日本財団 助成事業
The Nippon Foundation

財団法人日中医学協会
2008年度共同研究等助成金－調査・共同研究－報告書

2009 年 3 月 15 日

財団法人　日 中 医 学 協 会　御中

貴財団より助成金を受領して行った研究テーマについて報告いたします。

添付資料：**研 究 報 告 書**

受給者氏名：余 福 勲 ㊞

所属機関名：長崎大学熱帯医学研究所

所 属 部 署：ウイルス学分野　職名：助教

所　在　地：〒852-8523長崎市坂本1－12－4

電話：095-819-7829　内線：7829

1．助 成 金 額：100万 円

2．研 究 テ ー マ　SARS(新型肺炎)の血清学的診断法確立並びに血清免疫学的研究

3．成 果 の 概 要（100字程度）

今回バキュロウイルス発現系を使って組換えN蛋白抗原を用いた間接IgG ELISAを開発した。本法は感染性が無く、安全で、特異性が高く、感度が高い診断系であり、SARS の診断、調査および疾病予防への応用が十分期待できる。

※発 表 論 文 等

結果をまとめ、国際雑誌に投稿する予定

4．研 究 組 織

日本側研究者氏名：余福勲　職名：助教

所属機関：長崎大学熱帯医学研究所　部署：ウイルス学分野

中国側研究者氏名：張奉学　職名：教授

所属機関：広州中医薬大学熱帯医学研究所　部署：ウイルス学分野

－日中医学協会助成事業－

SARS(新型肺炎)の血清学的診断法確立並びに血清免疫学的研究

研究者氏名　余福勲
所属機関　長崎大学熱帯医学研究所ウイルス学分野助教
共同研究者名　張奉学
所属機関　中国広州中医薬大学熱帯医学研究所教授

Abstract

Severe acute respiratory syndrome (SARS) is a newly emerged human disease associated with pneumonia originated from Guangdong province of China. The disease has affected 30 countries in 5 continents, with more than 8400 cases and more than 910 deaths during the outbreak in 2003. SARS has been identified as an emerging zoonotic diseases with bats are the most possible natural reservoirs. Hence the threat of re-emergence of SARS persists although there is no outbreak of SARS-CoV after 2003. Since there are no suitable antiviral drugs and effective vaccine for SARS virus, active surveillance is the key measure for preventing future SARS outbreaks. Sensitive and specific sero-diagnosis of SARS-CoV infection in wild animals and human is critical for SARS surveillance.

We reported that an N-terminus truncated E.Coli originated recombinant N protein based IgG ELISA was more sensitive compared with whole virus based ELISA system, but has low titer of false positive reaction when evaluated with healthy volunteer sera (10). The cross reaction of recombinant SARS-CoV with other corona viruses were also reported (7,8).Leung DT et al reported that the cross reactivity of recombinant N protein was originated from the E.Coli bacteria, not from the SARS-CoV(3). To confirm if the low rate of cross reactivity was really caused by the expression of N protein in E.Coli and to develop a safe, sensitive and specific sero-diagnosis method for surveillance and clinical use, we expressed and purified the whole length and N-terminus truncated SARS-CoV N protein in insect Tn5 cell line by using the BAC-TO-BAC baculovirus expression system. Indirect IgG ELISA systems for human and bat sera were developed using the recombinant N proteins as antigen.

Compared with E.Coli derived N and $N_{\Delta 121}$ based IgG ELISA ,insect cell derived N and $N_{\Delta 121}$ based IgG ELISA have the same sensitivity in detecting SARS patient sera, out of 37 patients, 36 were positive, positive rate was 97.3%. When evaluated with healthy human sera, insect cell derived N based ELISA was more specific than E.Coli derived N based ELISA while the two $N_{\Delta 121}$ based ELISA has similar specificity, indicating the E.Coli origin was only partially responsible for the low titer of non-specific reaction.

