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		Identification and Characterization of Ankylosing Spondylitis Associated K. pneumoniae Antigens		

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	Western reagents	set	5	5,000	25,000	
	E. coli expression system	set	1	60,000	60,000	
	Restriction enzyme	set	10	2,450	24,500	
	PCR matrix mixture	set	6	10,000	60,000	
	colony hybridization membrane	box	20	3,500	70,000	
	DNA sequencing	Rxn	800	450	360,000	
	Domestic travel and symposium attendance		1	10,000	10,000	
	Lab maintenance		1	40,000	40,000	
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	Antibody	set	5	10,000	50,000	
	Restriction enzyme	set	10	2,450	24,500	
	PCR matrix mixture	set	6	10,000	60,000	
	ELISA plates	box	20	6,000	120,000	
	chemicals	set	1	80,000	80,000	
	plastic (tips, tubes, plates)	case	20	5,000	100,000	
	Western reagents	set	5	5,000	25,000	
	mice		300	80	24,000	
	affinity column	set	20	20,000	400,000	
	Domestic travel and symposium attendance		1	10,000	10,000	
	Lab maintenance		1	40,000	40,000	
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	Restriction enzyme	set	10	2,450	24,500	
	PCR matrix mixture	set	6	10,000	60,000	
	chemicals	set	1	80,000	80,000	
	plastic (tips, tubes, plates)	case	20	5,000	100,000	
	Western reagents	set	5	5,000	25,000	
	ELISA plates	case	20	6,000	120,000	

	tissue staining	set	1	50,000	50,000	
	collagen	bottle	5	10,000	50,000	
	proteomics analysis		10	20,000	200,000	
	Domestic travel and symposium attendance		1	10,000	10,000	
	Lab maintenance		1	40,000	40,000	
					759,500	

關鍵詞：僵直性脊椎炎，克雷白氏肺炎菌，IVIAT，HLA-B27

中文摘要：

　　僵直性脊椎炎（Ankylosing Spondylitis; AS）是一種主要侵襲脊椎的發炎性疾病。文獻報導以及研究發現 AS 患者血清具有對抗克雷白氏肺炎菌（*Klebsiella pneumoniae*）的抗體反應，而在 HLA-B27 分子以及細菌抗原之間具有分子相似（molecular mimicry）片段。在本計畫中我們將建構 IVIAT (In Vivo Inducible Antigen Technology)系統，利用此系統搜尋對 HLA-B27 陽性 AS 病患的血清具有免疫反應的 *K. pneumoniae* 抗原。接著再以 Bioinformatics 的軟體工具以及 ELISA 分析這些 *K. pneumoniae* 抗原對 AS 的免疫反應性以及其抗原特異區域。為能進一步了解這些抗原引發的免疫反應在 AS 病程中的重要性，我們將大量表達這些具有高度免疫反應性的 *K. pneumoniae* 抗原蛋白，再利用純化所得的蛋白置備特異的小鼠抗血清。藉由免疫組織染色分析法（immunohistochemical analysis），這些具有 *K. pneumoniae* 抗原特異性的小鼠抗血清將可幫助我們找到存在於 AS 患病組織中的自體免疫因子。此外，我們亦會針對 *K. pneumoniae* 抗原與人類組織膠原蛋白之間可能存在的交叉反應性（cross-reactivity）進行測試。我們相信藉由本研究計畫的完成，對於 *K. pneumoniae* 感染在 AS 疾病進程中所扮演的角色將有更清楚的了解；而這些在 *K. pneumoniae*，HLA-B27，以及 AS 患病組織中彼此共有的 peptide 分子將可提供為開發自體免疫藥物的設計平台。

Abstract:

Ankylosing spondylitis (AS) is an inflammatory disorder that primarily affects the axial skeleton. Some evidences indicated that *Klebsiella pneumoniae* might be related to the trigger or pathogenesis of AS. Anti-*Klebsiella* antibodies and the presence of molecular mimicry between the HLA-B27 molecule and bacterial antigens had been reported. In this project, we will initiate an IVIAT (in vivo inducible antigen technology)-based approach to identify *K. pneumoniae* antigens that are immunoreactive to the serum of HLA-B27 positive AS patients. Antigenicity and immunoreactivity of the AS-associated *K. pneumoniae* antigens identified will be determined using bioinformatics analysis and ELISA assay. The highly AS-responsive *K. pneumoniae* clones will be selected for large-scale expression and generation of polyclonal antibodies. Tissue-specific antigens present in the infected sites of AS patients that may serve as targets for autoreactive T cells will be identified by immunohistochemical analysis with the generated *K. pneumoniae* antibodies. Furthermore, the cross-reactivity between AS-associated *K. pneumoniae* antigens and human collagens will be examined. The possibly coexisted epitopes present in the *K. pneumoniae* antigens, HLA-B27, and infected tissues such as human collagens, will be analyzed using bioinformatics tools. We expect the results obtained from this study will contribute significantly toward the understanding of the pathogenic role of *K. pneumoniae* in the development of AS disease. Potential useful peptide targets will be obtained which may lead to the identification of novel anti-autoimmune drugs.

Research Plan

I. Main research results:

The P.I., Dr James Cheng-Chung Wei, is a attending physician and lecturer of Chung Shan medical university. After his training of rheumatology and clinical immunology at Taichung and Kaohsiung veterans general hospital, he went to Royal National Hospital for Rheumatic Diseases (RNHRD) for clinical fellowship under Dr Andrei Calin, the world's famous master in ankylosing spondylitis (AS) in 2000. In 2001, he went to UCLA rheumatology department as a research fellowship under Dr David Yu to investigate the pathogenesis of spondyloarthropathy. Before present position, he was the chief of family medicine department of Chia Yi veterans hospital. He organized the ankylosing spondylitis caring society in Taiwan and had been the president of the society.

