財團法人明日醫學基金會專題研究計畫申請書

一、基本資料： 申請條碼：

本申請案所需經費(單選)	■A 類(執行計畫所需經費) ☐B 類(研究主持費，限人文處計畫，不須填寫表 C002 及 C004 至 C009)				
計畫類別(單選)	■一般型研究計畫 ☐特約研究計畫 ☐新進人員研究計畫 ☐其他				
研究型別	■個別型計畫 ☐整合型計畫				
申請機構/系所(單位)	長庚大學醫學院微生物及免疫學科				
本計畫主持人姓名	賴志河	職稱	教授	身分證號碼	
本計畫名稱 中文	探討幽門螺旋桿菌基因體異質性導致宿主致病性的機制				
本計畫名稱 英文	Genomic heterogeneity contributes to the pathogenicity of *Helicobacter pylori* in the host				
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整合型總計畫主持人				身分證號碼	
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研究性質	■純基礎研究 ☐導向性基礎研究 ☐應用研究 ☐技術發展				
本計畫是否為國際合作計畫	■否； ☐是，合作國家：_____，請加填表 I001～I003				
本計畫是否申請海洋研究船	■否； ☐是，請務必填寫表 C014。				
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三、主要研究人力：

（一）請依照「主持人」、「共同主持人」、「協同研究人員」及「博士後研究」等類別之順序分別填寫。

類 別	姓名	服務機構/系所	職稱	在本研究計畫內擔任之具體工作性質、項目及範圍	*每週平均投入工作時數比率(%)
主持人	賴志河	長庚大學/醫學院/微生物及免疫學科	教授	研究之規劃及推動、實驗設計、整理數據、撰寫研究成果與發表論文	70%

※註：每週平均投入工作時數比率係填寫每人每週平均投入本計畫工作時數佔其每週全部工作時間之比率，以百分比表示（例如：50%即表示該研究人員每週投入本計畫研究工作之時數佔其每週全部工時之百分五十）。

Abstract

Helicobacter pylori is a spiral-shaped, Gram-negative bacterium that primarily colonizes the human stomach. Persistent *H. pylori* infection leads to chronic gastritis, peptic ulcers, and gastric cancer. Multiple *H. pylori* isolates can colonize different regions of the stomach within the same host. These strains exhibit significant genetic diversity, enabling them to express distinct virulence factors that contribute to disease progression. However, the differences in whole-genome sequences of *H. pylori* strains isolated from different gastric sites within the same host and their effects on gastric epithelial cells remain poorly understood. This study aimed to analyze the whole-genome sequences of two pairs of *H. pylori* strains isolated from the gastric antrum and corpus of two patients. We will investigate the genetic diversity of these isolates, with a particular focus on pathogenic genes such as adhesins and cytotoxins. Furthermore, we will examine how differences in these gene sequences influence the pathogenicity and inflammatory responses of human gastric epithelial cells.

Keywords: *Helicobacter pylori*; virulence factor; genome; heterogenicity; pathogenicity

A. Introduction

H. pylori is a microaerophilic, gram-negative bacterium with a spiral shape that colonizes the human stomach. The bacterial presence is closely linked to an elevated risk of developing peptic ulcer disease and gastric adenocarcinoma following persistent infection over a long period [1]. DNA fingerprinting studies have revealed substantial genetic diversity among different *H. pylori* clinical isolates, surpassing that of many other studied bacteria [2].

The pathogenicity of *H. pylori* is predominantly attributed to its array of virulence factors [3]. Additionally, this genetic heterogeneity equips *H. pylori* with the ability to generate multiple genetic variants during adhesion and colonization [4]. This is evidenced by allelic variations in genes such as *vacuolating cytotoxin A* (*vacA*) and the presence of non-conserved DNA segments between strains, including the *cag*-pathogenicity islands (*cag*-PAI). [5, 6]. Consequently, *H. pylori* exhibits the capacity to express various virulence factors and to trigger adaptive mechanisms within the stomach, all of which contribute to its pathogenicity and potential progression of associated diseases [7].

Despite these findings, a comprehensive exploration of the variations in whole genome sequences of different *H. pylori* isolates within the same host, and their impact on gastric epithelial cells remains to be explored. In this study, we will investigate the whole genome sequences of two pairs of strains (v225/v226 and v290/v291) isolated from the gastric antrum and corpus of two patients, respectively. We will further explore the genetic heterogeneity and pathogenicity of two pairs of *H. pylori* isolates. The specific aims of this study are:

1. To analyze the whole genomes of two pairs of clinical isolates using next-generation sequencing (NGS) to assess genetic heterogeneity.
2. To investigate differences in the sequences of key pathogenic genes between the two pairs of strains.
3. To evaluate the impact of genetic variations on the pathogenicity and inflammatory responses of human gastric epithelial cells.

