

Analysis of similarity of the S1 gene in infectious bronchitis virus (IBV) isolates in Shanghai, China

Análisis de similaridad del gen S1 de aislados del virus de la bronquitis infecciosa (IBV) en Shanghai, China

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RESUMEN

El objetivo del estudio fue analizar la similaridad del gen S1 de 5 nuevas cepas virales del virus de la bronquitis infecciosa (IBV) aislado en Shanghai, China, para lo cual se usaron un par de partidores específicos, los cuales fueron designados en base a las secuencias publicadas del gen S del IBV. Los 5 segmentos completos de cDNA del gen S1 fueron amplificados de las 5 cepas aisladas con RT-PCR. Se pudo confirmar que las secuencias de nucleótidos de genes S1 en las 5 cepas aisladas de gallinas y palomas tienen una longitud de 1626 bp y codifican 541 aminoácidos residuales. Al comparar con la secuencia de las cepas de referencia de IBV seleccionadas publicadas en GenBank, las 4 cepas de gallinas exhibieron 77,4%-82,9% de identidad en la secuencia del gen S1, con una similaridad deducida de la secuencia proteica de 74,7%-82,6%, mientras que la cepa de paloma exhibió un 79,3%-99,6% de similaridad en la secuencia del gen S1 con una identidad deducida de la secuencia proteica de 81,6%-99,6%. La secuencia del sitio de división de la proteína S1 de estos aislados contiene 5 aminoácidos básicos consecutivos, llamados Arg-Arg-Phe-Arg-Arg (RRFRR), similares a la mayoría de las cepas IBV. El análisis de los resultados indicó que el rango de variabilidad de la estructura del gen S1 es alto y corresponde al carácter biológico de IBV y, molecularmente, las cinco cepas aisladas en Shanghai se relacionan cercanamente con las cepas IBV de referencia. Se concluyó que las cinco cepas aisladas pertenecen al IBV, aun cuando una fue aislada desde palomas.

Palabras clave: virus, bronquitis infecciosa, RT-PCR, gen S1, variación.

Key words: infectious bronchitis virus, RT-PCR, S1 gene, variation.

INTRODUCTION

Coronaviruses have the largest RNA virus genomes and replicate by means of the specific enveloped transcription. Moreover, its mismatching percent and the combination percent is very high, so the virus can mutate easily (Moore *et al* 1998). Thus the viruses' antigenicity and pathogenicity are changed by these factors. Pathogenicity becomes more and more complicated and the cross-protective immunity between different serotypes is poor, causing immunity defeat (Johnson *et al* 2003). The alteration of its pathogenicity not only changes the tissue tropism but also widens the host range between species (Guy *et al* 2000). Recently, the study of avian coronavirus indicated that it has a wide host range, from francolin, sea-bird, greylag goose, mallard to columba livia besides fowl and turkey (Jonassen *et al* 2005). After identifying SARS as a coronavirus, it has been supposed that the SARS-CoV is also the result of the combination of coronaviruses with all kind of different genes (Ksiazek *et al* 2003). Therefore, it is important to study

the variation rules of the representative infectious bronchitis virus (IBV) strains, since it would help to develop husbandry and protect the security of sanitation (Kapczynski *et al* 2003).

The main infectious object of the avian coronavirus is poultry, with the IBV being the most representative (Breslin *et al* 1999). IBV causes a highly infectious disease called infectious bronchitis (IB) which is prevalent in all countries causing severe economic losses to the poultry industry and it is regarded as the most important poultry disease. There are more than 30 serotypes of IBV and dozens of virulence strains, with a variation being developed from time to time (Casais *et al* 2003). At the same time, tissue tropism of IBV has evolved, transferring from respiratory and urinary organs to digestive tracts and generative organs. The accumulation of variation in tissue tropism of IBV increases the probability of interspecies transmission between animals (Ignjatovic *et al* 2002).

The S1 protein is a structure protein exposed to the surface of IBV and its variation is rather common. The variation contributed to the change of antigenic epitopes on neutralizing site, causing the variation of tissue tropism of IBV at same time (Gallagher *et al* 2001). The S1 protein also decides the toxicity and pathogenicity of the

Aceptado: 28.11.2006.