Dr Wei's research interests included clinical and basic researches in ankylosing spondylitis. He conducted the first HLA-B27 subtyping study. HLA-B27 subtypes in Chinese with ankylosing spondylitis and normal controls. J. of Rheumatology. Vol 25, suppl 54: 27, 1998 Aug. He also find the association of AS with HLA-B60 and B61. HLA-A, B ANTIGENS IN HLA-B27 NEGATIVE ANKYLOSING SPONDYLITIS PATIENTS IN TAIWAN. Journal of Rheumatology – Suppl 59:1-62, 2000 Aug, complete paper submitted in 2003 In 2001, he and collaborators develop a new immunomagnetic separation (IMS)-sandwich ELISA technique to detect HLA-B27 antigen. (Development of immunomagnetic separation (IMS) - sandwich ELISA for the HLA-B27 antigen detection. J. Immunol Methods. 2001 Aug.) In 2003, he published a pilot clinical trial on thalidomide in AS patients.

Thalidomide for Severe Refractory Ankylosing Spondylitis. Journal of Rheumatology 2003;30:2627-31 . He was also invited to write a review article for Clinical & Experimental Rheumatology Thalidomide in Ankylosing Spondylitis. Clinical and Experimental Rheumatology. 2002. supp 28, s-158-61

Recently, he is working on traditional Chinese medicine trial in AS patients and the validation of some clinical indices in AS.

The co-PI has been working on the pathogenesis of *Klebsiella pneumoniae*. Her group is familiar with bacterial genetic analysis techniques and

has established several virulence gene identification approaches, including DNA subtractive hybridization, in vivo expression technology (IVET), and signature-tagged mutagenesis. More than 40 virulence-associated genes have been identified with these techniques. Four articles concerning the results have been published (23-26).

We believe that all these previous experience and efforts will ensure the success of this project.

II. Preliminary study:

1. Collection of AS sera and biopsies

Patients population

Patients in Chung Shan medical university fulfilling the modified New York criteria(1984) for AS were recruited after written informed consent was obtained. Patients' seri and tissues from synovium and enthesis were sampled simultaneously with the clinical indices and laboratory tests. Seri and tissue samples were stocked in -80 degree refrigerators.

Clinical assessments were performed by the same investigator including patient-reported global pain intensity over the past 7 days (recorded using a 100-mm VAS) and patient-reported activity and functional impairment (according to the Bath Ankylosing Spondylitis Disease Activity Index [BASDAI] and Bath Ankylosing Spondylitis Functional Index [BASFI]), Finger to floor test, Schober test, chest expansion test, occipital to wall test, lateral bending test. Laboratory assessment include complete blood count (CBC), liver and renal function, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and immunoglobulin A (IgA) were monitored at each visit of clinical assessments.

Control groups. Seri samples from healthy normal control and some healthy HLA-B27 carrier subjects were also collected as the control groups.

2. Western analysis of AS immunoreactivities against *K. pneumoniae*. We are interested in the humoral immune responses observed in HLA-B27 positive AS patients that are possibly elicited by the infection of *K.*

pneumoniae. We performed a preliminary Western analysis by using sera of HLA-B27 positive AS patients as the first antibodies. As shown in Fig. 1, total proteins (30 μg) of the cell lysates prepared from *K. pneumoniae* CG43 (serotype K2), *E. coli* XL-1 and *S. typhimurium* LT2 were separated by SDS-PAGE. After transferred onto PVDF membranes, sera from HLA-B27 positive healthy individuals or from HLA-B27 positive AS patients were added at 4°C for hybridization overnight. Sera from HLA-B27 negative healthy individuals were used as a control in parallel. Alkaline phosphatase (AP)-conjugated anti-human IgG (H+L) antibodies (Promega) were added for another 1 hour at room temperature. AP activity was detected using CDP-Star chemiluminescence reagent (PerkinElmer) and exposed to a BioMax X-film (Kodak). Since epitopes expressed on the bacterial surface are more likely to be involved in the immune responses, we extracted the extracellular components of *K. pneumoniae* CG43 for Western analysis (lane 1; Fig. 1). Overnight-cultured *K. pneumoniae* cells (500 μl) was mixed with 100 μl of 1 % Zwittergent 3-14 detergent (Sigma) in 100 mM citric acid (pH 2.0), and incubated at 50°C for 20 min. After centrifugation, 250 μl of the supernatant was transferred to a new tube and extracellular components including capsular polysaccharides were precipitated with 1 ml of absolute ethanol. The pellet was dissolved in 200 μl distilled water.

In comparison with the immunoreactivity of the HLA-B27 negative sera, a significant response to the extracellular components of *K. pneumoniae* was observed in that of HLA-B27 positive sera either from healthy person or AS patients (as pointed with an arrow in Fig. 1). Except *K. pneumoniae*, a cross-reactivity to the bacterial lysates of *E. coli* and *S. typhimurium* was observed in sera of HLA-B27 positive AS patients. Based on this preliminary result and enormous AS references reported by other groups, we hypothesize that *K. pneumoniae* has a pathological role in the development of AS disease with an involvement of HLA-B27.

III.   Background and Significance:

Ankylosing spondylitis (AS) is the most prominent component of a group of inflammatory arthritides, collectively named as "spondyloarthropathies (SpAs)". It is a chronic inflammatory disorder that involves mainly the lumbar spine and sacroiliac joints and less frequently affects the peripheral joints and eye structures such as the uvea. The inflammation can lead to fibrosis and ossification, where bridging spurs of bone known as "syndesmophytes" form, especially at the edges of the intervertebral discs. This form of ossification is primarily seen at the sacroiliac joints and lumbar spine from where it ascends. In extreme cases it can effectively solidify the whole of the vertebrate column. The disease in severe cases can progress to form the classic "bamboo spine" (12). Symptoms of AS first appear most frequently in young men between the ages of 10 and 40. The disease is less common in women, who often have milder symptoms that are more difficult to diagnose.

The causation of AS is largely unknown, but genes and heredity do play a role. The association between AS and HLA-B27 has been well established. The frequency of HLA-B27 in patients with AS ranges from 81 to 96% with control frequencies ranging between 4 and 12%. Rats transgenic for B27 spontaneously develop a chronic inflammatory disease that resembles, both clinically and histologically, the human SpA, while control rats transgenic for HLA-A2 do not develop such an illness (6, 14, 27). The prevalence of AS correlates with the presence of HLA-B27 in all ethnic groups has been studied. African Blacks of unmixed ancestry lack B27 genes and AS is very rare among them. Certain North American Indian tribes who have a high frequency of B27 also have a high prevalence of AS (21). The observations signify the importance of HLA-B27 in the development of AS.