In surveillance of human sera from different age groups in Guangzhou, China, the low titer of SARS-CoV IgG positive rate increased as the age increases, implying the low titer of cross reactivity was caused by other closely related corona viruses.

Key words

SARS-CoV, recombinant N protein,sero-diagnosis,ELISA

Introduction

Severe acute respiratory syndrome (SARS) is a newly emerged human disease associated with pneumonia. The outbreak is believed to have originated in November, 2002 in the Guangdong province of China with several hundred of cases of severe atypical pneumonia (1,10). The disease quickly affected 30 countries in 5 continents, with more than 8400 cases and more than 910 deaths (9). Several laboratories have unraveled the genetic Information of the SARS virus. The genome size of the SARS coronavirus is ~29 kb and has 11 open reading frames, composed of a stable region encoding an RNA-dependent RNA

polymerase with 2 open reading frames, a variable region representing 4 coding sequences for viral structural genes [spike (S protein), envelope (E protein), membrane (M protein), and nucleocapsid (N protein)], and 5 putative uncharacterized proteins (5, 6).

The rapid identification of highly similar viruses in masked palm civet and raccoon dog in the live-animal markets provided strong evidence of an animal origin of SARS-CoV and played an important role in the prevention of further outbreaks (2, 4). However, subsequent epidemiological studies on civets from market, farm and wild populations demonstrated that there was no widespread infection among wild or farmed civets, implying that wild animal(s) other than civets may serve as the natural reservoir(s) of SARS-CoV. In efforts to search for the origin of SARS-CoV, several research groups reported that bats may be the natural reservoirs of SARS-CoV (2, 4). Hence the threat of re-emergence of SARS persists although there is no outbreak of SARS-CoV after 2003.

Currently, there are no suitable antiviral drugs and effective vaccine for SARS virus. Therefore active surveillance is the key measure to prevent future SARS outbreaks. Sensitive and specific sero-diagnosis of SARS-CoV infection in wild animals and human is critical for the active surveillance.

We reported that a N-terminus truncated E.Coli originated recombinant N protein based IgG ELISA was safe and more sensitive compared with whole virus based ELISA system, but has low titer of false positive reaction when evaluated with healthy volunteer sera (10). The cross reaction of recombinant SARS-CoV with other corona viruses were also reported (7,8).Leung DT et al reported that the cross reactivity of recombinant N protein was originated from the E.Coli bacteria, not from the SARS-CoV(3). To confirm if the low rate of cross reactivity was really caused by the expression of N protein in E.Coli and to develop a safe, sensitive and specific sero-diagnosis method for surveillance and clinical use, we expressed and purified the whole length and N-terminus truncated SARS-CoV N protein (N and $N_{\Delta 121}$)in insect Tn5 cell line by using the BAC-TO-BAC baculovirus expression system. Indirect IgG ELISA systems for human and bat sera were developed using the recombinant N proteins as antigen. SARS patients sera and healthy human sera were used to evaluate the newly established systems and sero-surveillance was conducted in Guangzhou, China, the original place of SARS.

Materials and Methods

Cloning, expression, and purification of the N proteins of SARS-CoV. The SARS-CoV N gene and truncated N gene were amplified by RT-PCR as previously described(10). The 1.3-kb and 0.9-kb PCR amplified DNA fragments were digested with BamHI and Sal I and cloned into the corresponding restriction site of pFastBacHT B vector. Competent *Escherichia coli* DH10BAC cells, containing bacmid (baculovirus shuttle vector plasmid) and helper plasmid, were used to generate recombinant bacmids according to the manufacturer's instructions (BAC-TO-BAC baculovirus expression system; Life Technologies). The recombinant bacmid DNA was transfected into insect cells using the CELLFECTIN reagent.Tn 5 cells were cultured at 27°C in Sf-900 II SFM. For each transfection, 9×10^5 cells were seeded in 35-mm wells of a six-well plate and allowed to attach for ~1 h. Lipid reagent and DNA were diluted separately into 100 µl of Sf-900 II SFM cells and then combined to form lipid-DNA complexes, which were then diluted to 1 ml with SFM and added to the cells. The cells were incubated for 5 h at 27°C after which the transfection medium was removed and replaced with fresh medium. These cells were analyzed for protein expression at 24 to 72 h post transfection. Positive recombinant viral supernatant was collected at 72 h post transfection and used for large scale culture of insect Tn5 cell inoculation.

