行政院國家科學委員會專題研究計畫 成果報告

克雷白氏肺炎桿菌經由腸道上皮入侵造成肝脾膿瘍的細胞感染機轉研究

研究成果報告(精簡版)

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公 開 資 訊 ：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 101 年 11 月 20 日

中 文 摘 要 ： 在台灣克雷白氏肺炎桿菌是主要造成社區感染原發性肝膿瘍的致病菌。克雷白氏肺炎桿菌移生至腸道是發展克雷白氏肺炎桿菌肝膿瘍不可或缺的步驟。因此，腸道移生的克雷白氏肺炎桿菌是如何穿越腸道障壁造成感染就是個令人感興趣的問題。此研究的主要目的是在探討克雷白氏肺炎桿菌穿越腸道障壁的致病機轉。藉由非侵入性活體影像系統可看到帶有冷光表現的克雷白氏肺炎桿菌以 5 × 107 菌量感染小鼠後，在感染後 2 和 6 小時能看出此菌在小鼠的腹部和肛門聚積。並且，這些受到感染的小鼠多有嚴重腹瀉，腸道切片也可看出腸道絨毛多處的腫脹、受損。由此可知，克雷白氏肺炎桿菌確實能移生至腸道造成感染且引發腸道發炎和損傷。同時，在小鼠感染後的 6 小時能發現肝臟溶解物內的 IL-6、IL-10 和 IL-17F 的表現量增加。利用穿透式電子顯微鏡也發現感染小鼠的肝細胞核膜的完整性受到破壞造成細胞質內的過氧化氫體進入核內。流氏細胞儀結果也發現腸系膜內的 B 細胞吞噬克雷白氏肺炎桿菌的比例在感染後 12 小時由之前的 7.4%明顯增加至 26.2%。除了 B 細胞之外，可發現在脾臟的巨噬細胞也有吞噬克雷白氏肺炎桿菌的能力。此次實驗的結果初步說明了克雷白氏肺炎桿菌與腸道和免疫系統的交互作用。此免疫細胞在克雷白氏肺炎桿菌造成散布感染的過程中所扮演的角色有待後續實驗的更近一步研究。

中文關鍵詞： 克雷白氏肺炎桿菌, 肝膿瘍

英 文 摘 要 ： K. pneumoniae has been noticed as the primary pathogen responsible for community-cquired pyogenic liver abscess in Taiwan. K. pneumoniae colonization in the intestine is a prerequisite for the development of KLA (K. pneumonia liver abscess). It is interest to know how the intestine-olonized K. pneumoniae render themselves the ability to get across an intestinal barrier. The aim of the study is to explore the mechanism by which K. pneumoniae invade the intestinal lining. By the aid of a non-nvasive in vivo imaging system (IVIS), signals of the luminescence-xpressed K. pneumoniae after an oral inoculation with a single dose of 5 × 107 CFU were detected at 2 hpi (hours post-infection) and 6 hpi that mainly located in the abdomen and anus of the infected mice. The intestines retrieved from the K. pneumoniae-nfected mice, particularly those suffered from severe diarrhea, showed several sites of

swelling and damages of the intestinal villi. K. pneumoniae was able to establish intestinal colonization and thereafter induce inflammation of the intestinal mucosa. Also, a significant increase in the liver lysate of infected mice on the production of IL-, IL-0, and IL-7F was noted at 6 hpi. TEM (Transmission Electron Microscopy) analyses indicated that intactness of nuclear envelop for the infected hepatocytes was severely affected and the leakage caused the enclosure of cellular components, such as peroxisomes, into the nucleus. FACS (Fluorescence-activated cell sorting) analyses showed that a subset of MLN (mesenteric lymph node) B cells might uptake DsRed-xpressed K. pneumoniae, as the percentage of DsRed+ and B220+ cells was significantly increased from 7.4% to 26.2% upon K. pneumoniae infection at 12 hpi. In addition to the B cells, K. pneumoniae was also internalized by macrophages in the spleen. Our findings suggest that the interaction of K. pneumonia with intestinal linings and immue system. The role of these immune cells in the dissemination of K. pneumoniae to distal organs is characterized by our ongoing studies.