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virus and it is closely linked to immunogenicity (Jackwood *et al* 1995). It also induces various virus neutralizing antibodies, hence the investigation of the S1 gene plays an important and representative role in the research of IBV's molecular biology and it is also important for the further investigation of the pathogenic mechanism and the use of vaccines to prevent IBV (Estevez *et al* 2004). In order to obtain adequate information for studying the relationship between the variational characteristic of the S1 gene structure, pathogenicity and tissue tropism of IBV, we analyzed the S1 gene structure isolated from infectious domestic poultry in Shanghai area. The identity in the S1 gene was also studied by comparing the S1 sequence with some reference strains and domestic epidemic strains of IBV.

MATERIAL AND METHODS

Samples. The samples used in order to isolate the virus were collected using cotton pledgets from the oral cavity and laryngotrachea of the diseased pigeons and fowl expressing main clinical signs of depression, weakness, wheezing, watery eyes, and tracheal rales. The diseased poultry came from four infected chicken farms and one pigeon farm in Shanghai and nearby territories. Later, the pledgets were put into the sterilized physiologic phosphate buffered saline.

Animals. SPF embryonated chicken eggs and healthy pigeons were purchased from Shanghai Academy of Agricultural Sciences and Shanghai Jiaotong University Laboratory Animal Technology Co., Ltd, respectively. Embryonated chicken eggs were used for isolation of field isolates and re-isolation attempt of coronavirus from pancreatic tissues of the experimental infected pigeons. Healthy pigeons were used for the pathogenicity experiment.

Reagent. *E. coli* DH5a and IBV strain M41 are stored in our laboratory. RT-PCR kit (AMV) and plasmid pMD18-T were purchased from Takara. Co., Ltd, Japan; 3S Spin DNA Agarose Gel Purification Kit, the RNA abstraction Trizol kit were purchased from Shenergy Biocolor Co., Ltd, Shanghai, China.

Virus isolation. Samples of the diseased fowl and pigeons were extracted and homogenized (25% w/v). After being subjected to freeze-thawing for three times, the suspension was centrifugated at 6000g at 4°C for 15 min, filtered through 0.45 µm membrane and dissolved in penicillin and streptomycin with a final concentration of 2000 unit/ml. Then the samples were inoculated into 10-day-old SPF-embryonated eggs (0.3 ml per embryo) by intra-allantoic route and underwent incubation at 37°C for virus isolation. After 72 h of incubation part of the eggs allantoic fluid was harvested and was used as inoculum

for further serial passage (0.2 ml per embryo). The other embryonated eggs were incubated for 120 h at 37°C to observe the pathological changes on embryos. The infected allantoic fluid was then examined by transmission electron microscopy (Laboratory of Shanghai Jiaotong University).

Viral RNA extraction and RT-PCR amplification of S1 gene. The harvested allantoic fluids containing isolates from the original material of field-infected animals and experimentally infected animals were used to prepare viral RNA. A total of 250 µl of the isolate was dissolved in 800 µl of Trizolblue reagent and RNA was isolated according to the manual. The RNA obtained was resuspended in 20 µl of RNAase-free water, and heated at 58°C for 10 min. Primers S1oligo 5' (5'-TGA AAA CTG AAC AAA AGA CAG A -3') and S1oligo 3' (5'-GGG CAA CTT GTT ACA TTT TCA C-3') were designed based on Massachusetts strain (M41) published in GenBank (accession number X04722) and used to amplify the whole S1 coding sequence of recent isolated viruses SH1, SH2, SH3, SH4 and PSH. The four isolates that came from fowl were named SH1, SH2, SH3 and SH4, while the one obtained from pigeon was named PSH. The RT mixture to synthesize cDNA contained genomic RNA, 20 pmol S1oligo3' primer and RNA PCR kit (AMV) Ver.3.0 (Takara, Japan). The RT reaction was carried out by incubating the mixture at 48°C for 30 min, heated for 5 min at 99°C, and then 5 min at 5°C to stop the reaction. The PCR was performed by predenaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 60 sec, annealed at 48°C for 40 sec, and polymerization at 72 °C for 120 sec. The final polymerization step was performed at 72°C for 10 min. The PCR products were analyzed on a 1.0% agarose gel.