The recombinant N and $N_{\Delta 121}$ protein fused to a histidine affinity tag at its N terminus was purified using nickel-nitrilotriacetic acid (Ni-NTA) resin. Briefly, Tn5 cells at a density of about 1.5×10^6/ml in shaker flasks

were infected with the recombinant baculovirus at a multiplicity of infection (MOI) of about 5 and incubated with shaking for 72 h at 27°C. The infected cells were harvested and the pellet was resuspended in lysis buffer (50 mM Tris-HCl [pH 8.5], 5 mM 2-mercaptoethanol, 100 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 1% Tween 20. After clarification by centrifugation at 30,000 x *g* for 15 min. The supernatant was loaded onto an Ni-NTA column, after washing the protein was eluted from the column with 5 ml of buffer C (20 mM Tris-HCl [pH 8.5], 5 mM 2-mercaptoethanol, 100 mM KCl, 10% [vol/vol] glycerol, 500 mM imidazole. The samples were collected in 0.5-ml fractions and analyzed by SDS-PAGE and Western blotting. Protein concentrations were determined according to the Bradford method using bovine serum albumin (Sigma) as the standard.

Western blot analysis. Western blot was performed as described previously (10). A semi-dry protein transfer method was used to immobilize the recombinant proteins to a polyvinylidene difluoride (PVDF) membrane. Briefly, protein separated in a 10% polyacrylamide gel was transferred to a PVDF membrane (Immobilon, Millipore) using a semidry electroblotter (Sartorius, Germany). The membrane was initially blocked overnight at 4 ^{o}C with Blockace, then reacted with Blockace diluted mouse anti-histidine (1:200) or patient serum(1:100) for 1h at 37 ^{o}C, incubated with rabbit anti-mouse IgG or goat anti-human peroxidase conjugate diluted 1:1000 with TBS-T for 1h. The reaction was visualized by DAB(Wako, Japan).

Production of bat hyperimmune sera. Two fruit bats were injected intraperitoneally with 100 µl (100 µg) of purified recombinant N protein emulsified with an equal volume of Freund's complete adjuvant (MP Biomedicals, USA). Two booster injections were given by the same dose of N protein in Freund's incomplete adjuvant (MP Biomedicals, USA) with 14 days intervals. One week after the final booster injection,bat immune sera were collected .

ELISA using recombinant nucleocapsid proteins. Indirect IgG ELISA systems were established using the recombinant N and $N_{\Delta 121}$ protein obtained from insect cell like previously described (10). 175 serum samples collected from healthy volunteers before the SARS outbreak and 150 serial serum samples collected from 37 SARS patients with pneumonia were used for the assessment of the newly established systems. The results were compared with ELISA using E.Coli originated recombinant systems. IgG ELISA for bat was established similarly with the second antibody using 1:10,000 diluted HRP-conjugated anti-bat IgG (Funakoshi, Japan).

Sero-surveilance in Guangzhou, China. Sera samples were collected from different age groups in outpatients in Guangzhou,China. Age 0~5 years: 100 samples; 5~10 years: 50 samples, 10~20 years: 50 samples; 20~40 years: 50 samples; above 40 years: 50 samples. The baculovirus expressed $N_{\Delta 121}$ protein based IgG ELISA was used to check the SARS-CoV IgG antibody. Questionnaires about pneumonia, hospitalization, occupation and life style were asked.