英文關鍵詞： Klebsiella pneumoniae, liver abscess

Background and significance

Klebsiella pneumoniae is a Gram-negative enterobacterium ubiquitous in nature with two classical habitats, the environment and the mucosal surfaces of mammals. As behaving like a saprophyte resided in nasopharyngeal or intestinal mucosa of humans, *K. pneumoniae* frequently involves in a wide range of clinical illnesses, such as pneumonia, urinary tract infections, suppurative infections, bacteremia, meningitis, and septicemia. Without immediate treatment, infections caused by this bacterium have a significantly high rate of mortality (1). During 1990s, *K. pneumoniae* has been noticed as the primary pathogen responsible for community-acquired pyogenic liver abscess (PLA) in Taiwan (2). Despite *K. pneumoniae*-caused liver abscess (KLA) was initially thought as a disease of regional distribution, it has now been continually reported from other Asian and Western countries and is considered an emerging disease worldwide (3). Distinct from *Escherichia coli*-associated liver abscess, KLA is generically cryptogenic without underlying hepatobiliary disorders and is frequently complicated in up to 10% of cases with septic metastatic lesions to other organs (2, 4-7). By virtue of its primary and invasive nature, KLA represents one of the most severe infections caused by *K. pneumoniae* (8-11).

The clinical impact of KLA has been well-documented; however, our knowledge regarding the molecular basis of how *K. pneumoniae* causes an infection particularly in the liver is rather restricted. To address this issue, we have established a KLA model with BALB/c mice in which 28 genetic loci of *K. pneumoniae* have been identified as KLA-related (12). The progression of KLA in this model can be divided into 4 stages: intestinal colonization, extraintestinal dissemination, hepatosplenic replication, and septic metastasis. Interestingly, the failure to develop KLA for more than two thirds of the KLA-attenuated mutants was attributed to their incapability to disseminate from the intestines, suggesting that a replicating pool of *K. pneumoniae* has to be established prior to invading the internal organs. This intestine-spleen-liver infectious pathway of *K. pneumoniae* is reminiscent of the dissemination route that has been reported for *Yersinia enterocolitica* and *Salmonella enterica* serovar Typhimurium (13). The oral-inoculated *Yersinia* resides in the lymphoid Peyer's patches (PP) and the mesenteric lymph nodes (MLN) within 24 h and then appears in the liver and spleen between 48-72 h (14). The ordered spread from the intestinal lumen into the PP and MLN and then to the spleen and liver is also the presumed model of *Salmonella* dissemination. However, the existence of pathways bypassing the PP and MLN is recently

reported for the intestinal translocation of *Y. pseudotuberculosis* (15) and *Salmonella* (16). There are at least three possible translocation mechanisms that bypass the PP-MLN. First, local micro-damages in the intestinal epithelium which may be caused by host or bacteria provide sites for translocation. The release of *Yersinia* effectors through a type III secretion system may break down the tight junctions between intestinal epithelial cells and may also cause apoptotic processes occurring at the top of intestinal villi, permitting translocation of bacteria (17). Second, villous-associated M cells that allow sampling intestinal contents are recently identified to be served as portals across the epithelium (18). Finally, phagocytic or dendritic cells (DC) interdigitated within the intestinal epithelium that capture luminal bacteria may provide a phagocytic route for translocation, as evident for *S.* Typhimurium. Upon the expression of chemokine receptor CXC3CR1, the transepithelial extensions of dendritic cells facilitate sampling of intestinal bacteria that in turn provide as sites for attachment by intestinal pathogens (19). Even these processes that lead to translocation across the intestine may not be particularly efficient though, the large loads of bacteria in systemic infection can be derived from as few as one intestinal clone (15); therefore, enteropathogens still hold the key to develop a successful systemic infection with the multiple strategies provided for initiating dissemination.

Our previous study demonstrates that establishment of *K. pneumoniae* colonization in the intestine is a prerequisite for the development of KLA (12). Because bacterial across the intestinal barrier is a bottleneck steps for a systemic infection, it is of particular interest to know how the intestine-colonized *K. pneumoniae* render themselves the ability to get across an intestinal barrier, evade the host immune responses, and finally gain growth advantages within specific niches in the liver. To address this issue, we aim in this project to answer the question “What is the mechanism by which *K. pneumoniae* invade the intestinal lining?”.

Specific aims

We aim in this project to answer the question "What is the mechanism by which *K. pneumoniae* invades the intestinal lining?". Specific aims toward this question are listed.