Pathogenicity test of pigeons virus isolated. 30-day-old normal healthy pigeons were randomly divided into 3 groups with 10 birds in each group. Each pigeon in group 1 was inoculated with 0.2 ml of the allantoic fluid containing the isolate PSH, while each pigeon in group 2 was inoculated with the IBV M41 strain via trachea and muscles, respectively. The third group was used as the negative control. All pigeons were placed in the isolation units in different rooms. The inoculated birds were observed for clinical signs and disease course, and were later killed for observation of pathological changes on tissues of pancreas, kidney, trachea and lung. These tissue samples were processed as described previously for virus re-isolation and pathological changes observation.

DNA sequencing and sequence analysis. PCR products of the 5 isolated strains were purified using 3S Spin DNA Agarose Gel Purification Kit according to the manual. Then the purified PCR products were cloned into the cloning vector (pMD18-T) (Takara, Japan) and transformed

into competent cells (TOP10) (Invitrogen). Cells carrying recombinant plasmid were selected on LB agar plates containing ampicillin (5 µg/ml) and X-gal (80 µg/ml). Plasmid DNA for sequencing was prepared by 3S Spin Plasmid Maxi Kit. Plasmid DNA was digested by Eco R1 and Pst1 restriction enzyme (Takara, Japan) and electrophoresed on 1% agarose gel to confirm the size of the insert. Sequencing was performed with the M13 forward and M13 reverse primers. DNA sequencing was performed using an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City). The alignment and phylogenetic analysis of S1 gene sequences and its deduced amino acid sequences were performed using the Clustal method by DNASTar (DNASTAR, Madison, WI, USA), DNAMAN and other sequence analysis software. The strains of IBV used in this study and their GenBank accession numbers are: Massachusetts 4 (M41) (X04722), Holland 120 (H120) (M21970), Holland 52 (H52) (AF352315), Beaudette (M95169, Connecticut (L18990), Holte (L18988), and Gray (L14069).

RESULTS

Isolation and propagation, morphology observation of virus. SPF embryonated chicken eggs were inoculated with the suspension containing suspected isolates and allantoic fluids of a part of the eggs was harvested for further passage in embryos after 72 h incubation at 37 °C. The other SPF chicken eggs were incubated for 120 h for observation of the gross lesions on embryos. By increasing the number of passages, the appearance of the dwarf egg and mortality rate gradually grew. In the third passage, the presence of pathological changes on embryos which were similar to those of IB could be observed. The gross lesions of the infected embryos were haemorrhages on the legs, mottled necrosis of the liver, swelling of the kidneys, dwarfing and curling. Electron microscopy was used to determine the size of the virus isolated which was 80-130 nm diameter. The virions were coronal, round-shaped and covered with an envelope connected to pedunculate projections, similar to the IBV, so it can be preliminarily concluded as IBV. No other virus was observed.

Animal pathogenicity test of PSH. 20 days after inoculating healthy pigeons with incubation fluid of this virus, symptoms as tracheal rale, depressed and ruffled feathers, and depression, were found in pigeons of group 1. Pancreas affections and kre-necrosis in lung occurred in all infected pigeons. In addition, punctate hemorrhages in bronchus and abnormal increase of phlegm was found. Anatomical results showed excess mucus in the pigeon's trachea with distinct hemorrhage and engorged swollen pancreas as well as flesh-like lesions in the lung. After chicken embryos were inoculated with a 0.3 ml dose of the pancreatic suspension of the experimentally infected

chickens, the infected embryos revealed similar lesions when compared to the ones inoculated with the original material of the field-infected pigeons. In end, the infected birds showed a 16.6 % mortality, while clinical signs and pathological changes did not appear in group 2 (M41) and control group. Part of the S1 gene was amplified by RT-PCR from the infected pigeons, and the result of sequencing proved to be true.

Detection of RT-PCR production. 8 µl RT-PCR product were detected on 1% agarose gel electrophoresis, and the results indicated that the length of the amplified product was about 1.6 kb, agreeing with the length of the expected sequence of the S1 of IBV (figure 1).