Infections of *Klebsiella pneumoniae* have been strongly suggested to be a triggering factor of AS. Elevated antibody titers against *Klebsiella* but not to other microbes could be detected in the sera patients with AS when compared to healthy controls (1, 5, 9, 11, 18, 20, 30, 32). Evidence for molecular mimicry between *Klebsiella* and HLA-B27 has been shown in several studies (4, 33). An amino-acid sequence homology was identified between HLA-B27 and the *K. pneumoniae* nitrogenase reductase enzyme, in that the sequence QTDRED is common to both molecules (28). Rat antisera raised against 16-mer synthetic peptides of *K. pneumoniae* nitrogenase reductase reacted with synovial biopsies

obtained from HLA-B27 positive AS patients (17). Furthermore, there is extensive evidence to suggest an association between the gut lesions in AS and the coexistence of *Klebsiella* as the likely triggering agent across the gut mucosa. HLA-B27 positive individuals, who are suffering from inflammatory bowel disease (IBD) such as Crohn's disease or ulcerative colitis, have a significantly greater chance of developing AS than HLA-B27 positive individuals not having IBD (34). In a most recent study, patients with AS and Crohn's disease were found to have elevated levels of IgM, IgG and IgA class-specific antibodies to *K. pneumoniae* and to collagens type I, III, IV and V. A positive correlation was observed between *K. pneumoniae* and collagen antibody levels in these patients (31). These observations support the hypothesis that an important link exists between the infections of *Klebsiella* and the development of AS.

  K. pneumoniae is present as a saprophyte in the human nasopharynx and intestinal tract. It is an important nosocomial pathogen, causes suppurative infection, pneumonia, urinary tract infection and septicemia, occurring particularly in immunocompromised individuals (7) and has a high fatality rate if untreated. Several virulence factors have been found important in the pathogenesis of *K. pneumoniae*, including capsular polysaccharides (3), lipopolysaccharides (8), adhesins, iron acquisition systems, and several secreted toxins. Despite of these, much of the pathogenesis events in *K. pneumoniae* are present unclear. In view of the importance of *Klebsiella* infections with the aetiopathogenesis of AS, we propose to initiate an IVIAT (in vivo inducible antigen technology)-based approach (10, 15, 16) that will lead to the identification of *K. pneumoniae* antigens involved in the development of AS disease.

IV. Specific aims:

  The study is to be completed in three years. The goal of this project is to identify and characterize the causative *K. pneumoniae* factors that are involved in the pathogenesis of ankylosing spondylitis (AS). The specific aims are listed chronologically as follows.

The first year

1. Construction of an inducible expression genomic DNA library of *K. pneumoniae*

2. Screening for AS-associated *K. pneumoniae* genes

The second year

1. Bioinformatic analysis of AS-associated *K. pneumoniae* genes

2. Expression and purification of *K. pneumoniae* antigens

3. Determination of AS immunoreactivity to the associated *K. pneumoniae* antigens

4. Generation and purification of reactive AS antibodies against *K. pneumoniae* antigens

The third year

1. Determination of the cross-reactivity between the AS-associated *K. pneumoniae* antigens and putative AS factors

    (1) Immunohistochemical analysis of AS synovial tissues.

    (2) Immunoreactivity analysis with human collagens.

2. Identification of immunodominant peptides of AS-reactive *K. pneumoniae* antigens

V. Research design and methods:

We here describe our tentative approaches stepwise as follows.

The first year

1. **Screening for AS-associated *K. pneumoniae* antigens by using an IVIAT-based approach.**   In vivo induced antigen technology (IVIAT) is a novel method designed to screen microbial genes that are expressed during an actual human infections rather than in an animal model (15). IVIAT is highly effective and has allowed many infection-associated microbial genes to be identified (10, 16). Based on the IVIAT approach, we will use pooled sera from HLA-B27 positive AS patients to probe *K. pneumoniae* genes that

specifically expressed in the development of AS disease. The experimental scheme is shown in Fig. 2.

(1) **Construction of an inducible expression library of *K. pneumoniae*.**
An inducible expression library of *K. pneumoniae* will be generated as follows. *K. pneumoniae* genomic DNA will be purified by a conventional sodium dodecyl sulfate (SDS)-protease method, partially digested with *Sau*3AI, and separated by electrophoresis with a low-melting-point agarose gel (Amersham). DNA fragments of ca. 0.5 to 1.0 kb will be cut from the agarose gel and extracted by using the QIAEX II gel extraction kit (Qiagen). The purified DNA inserts will be ligated into the pET30abc expression vectors (Novagen), which will have been cut by *Bam*HI and treated with shrimp alkaline phosphatase (Roche). The resulting ligation mixture will be electroporated into electrocompetent DH5α cells (Gibco-BRL). The transformants will be spread onto LB agar plates containing 50 μg/ml of kanamycin. After overnight incubation, colonies on the plates will be scraped and frozen at –70°C until use. An aliquot of the resulting library will be grown in LB broth, and plasmids will be isolated. The isolated plasmid library DNA will be subsequently electroporated into the expression host *E. coli* BL21(DE3) (Novagen).

(2) **Screening for AS-associated *K. pneumoniae* genes with sera from HLA-B27 positive AS patients.** As illustrated in Fig. 1, the *K. pneumoniae* expression library constructed in *E. coli* BL21(DE3) will be plated onto BHI agar plates containing 50 μg/ml of kanamycin at a density of ca. 1000 colonies per plate. The colonies will be replica plated onto two BHI agar plates with supplement of kanamycin and 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to induce the expression of inserted DNA fragments. The colonies will be exposed to chloroform vapors to partially lyse the bacteria and the exposed proteins will be adsorbed onto overlaid HyBond nitrocellulose membranes (Amersham). The membranes will be carefully removed and saturated with a 5% skim milk solution in phosphate-buffered saline (PBS) containing 0.5% Tween 20. Comparative colony Western blot analysis will be performed

with the pooled serum of HLA-B27 positive AS patients and the HLA-B27 negative healthy control, respectively as the primary antibody. Peroxidase-conjugated rabbit antihuman immunoglobulin G (IgG), IgA and IgM (Promega) will be used as the secondary antibody. The positive signals will be detected by using an enhanced chemiluminescence (ECL) and Hyperfilm ECL (Amersham). Reactive clones, which are positive in the blotting with AS sera and negative in the control blotting by parallel, will be collected and retested for reactivity by another round of colony immunoblot analysis. Each colony of the resulting reactive clones will be grown overnight in 96-well culture plates and then replica plated onto BHI agar plates containing kanamycin with or without IPTG. Only the clones showing strong positively at the secondary screening will be regarded to contain putative AS-associated antigens. Inserted DNAs in the positive clones will be sequenced.

