in addition to contribution of human activities.

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VI.Publication list for this work:

The manuscript has been submitted to Emerging Infectious Diseases, Under reviewer

3. Prevention of Respiratory Infectious Diseases

Title: Potential emerging respiratory infection pathogens discovery based on national influenza surveillance network.

Name of researcher: Yue-Long Shu

Affiliation: National Institute for viral diseases control and prevention, China CDC, Beijing China

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. Generally 10-15 specimens from influenza like illness patients being collected by each sentinel hospital per week. The basic proposal for this project is that the samples for influenza negative will be further tested by multiple pathogens detection method based on liquid chip Luminex systems that is able to distinguish

about 20 kinds of respiratory RNA and DNA pathogens (QIAGEN). If the samples are negative, they will be subjected into the mix cells of MV1 LU (mink lung) and A549 cell suitable for isolation of most of respiratory viral pathogens. The positive isolates will be applied in Biosystem solid sequence technique for novel pathogen sequencing.

I. Purpose:

Respiratory tract infections (RTI) are among the most common infectious diseases of humans 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