1 To determine the histopathology of *K. pneumoniae*-colonized intestinal mucosa.

2 To explore the interaction of *K. pneumoniae* with intestinal linings.

3 To investigate the disseminating route toward the spleen and liver.

Results and discussion

1. **Intestinal colonization of *K. pneumoniae*.** In most of the *K. pneumoniae*-infected mice, episodes of diarrhea were noted in prior to the development of septicemia. By the aid of a non-invasive in vivo imaging system (IVIS), signals of the luminescence-expressed *K. pneumoniae* after an oral inoculation with a single dose of 5×10^7 CFU were detected at 2 hpi and 6 hpi that mainly located in the abdomen and anus of the infected mice (Fig. 1A). The intestines retrieved from the *K. pneumoniae*-infected mice, particularly those suffered from severe diarrhea, showed several sites of swelling (Fig. 1B) and damages of the intestinal villi (Fig. 1C). To detect the distribution of *K. pneumoniae*, the mouse tissues were harvested, paraformaldehyde-fixed, parafilm-embedded, sectioned, and the tissue sections were subjected to HE stain and immunohistochemistry (IHC) analyses with *K. pneumoniae*-specific antibodies. As compared to the result from tissue sections of the intestines retrieved from the control mice (Fig. 1D), a significant amount of *K. pneumoniae* were located on the mucosal surface of small intestines (Fig. 1E). These results indicated that *K. pneumoniae* was able to establish intestinal colonization and thereafter induce inflammation of the intestinal mucosa.

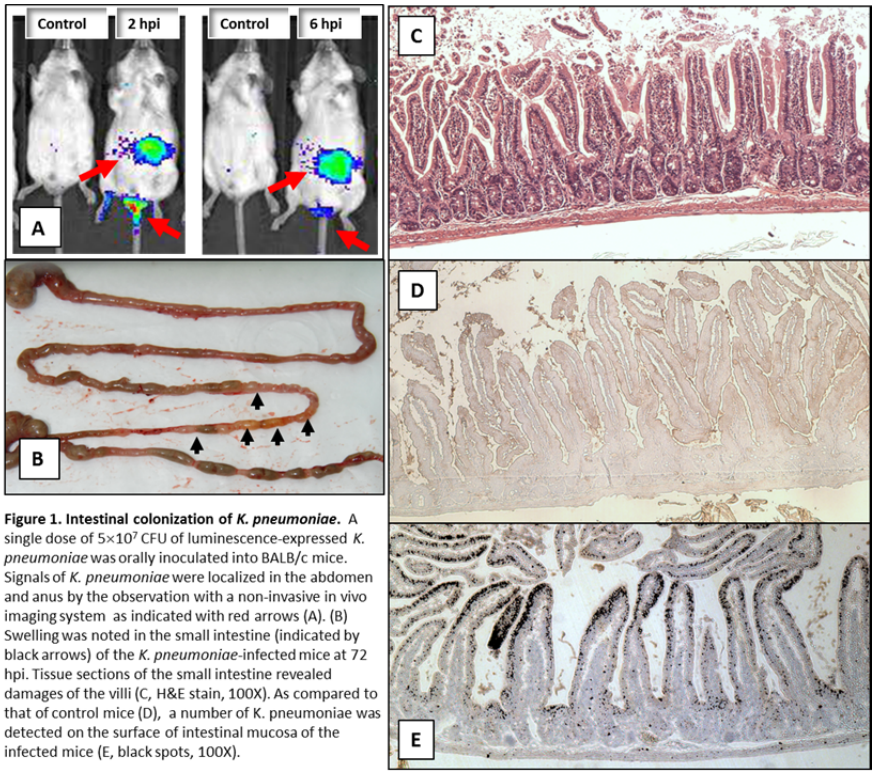

Figure 1. Intestinal colonization of *K. pneumoniae*. A single dose of 5×10^7 CFU of luminescence-expressed *K. pneumoniae* was orally inoculated into BALB/c mice. Signals of *K. pneumoniae* were localized in the abdomen and anus by the observation with a non-invasive in vivo imaging system as indicated with red arrows (A). (B) Swelling was noted in the small intestine (indicated by black arrows) of the *K. pneumoniae*-infected mice at 72 hpi. Tissue sections of the small intestine revealed damages of the villi (C, H&E stain, 100X). As compared to that of control mice (D), a number of K. pneumoniae was detected on the surface of intestinal mucosa of the infected mice (E, black spots, 100X).