Analysis of S1 gene sequence. The nucleotide sequences of S1 genes of all the 4 strains from chickens, and the isolated strain from pigeon, had a length of 1626 bp and encode 541 amino acid residues. Compared to the sequence of selected reference strains IBV strains - M41, H120, H52, Beaudette, Holte, Gray and Connecticut published in GenBank (table 1), the 4 strains from chickens (SH1, SH2, SH3, SH4) exhibited 77.4%-82.9% identity in the S1 gene sequence, with the deduced identity of the protein sequence being 74.7%-82.6%. The details of these results are as follows: SH1 S1 gene sequence identity was 79.2%-81.9% and amino acids identity was 75.1%-79.9%; SH2 was 78.2%-82.4% and 75.4%-79.6%, SH3 was 77.4%-82.9% and 74.7%-82.6%, SH4 was 77.9%-82.1% and 74.7%-79.3%. The strain from pigeon (PSH) exhibited 79.3%-99.6% identity in S1 gene sequence, with the deduced identity of the protein sequence being 81.6%-99.6%.

Compared to TCoV (Turker coronavirus)(Gh and G1) strains, the deduced amino acid sequence of S1 protein of the isolate showed closer phylogenetic position to those

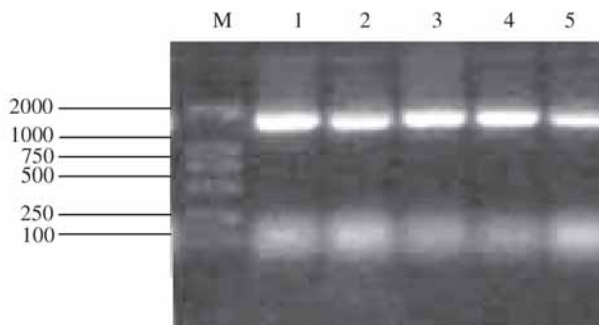


Figure 1. The RT-PCR electrophoresis result of IBV SH1 S1 gene.

Resultado de electroforesis del gen IBV SH1 S1.

M = Mark DL-2000. 1 = RT-PCR production of SH1. 2 = RT-PCR production of SH2. 3 = RT-PCR production of SH3. 4 = RT-PCR production SH4. 5 = RT-PCR production of PSH.

Table 1. Identity of gene sequence and deduced amino acid of S1 among the avian coronavirus strains.
 Identidad de secuencia del gen y aminoácido deducido de S1 entre las cepas de coronavirus aviar.

	SH1	SH2	SH3	SH4	PSH	M41	Beau	Conn	Gray	H120	H52	Holte	Gh	G1
SH1		89.1	80.4	88.6	87.3	81.9	81.2	81.2	79.3	81.5	81.2	79.2	45.1	44.3
SH2	87.1		82.6	99.3	99.8	82.4	82.0	81.8	79.4	81.7	81.8	78.2	44.6	43.7
SH3	80.8	82.8		82.2	99.3	82.9	82.2	82.5	78.0	82.5	82.8	77.4	44.5	43.9
SH4	86.1	98.9	81.7		99.4	82.1	81.5	81.4	79.3	81.6	81.3	77.9	44.6	43.7
PSH	87.3	99.6	99.6	99.4		79.4	79.3	79.8	79.6	79.4	79.3	79.8	37.4	37.8
M41	82.6	79.9	81.6	78.3	84.4		97.8	97.2	83.7	97.6	97.2	79.9	49.3	48.5
Beau	79.4	79.2	81.1	78.3	84.0	95.1		97.0	83.7	97.4	97.4	79.9	49.8	48.9
conn	79.2	78.9	81.1	77.9	82.6	95.4	95.6		83.6	99.3	99.1	79.6	48.6	47.6
Gray	77.2	77.9	76.5	77.4	89.3	80.2	80.9	80.4%		83.3	83.4	82.3	43.4	42.7
H120	79.9	79.6	82.6	79.3	89.3	96.1	96.3	99.1	80.6		99.6	79.5	48.7	47.9
H52	79.7	79.4	82.5	79.1	84.4	95.2	96.1	98.5	80.2	99.1		79.6	49.1	48.2
Holte	74.7	75.1	74.7	74.7	81.6	75.5	74.8	75.0	77.5	74.9	74.9		43.5	43.2
Gh	24.2	24.4	25.2	24.2	24.5	27.1	27.0	26.4	24.9	26.8	22.7	25.0		95.6
G1	24.5	24.7	25.5	24.7	24.8	27.0	26.9	27.1	25.0	26.8	26.6	25.9	93.6	

IBV strains. All the 5 isolates had a nucleotide sequence similarity between 77.4% and 99.8% with IBV strains and they had only nucleotide sequence similarity between 37.4% and 49.1% with TCoV (Gh and G1) strains. The 5 isolates had amino acid sequence similarity between 74.7% and 99.6% with IBV strains and an amino acid sequence similarity between 22.7% and 27.1% with TCoV (Gh and G1) strains.