Results

Expression and purification of recombinant SARS-CoV N proteins. To produce recombinant SARS-CoV N proteins, the sequence coding for amino acid residues 5-422 and 122-422 of the nucleocapsid protein was amplified by RT-PCR and cloned into the BamHI and SalI sites of pFastBacHT B vector in frame and downstream of the six histidine tag. The recombinant proteins were expressed in Tn5 insect cells and purified by Ni-NTA affinity column under natural conditions. Analysis of purified recombinant protein by SDS-PAGE and Coomassie blue staining revealed a single protein band of 52 kD and 40 kD respectively. The resultant recombinant protein bands are 8 kD bigger than the recombinant protein obtained from E.Coli because of the extra amino acid originated from the pFastBacHT B vector. The identity of the recombinant

SARS-CoV N and SARS-CoV $N_{\Delta 121}$ protein was further confirmed by western blot with mouse antiserum to histidine and SARS patient serum.

Assessment of recombinant N and $N_{\Delta 121}$protein ELISA using healthy volunteers sera. Serum samples collected from 175 health volunteers before the SARS outbreak were used to for the assessment of indirect IgG ELISA test for the insect cell derived recombinant SARS-CoV N and $N_{\Delta 121}$ protein compared with E.Coli derived N and $N_{\Delta 121}$ based IgG ELISA. In E.Coli derived N based ELISA, 38 out of the 175 serum samples has a titer of more than 100, ranging 100~3,200, while in insect cell derived N ELISA, 28 out of the 175 serum samples has a titer of more than 100, ranging 100~400. In E.Coli derived $N_{\Delta 121}$ ELISA, 11 out of the 175 has a titer above 100, the highest titer was 400, while in insect cell derived $N_{\Delta 121}$ ELISA, 10 out of the 175 has a titer above 100, the highest titer was 400. The positive samples were all confirmed by western-blot. Insect cell derived N based ELISA was more specific than E.Coli derived N based ELISA while the two $N_{\Delta 121}$ based ELISA has similar specificity, indicating the E.Coli origin was only partially responsible for the low titer of non-specific reaction.

Assessment of recombinant N and $N_{\Delta 121}$ protein ELISA using SARS patients sera. Serial serum samples collected from 37 SARS cases were assessed for the insect cell derived N and $N_{\Delta 121}$ ELISA compared with E.Coli derived N and $N_{\Delta 121}$ based IgG ELISA . In all ELISA systems, 36 patients showed specific IgG seroconversion, the titer ranged from 600 to 204,800. The positive rate was 97.30%. All the positive samples were confirmed by western-blot. Among the 36 IgG positive patients, the IgG seroconversion rate to the N protein was 22.22% in less than 7 days after the onset of fever, 69.44% in less than 2 weeks and reached 100% in less than 3 weeks.

In the recombinant $N_{\Delta 121}$ –based IgG ELISA, the highest titer of the 175 health volunteers is 400, the SARS patient's sera titer is 600 or above, so we set the cut-off titer of our $N_{\Delta 121}$ –based IgG ELISA as 400 to ensure a 100% specificity of the assay system.

Human sera surveillance in Guangzhou province, China. The SARS-CoV IgG titer in different age groups in Guangzhou was determined by the baculovirus expressed $N_{\Delta 121}$ protein based IgG ELISA. In individuals aged 0~5 years group, out of 100 people, no SARS-CoV IgG was detected. In age 5~10 years group, 2 out of 50 people (4%) have IgG titer 100~200. In age 10~20 years group, 3 out of 50 people (6%) have IgG titer 100~400. In age 20~40 years group, 4 out of 50 people (8%) have IgG titer 100~400. In age 40 years group, 4 out of 50 people (8%) have IgG titer 100~400. The positive people had no past history of SARS like disease and close contact with wild animals.