2. **Inflammation and cell damage induced by *K. pneumoniae* infection.** In our model, upon the occurrence of extraintestinal dissemination, multiplication of *K. pneumoniae* in niches within the liver leads to the formation of microabscesses. Therefore, to elucidate the mechanism of KLA pathogenesis, it is necessary to understand what happen to the liver after the arrival of *K. pneumoniae*. Because blood from the intestine which is rich in microbial products converges in liver with that of the systemic circulation, the liver has developed a distinctive local immune environment to deal with antigens derived from the vast amount of commensal microbes. Innate lymphocytes, including both natural killer (NK) cells and natural killer T (NKT) cells are unusually abundant in the liver. Multiple populations of nonhematopoietic liver cells, including sinusoidal endothelial cells, stellate cells located in the subendothelial space, and liver parenchymal cells, have their roles in antigen presentation. These liver-specialized antigen presenting cells (APCs) present antigen in the context of immunosuppressive cytokines and inhibitory cell surface ligands, and thereby immune responses to the liver antigens often result in tolerance. The tolerogenic immune environment created for antigen-specific T-cells, activation of Kupffer cells, recruited macrophages, and inflammatory cells may incur the production of cytokines and chemokines that lead to prolonged inflammation and hepatocyte damage. To unravel the immune response in the liver to *K. pneumoniae* infection, the level of various inflammation-associated cytokines were determined by ELISA. As shown in Fig. 2, a significant increase on the production of IL-6, IL-10, and IL-17F was noted at 6 hpi. Local production of IL-17 is a pivotal factor in effective host defense against Gram-negative bacteria that may be caused by the PRRs-TLRs interaction. IL-17 (Fig. 2C) induces and mediates proinflammatory responses, including the production of cytokines, such as IL-6 (Fig. 2A), and chemokines, which thereafter recruit monocytes and neutrophils to the site of inflammation. However, the IL-17 provoked inflammation might be countered by the action of IL-10, as its production was also enhanced by *K. pneumoniae* infection (Fig. 2B). As a consequence of the

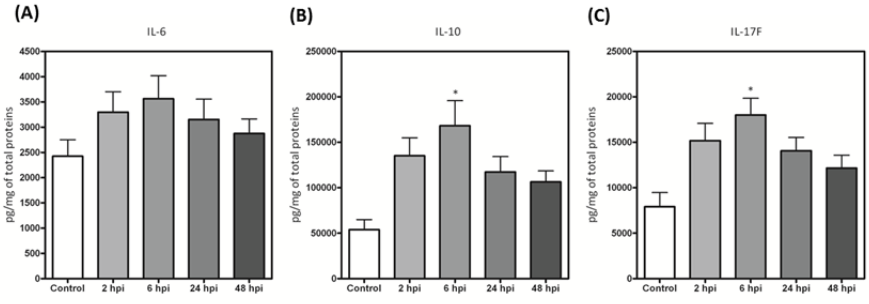

Figure 2. Induction of inflammatory cytokines upon *K. pneumoniae* infection. At 2, 6, 24, and 48 hpi, the amount of cytokines were measured by ELISA in liver lysates, which were retrieved from the control or the *K. pneumoniae*-infected mice and were normalized with the total protein amount. The level of IL-6 (A), IL-10 (B), and IL-17F (C) are expressed as pg/mg of tissues. Data represent average ± SD from 10 mice. $*p < 0.05$ by Student's *t* test.

complicated local immune responses in the liver, which remains to be clarified, damages on the liver cells, particularly the parenchyma cells, were developed during the KLA progression. As shown in Fig. 3, compared to the control mice (A-C), the major component of nuclear envelop, lamin A, was dispersed all over the cytoplasm of the *K. pneumoniae*-infected hepatocytes (D and E). TEM analyses indicated that intactness of nuclear envelop for the infected hepatocytes was severely affected and the leakage caused the enclosure of cellular components, such as peroxisomes, into the nucleus (F).