The PSH was more related to group 3 coronaviruses (IBV, TCoV) than to members of group 1 (TGEV, PEDV, FCoV, CCoV and HCoV 229E) and group 2 (HCoV OC43, BCoV and MHoV) coronaviruses. In addition, higher identity could be observed between PSH and IBV strains compared with TCoV strains. The deduced amino acid sequence of S1 gene of the isolate PSH was most similar to that of field IBV isolates SH1 (87.3%), SH2 (99.6%), SH3 (99.6%) and SH4 (99.4%) which were all isolated synchronously from the diseased flocks in Shanghai, China and showed lower identities to other IBV strains and TCoV strains M41 (79.4%), 225 H120 (79.4%), H52 (79.3%), Beaudette (79.3%), Connecticut (79.8%), Gray (79.6%), Holte (79.8%) and Gh (37.4%), G1(37.8%).

Also, there is a low identity among these strains when compared to nucleotide sequence of SH1, SH2, SH3, SH4 and amino acid sequence deduced by those with reference IBV strains - M41, H120, H52, Beaudette, Holte, Gray, Connecticut in Genbank. Nucleotide sequence identity is 77.4%-82.9%, and amino acid sequence identity is 74.7%-82.6%. Comparatively, these four strains have relatively high identity as 81.2%-82.9% with breathing pattern strains - M41, Beaudette, H120, H52, while they have low identity as 77.4%-79.4% with Gray, Holte.

The cladogram analysis indicated that SH1, SH2, SH3, SH4 are far from vaccine and other serotype strains in evolution (figure 2). There is low identity among strains. The highest gene sequence identity is 82.9% and the lowest is 77.4%. SH1, SH2, SH4 have high identity when comparing the strains from different areas, however, SH3 has low identity with other strains. The

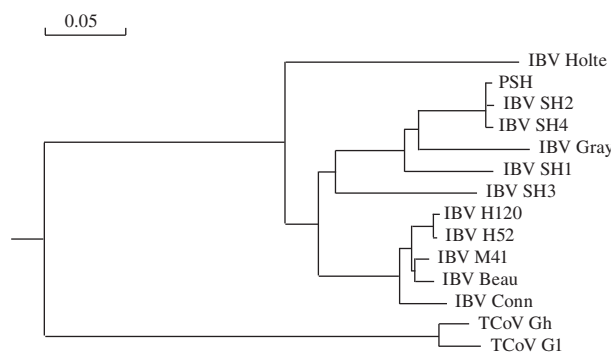


Figure 2. Cladogram based on the deduced amino acid sequence of spike glycoprotein of avian coronavirus strains.

Cladograma basado en la secuencia de aminoácidos deducida de glicoproteína de la cepa coronavirus aviar.

Abbreviations:
 IBV = infectious bronchitis virus; S gene = spike glycoprotein gene; TCoV = turkey coronavirus; SPF = specific-pathogen-free; RT-PCR = reverse transcription-polymerase chain reaction; HCoV = human coronavirus; TGEV=porcine transmissible gastroenteritis virus; CCoV = Canine coronavirus; FCoV = feline infectious peritonitis virus; MHoV = murine hepatitis virus; BCoV = bovine coronavirus; PEDV = Porcine Epidemic Diarrhea virus SARS = Severe Acute Respiratory Syndrome coronavirus.

identity between SH2 and SH4 is 99.3%. A high degree of sequence identity (79.3-99.8%) was observed between the S1 sequence of PSH and the published sequences of infectious bronchitis virus.

A further analysis of the results also indicated that the cleavage site sequence of S1 protein of these isolates contains 5 consecutive basic amino acids, namely Arg-Arg-Phe-Arg-Arg (RRFRR), similar to most of the IBV strains.