The second year

2. **Characterization of AS-associated *K. pneumoniae* antigens**. By using bioinformatics analysis and ELISA assay, we will determine the antigenicity and immunoreactivity of the *K. pneumoniae* genes identified in the first year. The experimental scheme is shown in Fig. 3.

(1) **Bioinformatics analysis of AS-associated *K. pneumoniae* genes.** The nucleotide sequences of the AS-associated K. pneumoniae genes will be analyzed in GenBank database and the genomic sequence database of *K. pneumoniae* MGH78578 provided by Washington University with the BLAST programs (2). Based on the homology searches, these AS-associated genes will be assigned to a gene cluster using a microbial genome classification system. Amino acid sequences of the putative AS-associated *K. pneumoniae* antigens will be deduced and used for a computer analysis of probable antigenic sites. Regions with a high antigenicity index according to Jameson and Wolf (19) and a high probability of containing turns based on secondary structure predictions (13) will be identified.

(2) **Determination of the immunoreactivity of the identified *K. pneumoniae* antigens with AS patient sera.** Levels of IgG, IgA and IgM antibodies of AS patients to each of the putative *K. pneumoniae* antigens will be evaluated by ELISA assay. These putative AS-associated *K. pneumoniae* genes will be expressed and purified by affinity chromatography with the His-Bind resin (Novagen). These purified *K. pneumoniae* antigen proteins will be coupled covalently to 96-well assay plates at 1 μg/well. Sera collected from AS patients will be diluted and incubated for 1.5 h at room temperature. Sera from healthy individual will be introduced to serve as the controls. After washing, 100 μl of anti-human class-specific IgG, IgA, or IgM conjugated to peroxidase will be added. After incubation, plates will be washed, and bound antibodies will be detected with 3,3-5,5- tetramethyl-benzidine and read at a wavelength of 450 nm. All sera will be tested in triplicate, and the results will be averaged. Differences in immunoglobulin class levels against the various *K. pneumoniae* antigens in the serum samples of AS patients will be assessed by comparing the proportion in each group having OD units greater than the 95% confidence limits for the population of controls (one-tailed test) using the χ^2 test, with Yates correction (significance will be taken at $P < 0.05$).

3. Generation and purification of AS-reactive antibodies

(1) **Generation of polyclonal antibodies for AS-associated *K. pneumoniae* antigens.** The AS-associated *K. pneumoniae* antigens that present a strong immunoreactivity to the AS sera will be selected to generate polyclonal antibodies for further studies. Female BALB/c mice (six to eight weeks of age) will be immunized with the following schedule. The mice will receive 25 μg of *K. pneumoniae* antigen i.p. on days 0 and 14 and pristine on days 3 and 17; on day 21 blood samples will be taken for assessing the course of immunization. At day 28, the mice will be given a third i.p. dose of *K. pneumoniae* antigen, and on day 31, they will be injected i.p. with a nonsecreting myeloma cell line. On day 42, ascites fluid will be collected and pooled. Immunoglobulins will

be purified by 50% saturation with ammonium sulfate (22). Whether the generated *K. pneumoniae* antibodies will cross-react with the infected tissue of AS patients will be subsequently assessed.

(2) Purification of *K. pneumoniae* reactive antibodies from AS sera by affinity chromatography. For a comparative analysis of *K. pneumoniae*-associated immune response elicited in an actual AS patient, we will purify the antibodies from pooled AS sera by using chromatography on immobilized *K. pneumoniae* antigens. The purified *K. pneumoniae* proteins that show strongly reactivity to AS sera (2 mg) will be coupled to 0.3 g of CH-Sepharose 4B (Pharmacia) in 0.1 M Na_2HCO_3/NaH_2CO_3 (pH 8.0) containing 0.5 M NaCl. Chromatography will be carried out at room temperature at a flow rate of 0.2 ml/min. Protein-A-purified antibodies obtained from AS sera, will be applied to the column for 2 h. The fraction not bound by the column will be saved for analysis. The column will be washed extensively with PBST (PBS/ 0.05% Tween-20) followed by the same buffer supplemented with 0.35 M NaCl. During elution of bound antibodies with 0.1 M glycine-HCl, pH 2.5, fractions of 0.2 ml will be collected and immediately neutralized with 10 µl 2 M Tris buffer and subsequently dialyzed against PBS.

The third year

- **Determination of cross-reactivity between the AS-associated *K. pneumoniae* antigens and the putative AS factors.** Possession of HLA-B27 has been strongly associated with the development of AS. Despite intensive research, the pathogenic role of HLA-B27 and its relevance with *Klebsiella* infection remain unclear. To understand the underlying mechanism of this cross-reactivity, we will (i) identify the tissue-specific antigen that the antibody raised against *K. pneumoniae* will deposit by using an immunohistochemical analysis of the synovial tissue from HLA-B27[+] AS patients; (ii) evaluate the immunoreactivity of AS-associated *K. pneumoniae* antibodies to human collagens.