Discussion

The predicted N protein of SARS-CoV is a highly charged basic protein of 422 amino acids with seven successive hydrophobic residues near the middle of the protein. Although the overall amino acid sequence homology among coronavirus N proteins is low, a highly conserved motif [FYYLGTGP] occurs in the N-terminal half of all coronavirus N proteins, including that of SARS-CoV. Other conserved residues occur near this highly conserved motif (6). It was reported that the most immunoreactive epitopic site in the SARS coronavirus N protein is located at the COOH terminus of the N protein.This made it possible theoretically to remove the N terminus part including the conserved motif to reduce the cross reactivity for SARS diagnosis. We expressed a truncated SARS-CoV $N_{\Delta 121}$ protein, which deleted the highly conserved motif [FYYLGTGP]. In our insect cell derived and E.Coli derived $N_{\Delta 121}$ ELISA, lower titer and lower positive rate were detected in healthy human sera, indicate that the SARS-CoV N protein has cross reactivity with other corona viruses and it is partially caused by the conserved motifs. Leung DT et al reported that the cross reactivity of recombinant N protein was originated from the E.Coli bacteria, not from the SARS-CoV(3). In our study, when evaluated

with healthy human sera, insect cell derived N based ELISA was more specific than E.Coli derived N based ELISA while the two $N_{\Delta 121}$ based ELISA has similar specificity, indicating the E.Coli origin was only partially responsible for the low titer of non-specific reaction.

Compared with E.Coli derived N and $N_{\Delta 121}$ based IgG ELISA ,insect cell derived N and $N_{\Delta 121}$ based IgG ELISA have the same sensitivity in detecting SARS patient sera, out of 37 patients, 36 were positive, positive rate was 97.3%. The titer was ranging 600~204,800.Among the 36 positive cases, the timing of IgG sero-conversion against $N_{\Delta 121}$ protein after onset of illness were 22.2% in the first week, 69.4% in the second week and reached to 100% in the third week. Indicated $N_{\Delta 121}$ based IgG ELISA is a sensitive assay system for SARS diagnosis. In the recombinant $N_{\Delta 121}$ –based IgG ELISA, the highest titer of the 175 health volunteers is 400, while the SARS patient sera titer is 600 or above, so we set the cut-off titer of our $N_{\Delta 121}$ –based IgG ELISA as 400. This cutoff titer ensures 100% specificity of SARS diagnosis.

In surveillance of human sera from different age groups in Guangzhou China, the low titer of SARS-CoV IgG positive rate increased as the age increases, implying the low titer of cross reactivity was caused by other closely related corona viruses. The positive people had no past history of SARS like disease and close contact with wild animals. We hypothesize that a yet unidentified SARS-CoV close related virus might be endemic in the study area. Further studies are needed to identify the origin of this low titer of reactivity. This result also demonstrated that a properly cut-off is very important for sero-diagnosis and care should be taken in diagnosis when facing isolated low titer of positive samples.

In conclusion, our newly developed SARS-CoV $N_{\Delta 121}$ protein-based IgG ELISA is a safe, specific and sensitive test for diagnosing SARS-CoV infection. It will contribute to the SARS diagnosis, surveillance, prevention and control.

References

1. Ksiazek TG, Erdman D, Goldsmith CS, et al. 2003.. N Engl J Med. 348:1953-1966.
2. Lau SK, Woo PC, Li KS,et al.Proc Natl Acad Sci U S A. 2005,27;102:14040-5.
3. Leung DT, van Maren WW, Chan FK,et al .J Virol. 2006,80:8920-8.
4. Li, W., Shi, Z., Yu, M. et al . Science 2005, 310, 676–679.
5. Marra, M. A., S. J. Jones, C. R. Astell, et al. 2003. Science 300:1399-1404.
6. Rota, P.A., M.S. Oberste, S.S. Monroe, et al 2003.. Science. 300(5624): 1394-1399.
7. Sun ZF, Meng XJ. 2004..J Clin Microbiol. 42:2351-2352.
8. Woo PC, Lau SK, Tsoi HW, et al. 2004. Lancet. 363(9412):841-845.
9. World Health Organization. 2003. Wkly. Epidemiol. Rec. 78:86-87.
10. Yu F, Le MQ, Inoue S, Thai HT, et al.Clin Diagn Lab Immunol. 2005,12:848-54.