B. Preliminary results

1. Whole genome sequence analysis of *H. pylori* isolates

We previously isolated *H. pylori* strains (v225/v226 and v290/v291) from two distinct gastric sites (antrum and corpus) and found a chimerism phenomenon in the *vacA* gene between corpus-isolated strains (v226 and v291) and antrum-isolated strains (v225 and v226) [8]. In the present study, we first investigated the differences in the genetic level and pathogenicity among two pairs of clinical isolates. We conducted the whole genome sequencing and analyzed the virulence genes between two different clinical *H. pylori* strains that had been isolated from two distinct gastric parts, antrum and corpus, respectively. The whole genome of two pairs of strains was sequenced by next-generation sequencing (NGS) and compared with the reference strain 26695 by using the Mauve. Our results showed the conserved genes between two pairs of strains (v225/v226 and v290/v291) in relative order (Fig. 1).

2. Comparison of virulence genes in *H. pylori* isolates

Next, we analyzed the virulence genes between two clinical strains isolated from the gastric corpus and antrum to analyze differences in virulence factors present among selected strains. As shown in Fig. 2, v290 was the only strain that did not contain virulence genes *sabA/hopP* in its genome. While *sabB/hopO*, α-(1,2)-fucosyltransferase (*futA*), and undetermined virulence factors were examined to be common deletions that appeared in all isolates.

2. Heterogeneity in virulence genes of *H. pylori* isolates

To have a comprehensive understanding of these differences in sequence identity of important virulence factors, we further analyzed multiple sequence alignments between different isolates' adhesion genes (*hop* family), urease genes, toxin genes (*cag-PAI* and *vacA*), and immune evasion proteins (*capJ*, *cgat*, etc.). Our results showed that the essential genes for adhesion molecules, such as *hop* family-*sabA* and *babA/B* were significantly different only in the pair of strains v290/v291 (Table 1). For instance, *sabA* gene was found only in v291, while the similarity in *babA* and *babB* between v290 and v291 were 63% and 59%, respectively. As a result, the gene sequences of these two strains in *babA/B* were significantly different. Moreover, the identity of virulence genes-*cag-PAI* in v225/v226 and v290/v291 strains comprised more than 90%, and in *vacA* accounted for 93% and 89%, respectively.