(1) **Immunohistochemical analysis.** The biopsy specimens of synovial tissue (ST) have been collected from AS patients and frozen at -80°C. Cryostat sections will be prepared and mounted on glass slides. The serial sections will be stained with the polyclonal antibodies generated against *K. pneumoniae* antigen or with the purified antibodies from AS patients. For control sections, the primary antibodies will be omitted or irrelevant mouse antibodies will be applied. Staining will be performed based on a three-step immunoperoxidase method (29). After the slides warmed to room temperature and air-dried, the sections will be washed between all steps with PBS. All incubations will be carried out at room temperature. The primary antibodies will be diluted in PBS-1% BSA. The HRP-conjugated secondary antibodies will be diluted in PBS-1% BSA supplemented with 10% normal human serum (NHS) as blocking serum. Endogenous peroxidase activity will be inhibited using 0.1% sodium azide and 0.3% hydrogen peroxide in PBS. The primary antibodies will be incubated for 1 h. HRP-conjugated goat anti-mouse or goat anti-human antibody will be added for 30 min, followed by incubation with HRP-conjugated swine anti-goat antibody for another 30 min. HRP activity will be detected with hydrogen peroxide as substrate and amino ethylcarbazole (AEC) as dye.

(2) **Examination of the cross-reactivity with human collagens.** Since a correlation between IgM and IgG antibodies against *K. pneumoniae* and collagen types I, III and IV has been reported (31), we will examine whether the *K. pneumoniae* reactive antibodies that generated and purified in the second year will crossly react with the human collagens. Collagen types I, II, III and IV will be purchased from Sigma and dissolved in NaHCO$_3$ (0.1 M) containing with NaCl (0.5 M) to a final concentration of 1 mg/ml. Flat-bottomed 96-well plates will be coated with 200 µl of collagen solution and incubated overnight at 4°C. Polyclonal antibodies generated from BALB/c mice or purified from AS patient sera will be added and incubated for 1.5 h at room temperature. After washing, 100 µl of anti-mouse or anti-human class-specific IgG, IgA, or IgM antibody conjugated with peroxidase will be added for

another 1 h. After incubation, plates will be washed, and bound antibodies will be detected and read at a wavelength of 450 nm.

- **Identification of cross-reactive peptides of the AS-associated *K. pneumoniae* antigens.** Currently the management of patients with AS includes two main approaches. The first one involves the use of one or more of non-steroid anti-inflammatory drugs (NSAIDs) that are mainly used to alleviate the pain and reduce the associated inflammation. The second approach involves physiotherapy and postural education to prevent the stiffness and deformities which are the main sequelae occurring in this disease. However, the use of NSAIDs is considered as non-specific measures, as they could not reverse or even halt the damaging pathological process of the disease. Furthermore, these drugs have various undesirable side effects. The use of other therapeutic measures to eliminate the *K. pneumoniae* infection or to reduce the level of autoantibodies will be beneficial for the AS patients. For this reason, we will identify the immunodominant region of the identified AS-reactive *K. pneumoniae* antigens that might bind to HLA-B27. It will provide candidate peptide sequences that can be served as a template for a selective immunotherapy by blocking or antagonizing the HLA-B27 interaction with autoreactive T cell clones.

VI. Anticipated results and achievements:

We anticipate achieving two major contributions through the study. First, although the IVIAT is not a brand new technology, the application of IVIAT in screening for cross-reactive bacterial antigens in an autoimmune disease is a new approach. The IVIAT system developed in this study can be considered as a platform and will be useful for future analysis of other autoimmune disease associated microbial factors, such as *Proteus* or *Mycobacterium* with rheumatic arthritis (RA), *Chlamydia* trachomatis with spondyloarthropathies (SpA), or certain viral infections with systemic lupus erythematosus (SLE). Second, through the IVIAT analysis, we anticipate to

identify 20-30 genes that putatively associated with the pathogenesis of AS. By determining their cross-reactivity with AS factors, potential useful peptide targets will be obtained which may lead to the identification of novel anti-autoimmune drugs.

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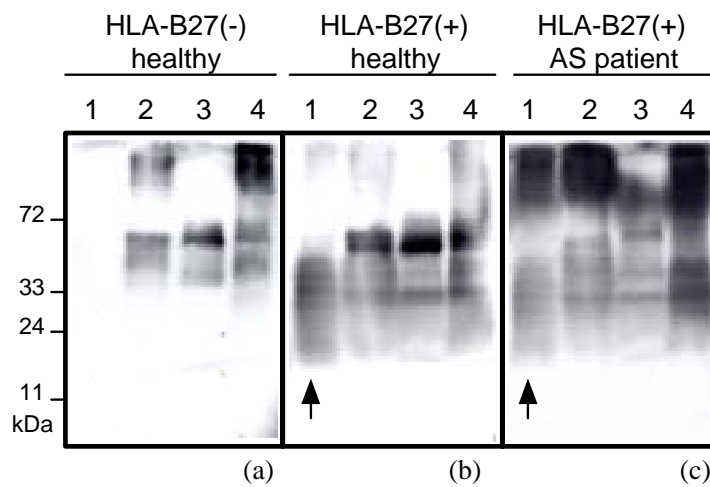
Figures:

Figure 1. **Western analysis.** The extracellular components of *K. pneumoniae* (lane 1) and the t otal cell lysates of *K. pneumoniae* (lane 2), *E. coli* XL-1 (lane 3) and *S. typhimurium* LT2 (lane 4) (30 ug/per lane) were separated by 12.5% SDSŠPAGE and transferred onto PVDF membranes. The membranes were hybridized with serum samples diluted at 1:2000. Representative results from three independent experiments were shown. Panel a: sera from HLA-B27 negative healthy; panel b: HLA-B27 positive healthy; panel c: HLA-B27 positive AS patients. Arrows indicate a specific antigenic response against *K. pneumoniae*.
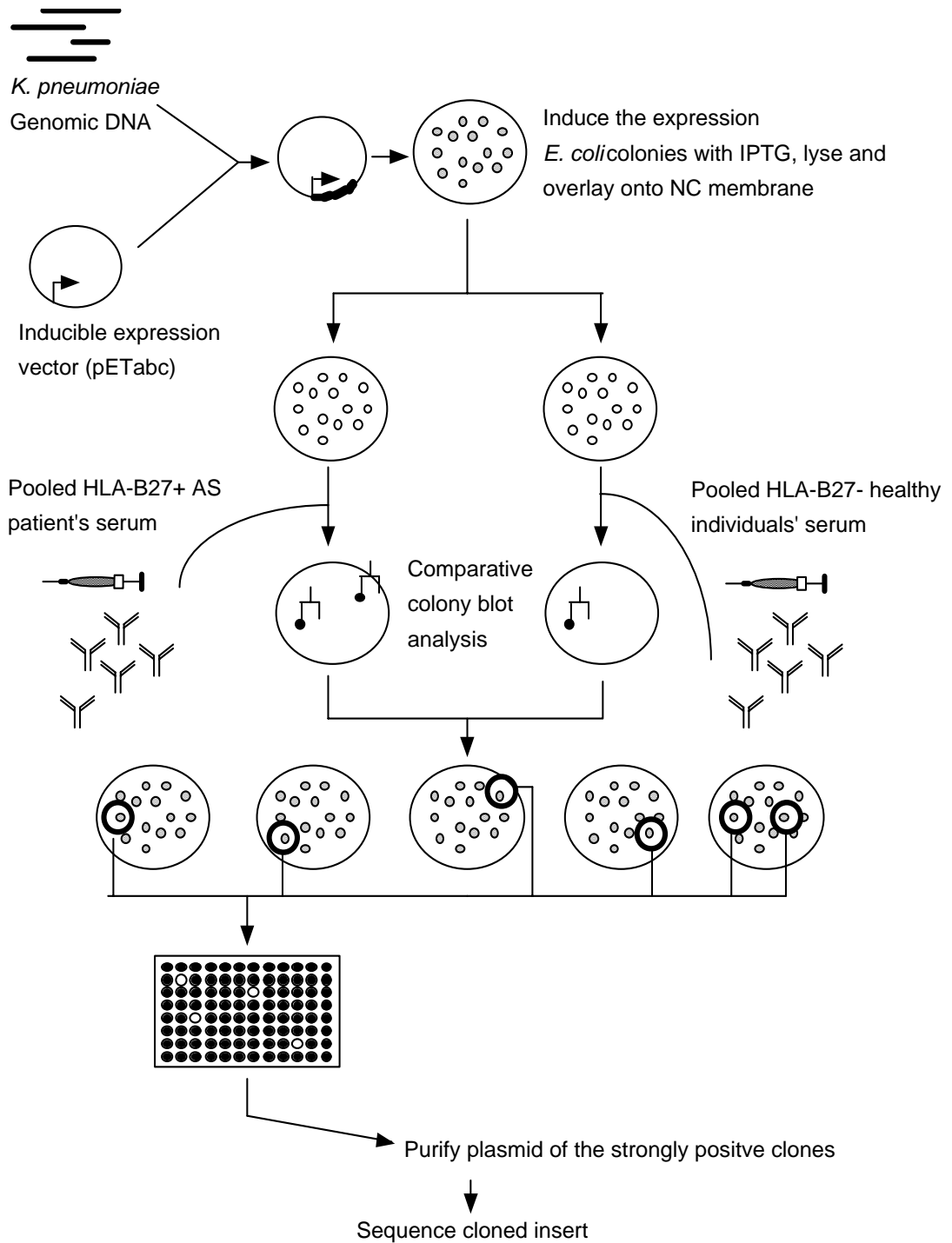
K. pneumoniae Genomic DNA

Inducible expression vector (pETabc)

Induce the expression *E. coli* colonies with IPTG, lyse and overlay onto NC membrane

Pooled HLA-B27+ AS patient's serum

Pooled HLA-B27- healthy individuals' serum

Comparative colony blot analysis

Purify plasmid of the strongly positve clones

Sequence cloned insert

Figure 2. Schematic illustration of the experimental design proposed in the first year.
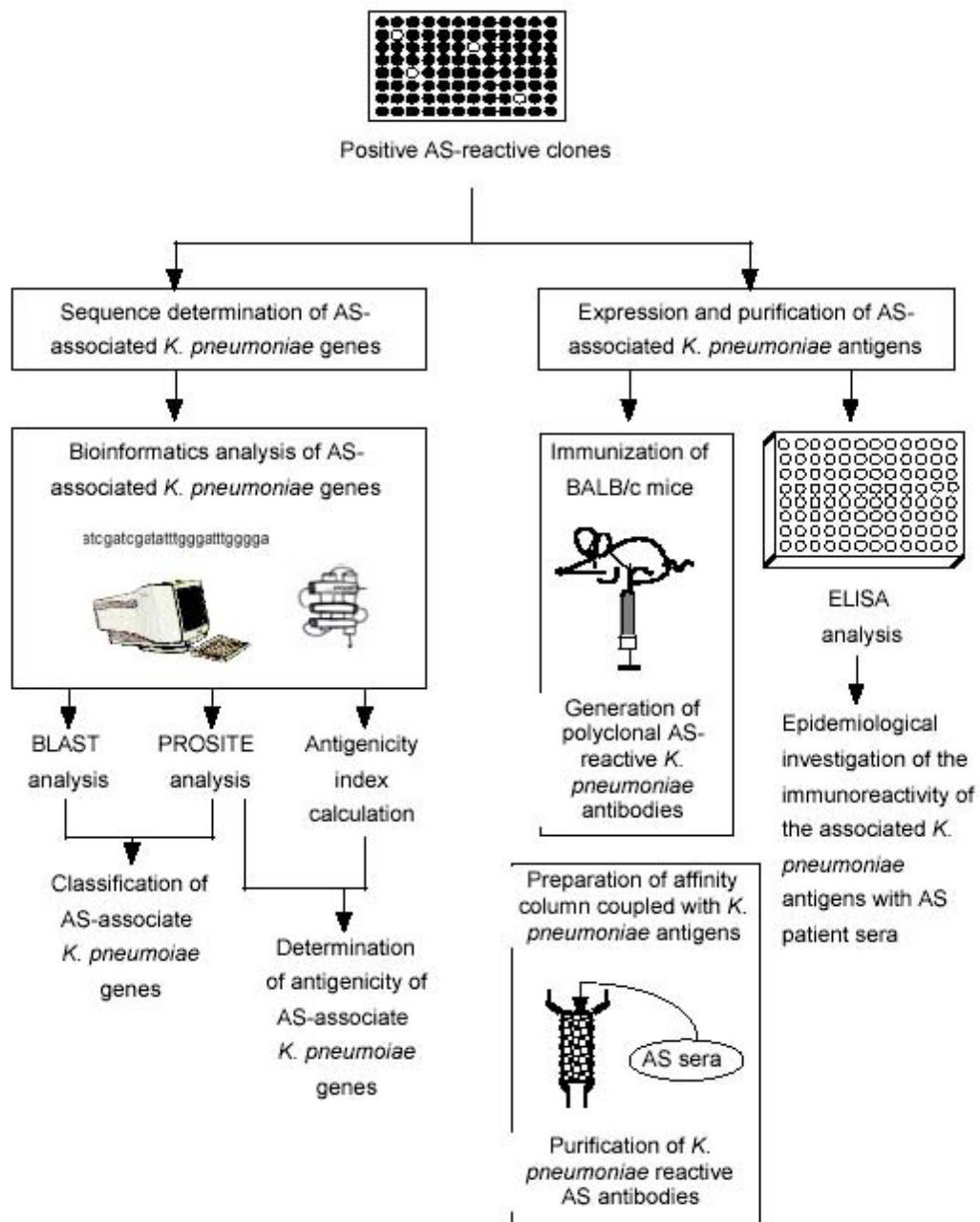
Figure 3. Schematic illustration of the experimental design for the second year.