DISCUSSION

The variation of the S1 strain genetic structure. It has been proved that the main molecular basis for the variation of IBV were point mutation, insertion, absence of viral gene and the genetic recombination between different strains (Lee *et al* 2000). More point mutations exist in the five strains isolated from Shanghai than in other strains when comparing the nucleotide sequence of SH1, SH2, SH3, SH4 and PSH with that of reference strains in GenBank. The cladogram analysis indicated that the five isolated strains are far from vaccine and other serotype strains in evolution and it also indicated that frequent use of vaccine strains H52, H120 in chicken farms may result in a combination of various virus genes (Zhou *et al* 2004). PSH isolate had a nucleotide sequence similarity between 77.9% and 99.8% with IBV strains and it had a nucleotide sequence similarity between 37.4% and 49.1% with TCoV strains. A high degree of sequence identity (79.3-99.8%) was observed between the S1 sequence of PSH and published sequences of avian infectious bronchitis virus (IBV). Compared with TCoV, the deduced amino acid sequence of S1 protein of the isolate PSH showed a close genetic relationship with IBV strains.

The hosts of IBV. An increasing number of researchers consider that the host range of IBV has been enlarging (Schikora *et al* 2003). Fowl, turkey and other bird species can be infected by IBV (Jonassen *et al* 2005). Barr *et al* (1988) firstly proved infection of IBV in the carrier pigeon, and he also found that this strain and the vaccine strain which had been generally applied in that place belonged to the same serotype. Soon after, Cavanagh *et al* (2002) reported an infection by coronaviruses and the close genetic relationship between IBV and TCoV through the analysis of genetic structure. Liu *et al* (2005) reported the infection of the peacock, partridge and mallard by IBV. The strain isolated in this work has the closest genetic relationship with IBV and it was isolated from recently infected fowl. This strain has 99.6% gene identity with the IBV SH2. Enzyme recognition site on spike protein of PSH is Arg-Arg-Phe-Arg-Arg (RRFRR), similar to that on spike protein (RF/SRR) of IBV (Jackwood *et al* 2001).

The pathogenicity character of IBV that affected pigeons is similar to that of IBV that affected fowl, which was the respiratory symptom. An animal regression experiment was conducted. Anhelation, tracheal rales, asthenia universalis and dysplasia was found in the affected pigeons. Besides the punctate hemorrhage in respiratory tract, increase of secretory juice and kre-necrosis in lung, typical pathological changes occurred in pancreas. Pancreas was swelling and bleeding and a high viral isolation rate was obtained from pancreas, which leads to the conclusion that IBV strains show tropism to pancreas. Further studies need to be carried out regarding the role pigeons play in the spreading of IBV in these areas and whether the pigeon is a natural host of IBV.

Based on these findings we concluded that molecularly, the five isolated strains from Shanghai have a close relationship with IBV reference strains, they belong to IBV although the PSH was isolated from pigeons.

SUMMARY

The aim of the study was to analyze the similarity of the S1 gene in 5 novel viral strains of infectious bronchitis virus (IBV) isolated in Shanghai, China, using a pair of specific primers which were designed based on the published sequences of the S gene of IBV. The 5 full-length cDNA segments of the S1 gene were amplified from the 5 isolated strains with RT-PCR. It was confirmed that the nucleotide sequences of the S1 gene in the 5 strains isolated from chickens and pigeon have a length of 1626 bp and encode 541 amino acid residues. Compared to the sequence of selected reference IBV strains published in GenBank, the 4 strains from chickens exhibited 77.4%-82.9% identity in the S1 gene sequence, with the deduced similarity of the protein sequence being 74.7%-82.6%, while the strain from pigeon exhibited 79.3%-99.6% similarity in the S1 gene sequence, with a deduced identity of the protein sequence of 81.6%-99.6%. The S1 protein cleavage site sequence of these isolates contains 5 consecutive basic amino acids, named, Arg-Arg-Phe-Arg-Arg (RRFRR) similar to most of IBV strains. The results indicated that the variability range of the S1 gene structure is high, corresponding with the biological character of IBV, and that molecularly the five isolated strains from Shanghai have a close relationship with the IBV reference strains. It was concluded that the five isolated strains belong to IBV, even though one of them was isolated from pigeons.

ACKNOWLEDGEMENT

This work was financially supported by a grant from the anti-SARS Science Foundation of Shanghai Jiao Da Onlly Co., Ltd. (Grant No. SARS03-03).

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