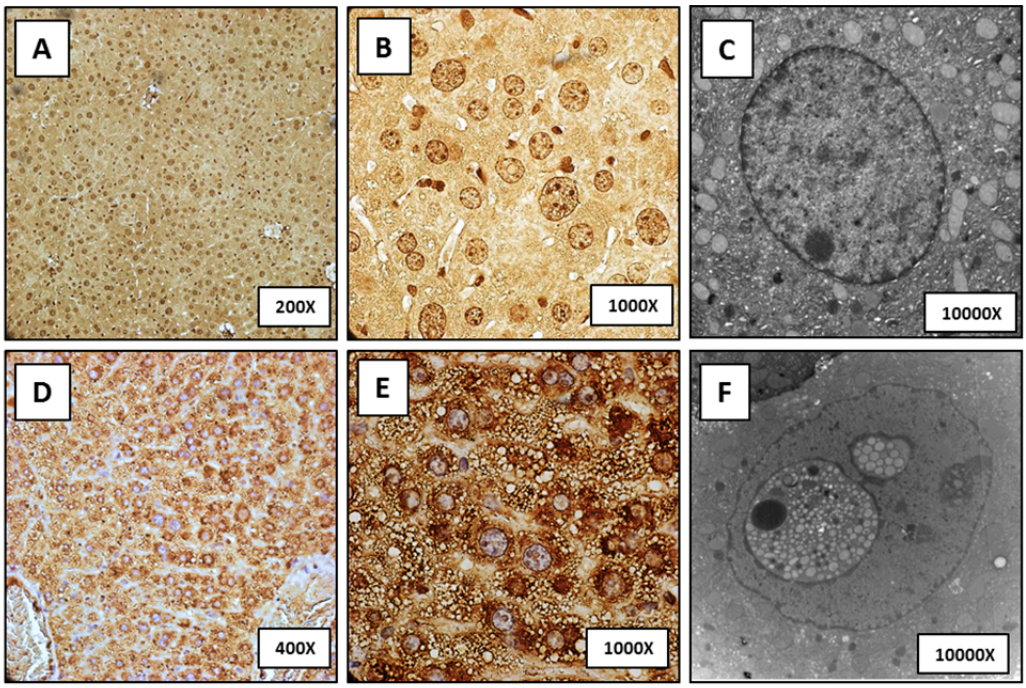

Figure 3. Cell damage of hepatocytes. The mouse liver was retrieved from control mice (A-C) or from the *K. pneumoniae*-infected mice (D-F) at 72 hpi. Tissue sections were prepared from paraffin-embedded block and were subjected to IHC analyses with anti-lamin A antibody to examine the nucleus integrity of hepatocytes. Compared to that of the control mice (A, and B), loss of cellular and nuclear integrity and accumulation of vacuoles was noted in the hepatocytes from the infected group (C, and E). TEM observation of liver tissues which were retrieved from the control group (C) or from the *K. pneumoniae*-infected mice (F).

3. **Internalization of *K. pneumoniae* by B cells and macrophages in spleen and MLN.** Once the enteropathogenic bacteria penetrate the intestinal linings as either free or host cell-associated organisms, what is the pathway routing bacteria to distal infection sites? Blood-borne bacteria can travel via the portal vein system, which transports intestinal blood directly to the liver. This allows the transit of unfiltered bacteria to other downstream blood-filtering organs, such as the spleen. Alternatively, bacteria entering through the lymphatics may first route to the MLN, drain into the thoracic duct and finally flow into the bloodstream. In either case, the colonization of liver and/or spleen indicates that bacteria are present in the blood at some point during the infectious course. To examine whether *K. pneumoniae* disseminates freely from the intestine or is associated with certain type of immune cells, groups of mice were orally inoculated with *K. pneumoniae* expressing DsRed

fluorescence (DsRed-Kp), which was generated by the introduction of pDsRed (Invitrogen) into *K. pneumoniae* CG43. FACS analyses showed that a subset of MLN B cells might uptake DsRed-expressed *K. pneumoniae*, as the percentage of $DsRed^+$ and $B220^+$ cells was significantly increased from 7.4% to 26.2% upon *K. pneumoniae* infection at 12 hpi (Fig. 4A). In addition to the B cells, *K. pneumoniae* was also internalized by macrophages in the spleen (Fig. 4B). The role of these immune cells in the dissemination of *K. pneumoniae* to distal organs is characterized by our ongoing studies.