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(2-3)		1. <u> </u> 2. <u> </u> 3. <u> </u>
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*SCI (Science Citation Index)   SSCI (Social Science Citation Index)   EI (Engineering Index)

(:http://www.isinet.com)   SCI

SSCI   EI   2002

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1. 89 1 (88.1.1~88.12.31)<u> </u>	1. 88 (88.1.1~88.12.31) <u> </u>
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4. 90 (91.1.1~91.12.31) <u> </u>	4. 91 (91.1.1~91.12.31) <u> </u>
5. 92 (92.1.1~) <u> </u>	5. 92 (92.1.1) <u> </u>

(RPI) (　B)

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	(　　　　　　　;　:　　　　)	(C)	(J)	(A)	C×J×A
1	**J.CC. Wei**, T.W. Chan, H-S. Lin, F. Huang, C-T. Chou. Thalidomide for Severe Refractory Ankylosing Spondylitis. **Journal of Rheumatology 2003**;30:2627-31**(SCI)**	3	5	5	75 75
2	F Huang, **JCC Wei**, M Breban. Thalidomide in Ankylosing Spondylitis. **Clinical and Experimental Rheumatology. 2002**. supp 28, s-158-61. **(SCI)**	3	4	3	S01 36
3	Chou CT, Tsai YF, Liu J, **Wei JC**, Liao TS, Chen ML, Liu LY. Development of immunomagnetic separation(IMS)- sandwich ELISA for the HLA-B27 antigen detection. **J. Immunol Methods. 2001**. 255(1-2):15-22. **(SCI)**	3	4	0.5	S02 6
4	魏正宗. 2003/12 抗腫瘤壞死因子製劑 **臺灣醫界,2003,** 46(12):699-704	1	1	6	S04 6
5	魏正宗.食物與關節炎的關係. **基層醫學 2001** 16:270-3	1	1	6	S05 6
6	魏正宗.魚油的證據醫學. **基層醫學 2002.**17:270-3.	1	1	6	S06 6
7	魏正宗.血清陰性脊椎關節病變.**當代醫學 2001.** 28:674-8	1	1	6	S07 6
8	簡志弘 **魏正宗**.自體免疫性肝炎. **中華民國風濕病雜誌 2000.**15(1), 29-32.	1	1	5	S08 5
9	**魏正宗,** 莊家銘..血管硬化新指標--同胱胺酸. **基層醫學 2003.** 17:300-2.	1	1	5	5

10	蔡宗宏, **魏正宗**. .紅斑狼瘡病患常見之感染. **基層醫學 2002.** 17:246-51.	1	1	5	5

(; :)					C×J×A
		(C)	(J)	(A)	
11					S11
12					S12
13					S13
14					S14
15					S15
(S16=S01+S02+S03+..+S14+S15)					S16 **146**
(RPI) (×100)/ =(S16×100)/					**12.9**

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		(C)	(J)	(A)	
11					S11

12					S12
13					S13
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15					S15
	(S16=S01+S02+S03+..+S14+S15)				S16 **156**
	(RPI)　(　×100)/		=(S16×100)/		**34.6**

a)   b)   c)

d)

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	M		
	15	1125	(15×75)
	12	900	(12×75)
	10	750	(10×75)
	8	600	(8×75)
	6	450	(6×75)
	4	300	(4×75)

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1   (original article   DNA   RNA   amino acid   )
2   (note   short communication   letter to editor   or accelerated publication)
3   (case report)   4   (review)   5   6   7
8   9

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SSCI   SCI   "SSCI"   (category)   (
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2-1. (J) SCI

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4   SCI   SSCI   Journal Citation Report 2002

2-2. **Proceeding**   **SCI**

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2-4.   **SCI SSCI EI**   **(J)**   0.5

3.   **(A)**

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( )	40
(/)	60

                 = (S01..S15)

       =(×100)／   =(S16×100)／

表1 　　　　　　　　　　　　　　　　　　　　　　　SCI 　　　　　　　　　　(J)

				SCI	(J)
1.	Journal of Biomedical Science (National Science Council, Taipei)			SCI	4.5
2.	（Journal of the Formosan Medical Association）		85-92	SCI	2.5
3.	（Journal of the Chinese Chemical Society）			SCI	2.5
6.	（Zoological Studies）		84-92	SCI	2.5
7.	（The Chinese Journal of Physiology）		84-92	SCI pending	2.5
4.	（Botanical Bulletin of Academia Sinica）		84-92	SCI	2
5.	（Journal of Food & Drug Analysis）		85-92	SCI pending	2
8.	Proceeding of the National Science Council, ROC, Part A: Physical Science and Engineering (　　2002,12)				1.5
9.	Proceeding of the National Science Council, ROC, Part B: Life Sciences (　　2002,12)				1.5
10.	Proceeding of the National Science Council, ROC, Part C: Humanities and Social Sciences (　　2002,12)				1.5

　　　　　　　SCI 　　　　　2002 JCR
Proceeding of the N. S. C., Part A, B, C 　　91 　12

表2 　　　　　　　　　　　　　　　　　　　　　　　　　(J)

				(J)
1.	Chinese Medical Journal (Taipei) (　　　　　　)		84-92	1.0
2.	Taiwania		85-91	1.0
3.	The Chinese Pharmaceutical Journal		84-86,88-91	1.0
4.	（Food Science and Agricultural Chemistry）		90-92	1.0

5.			84-92	1.0
6.		8	84-90,92	1.0
7.			84-92	1.0
8.	The Kaohsiung Journal of Medical Sciences		84-92	1.0
9.	Chang Gung Medical Journal( )		85-92	1.0
10.	Acta Abaesthesiologica Cinica		84,85,88-92	1.0
12.			88,90-92	1.0
13.	The Journal of Nursing Research		86-88,91-92	1.0
15.			84-86,90-91	1.0

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	(　　　　　　；　：　　　　)	(C)	(J)	(A)	C×J×A
1. 1	<u>**Wang LY**</u>, Chen CJ, Zhang YJ, Tsai WY, and Regina RM*. 4-aminophenyl-DNA damage in liver tissue of Hepatocellular carcinoma patients and controls. <u>**American Journal of Epidemiology**</u> 1999;147:315-323.	3	5	5	S01 **75**
2 1	Regina RM, Hatch M, Chen CJ, Levin B, Zhang YJ, Tsai WY, and <u>**Wang LY**</u> *. Aflatoxin exposure and Risk of hepatocellular carcinoma in Taiwan. <u>**International Journal of Cancer**</u> 2000; 67:620-625.	3	5	5	S02 **75**
3 1	<u>**Wang LY**</u>*, Lan C. Environmental factors of motor accidents in Hualian County, Taiwan. <u>**Accident Analysis and Prevention**</u> 2002;15:245-251. (**SSCI; Category: Transportation; Ranking: 2/11**)	3	4	5	S03 **60**
4 2	Curigliano G, Zhang YJ, <u>**Wang LY**</u>, Flamini G, *et al.* Immunohistochemical quantitation of 4-aminobiphenyl-DNA adducts and p53 nuclear overexpression in T1 bladder cancer of smokers and nonsmokers. <u>**Carcinogenesis**</u> 2001;17:911-916.	2	5	1	S04 **10**
5 1	Chen CJ*, <u>**Wang LY**</u>, Lu SN, Wu MH, *et al.* Elevated aflatoxin exposure and increased risk of hepatocellular carcinoma. <u>**Hepatology**</u> 2001;24:38-42.	3	7.5	3	S05 **67.5**
6 1	(　　　　　　　　　). Epidemiologic studies for aflatoxin exposure, cigarette smoking, genetic polymorphisms of xenobiotic-metabolizing enzymes and hepatocellular carcinoma, 1999.				S06 **15**
7	(　　　　)				S07
(S16=S01+S02+S03+..+S14+S15)					S16 **302.5**
(　×100)／　　　　　=(S16×100)／					**67.2**

基因重組實驗申請同意書

凡進行基因重組實驗須由計畫主持人或實驗負責人填寫本表，送生物實驗安全委員會覈實同意並簽名後，發還申請人並保留影本。向有關機構申請研究計畫經費時，將影本隨附於計畫書備查。研究計畫核准後，所進行之基因重組實驗須與填寫內容相符，如實驗變更至更高安全等級，須再另填寫「申請同意書」報請生物實驗安全委員會同意。

研究計畫名稱：Identification and Characterization of Ankylosing Spondylitis associated *Klebsiella pneumoniae* antigens

計畫主持人： 魏正宗　職稱：主治醫師　電話及傳真：04-24739595 ext 4724 FAX:04-24637389

執行機構、系所： 中山醫學大學過敏免疫風濕科

1、實驗內容：　是否進行基因重組之實驗？ ---------■是
　　　　　　　是否進行微生物培養的實驗？ --------■是
　　　　　　　是否進行基因轉殖之動物實驗？ ------□是
　　　　　　　是否進行基因轉殖之植物實驗？ ------□是
　　　　　　　是否為自交植物？ ----------------□是

2、重組基因來源、宿主之安全等級及名稱（參考基因重組實驗守則附表二）

　　　a.重組基因來源名稱：*Klebsiella pneumoniae*
　　　□第一級危險群，■第二級危險群，□第三級危險群，□第四級危險群，□動物，□植物

　　　b.進行重組基因之微生物或病毒宿主名稱：*Klebsiella pneumoniae*
　　　□第一級危險群，■第二級危險群，□第三級危險群，□第四級危險群

　　　c.進行重組基因之細胞、植物或動物宿主名稱：＿＿＿＿＿＿＿＿＿＿＿＿

3、基因轉殖實驗設備及轉殖方法

　　　a.具備之基因轉殖之動物實驗設備：□SPF 設備;　□IVC 設備;
　　　　　　　其他_名稱＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

　　　b.具備之基因轉殖之植物實驗設備：□生長箱;　□溫室;　□農場;
　　　　　　　其他_名稱＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

　　　c.基因轉殖方法：□virus;　□microinjection;　□liposome;　□gene gun;　□＿＿

4、進行本研究所需之安全等級：□P1　■P2　□P3　□P4

5、進行本研究之實驗室 臨床病毒研究中心　其生物安全等級：□P1　■P2　□P3　□P4

計畫主持人(申請人)簽名：＿＿＿＿＿＿ 醫字第1868 92 年 12 月 14 日

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生物實驗安全委員會查覈欄 (以上基因重組實驗資料，由生物實驗安全委員會查覈人覈實同意並簽名後，發還申請人並保留影本。任一項目不合適或不完備，則退還請申請人改善或更正)

本項基因重組實驗查覈結果：☑同意進行　　□不同意進行

附註意見(無者免填)：

生物實驗安全委員會負責人(或查覈人)簽名：＿＿＿＿＿ 92 年 12 月 18 日