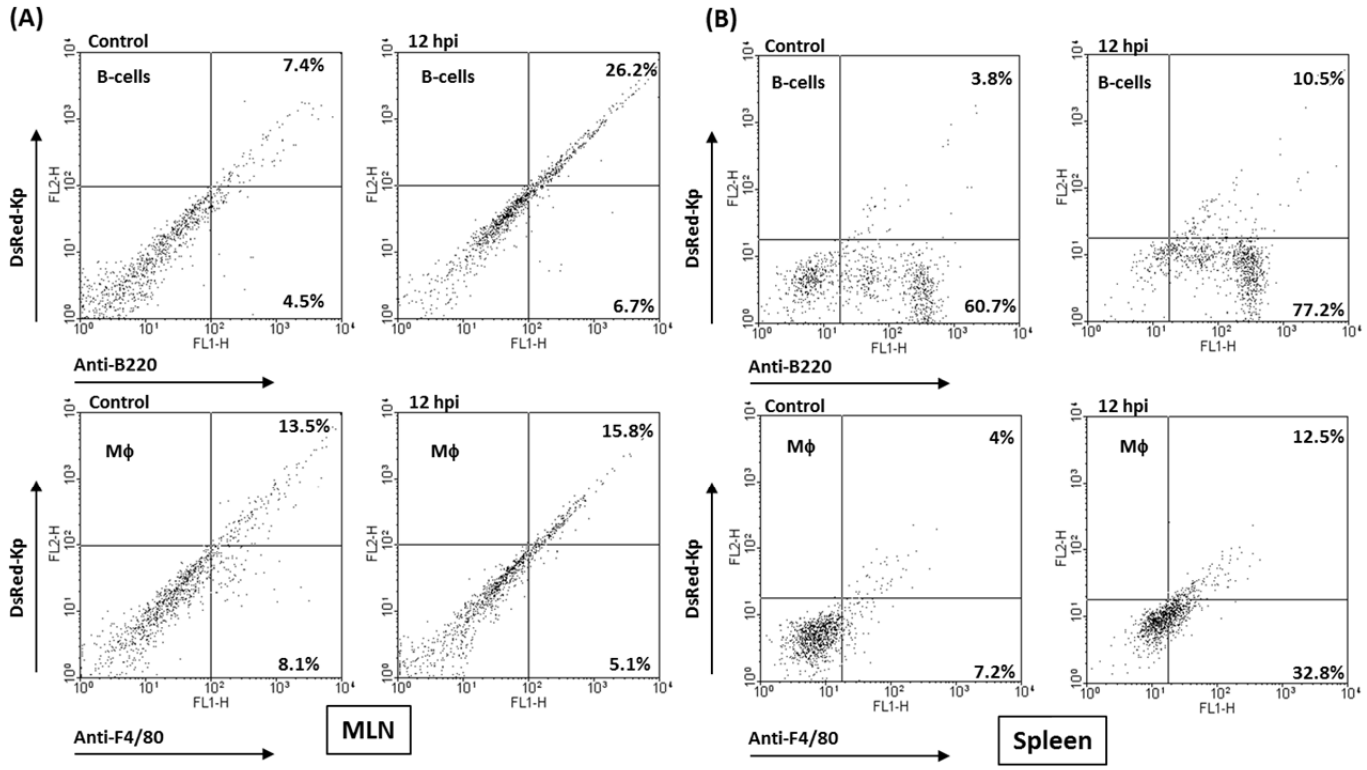

Figure 4. In vivo uptake of *K. pneumoniae* by B cells and macrophages. Eight-to-ten week old BALB/c mice were orally inoculated with 5×10^7 CFU of *K. pneumoniae* CG43-expressing DsRed fluorescence (DsRed-Kp). At 12 hpi, spleen and MLN were retrieved from the control and infected mice. Total cells isolated from spleen (A) or MLN (B) pooled from three mice were stained with FITC-conjugated anti-B220, anti-CD3, and anti-F4/80 antibodies. DsRed versus FITC scatter plots are depicted.

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國科會補助計畫衍生研發成果推廣資料表

日期:2012/11/20

國科會補助計畫	計畫名稱: 克雷白氏肺炎桿菌經由腸道上皮入侵造成肝脾膿瘍的細胞感染機轉研究
	計畫主持人: 盧敏吉
	計畫編號: 100-2320-B-040-016- 學門領域: 微生物及免疫學

無研發成果推廣資料

100 年度專題研究計畫研究成果彙整表

計畫主持人：盧敏吉	計畫編號：100-2320-B-040-016-
計畫名稱：克雷白氏肺炎桿菌經由腸道上皮入侵造成肝脾膿瘍的細胞感染機轉研究	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	
		博士生	1	1	100%		
		博士後研究員	0	0	100%		
		專任助理	2	2	100%		
國外	論文著作	期刊論文	0	1	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：□已發表 □未發表之文稿 ■撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：(以 100 字為限)

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）(以 500 字為限)

研究克雷白氏肺炎桿菌的致病機轉有助於預防此菌造成的感染. 並提升致病後的病人存活率