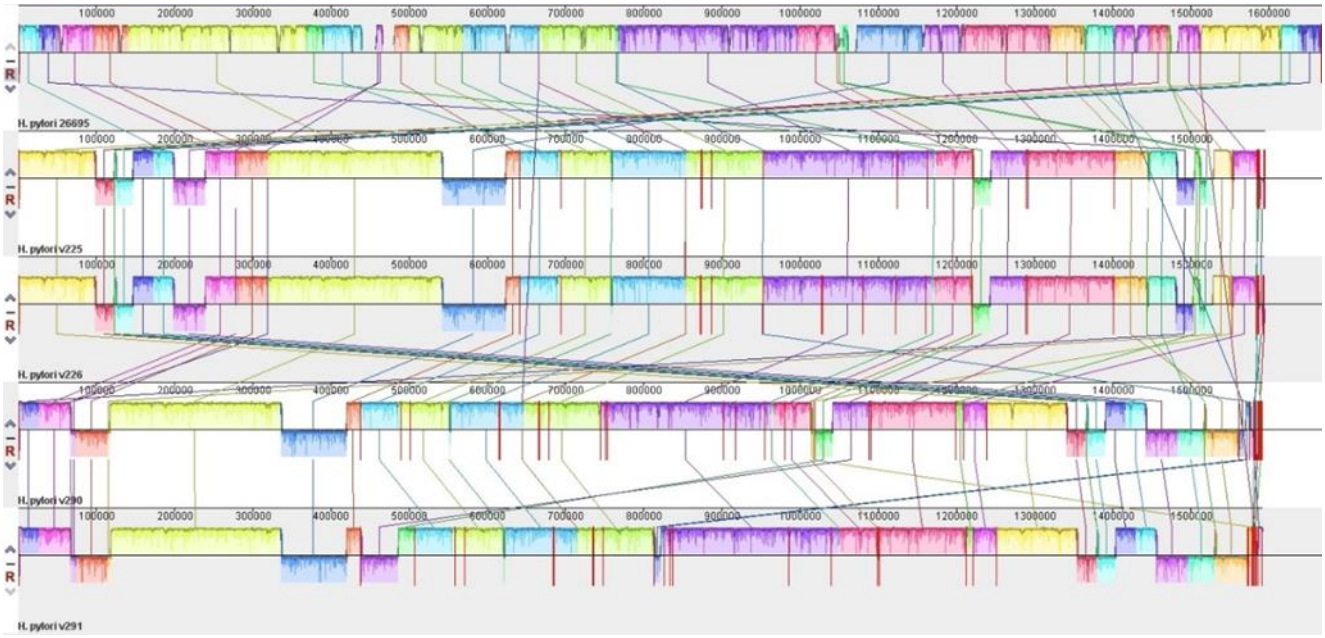

Figure 1: Genomic divergence among clinical isolates v225/v226 and v290/v291. Each genome is displayed horizontally, with homologous segments (locally collinear blocks, LCBs) between genomes connected by lines and represented in matching colors. Regions inverted relative to *H. pylori* 26695 are shown below the centerline of the genome.

Figure 2: Analysis of virulence genes in *H. pylori* clinical isolates. The reference strain *H. pylori* 26695 (hpNEAfrica1) and clinical isolates v225/v226 and v290/v291 were analyzed for virulence genes associated with acid resistance, bacterial adhesion, immune evasion, motility, secretion systems, and key toxins. Black blocks indicate deleted genes.

Table 1. Genetic comparison between clinical isolates v225/v226 and v290/v291

	Gene [a]	Gene no.[b]	% identity of amino acid	
			v225 vs v226	v290 vs v291
Adhesion	Hop family (major outer membrane protein family)			
	hopZ	HP0009	100	98
	hopD	HP0025	100	99
	hopM	HP0027	100	99
	hopA	HP0229	100	96
	hopF	HP0252	99	100
	hopG	HP0253/0254	100	99
	hopJ	HP0477	none	none
	hopH	HP0638	100	100
	hopE	HP0706	100	99
	hopC	HP0912	100	97
	hopB	HP0913	100	93
	hopK	HP0923	none	none
	hopI	HP1156	99	97
	hopL	HP1157	99	97
	hopQ	HP1177	96	90
	hopN	HP1342	none	none
	hopU	HP0317	none	none
	sabA (hopP)	HP0725	100	none (only 291)
	sabB (hopO)	HP0722	none	none
	babA (hopS)	HP1243	99	63
	babB (hopT)	HP0896	100	59
Motility	Flagellum A (flaA)	HP0601	100	99
	Flagellum B (flaB)	HP0115	100	99
Acid resistance	ureH	HP0067	100	100
	ureG	HP0068	100	99
	ureF	HP0069	100	100
	ureE	HP0070	99	100
	ureI	HP0071	100	99
	ureB	HP0072	100	100
	ureA	HP0073	100	99
	lipoprotein signal peptidase (lspA)(ureC)	HP0074	100	98
	Phosphoglucosamine mutas (glmM)(ureD)	HP0075	100	100
Toxin	cagPAI			
	cag1 (cagζ)	HP0520	100	100
	cag2 (cagε)	HP0521_pseudo	none	none
	cag3 (cagδ)	HP0522	100	100
	cag4 (cagγ)	HP0523	100	100
	cag5 (cagβ)	HP0524	100	100
	cagα	HP0525	100	100
	cagZ	HP0526	100	99
	cagY	HP0527	100	91
	cagX	HP0528	100	100
	cagW	HP0529	100	100
	cagV	HP0530	100	100
	cagU	HP0531	100	100
	cagT	HP0532	100	100
	cagS	HP0534	100	100
	cagQ	HP0535	100	100
	cagP	HP0536	100	100
	cagM	HP0537	100	100
	cagN	HP0538	100	100
	cagL	HP0539	100	100
	cagI	HP0540	100	100
	cagH	HP0541	100	100
	cagG	HP0542	100	100
	cagF	HP0543	100	100
	cagE	HP0544	100	100
	cagD	HP0545	100	96
	cagC	HP0546	100	91
	cagA	HP0547	99	98
	vacA	HP0887	93	89
Others	capJ	HP0421	100	98
	CGAT	HP0499	100	96
	acyl_carrier_protein_(ACP)-dehydratase CoA	HP0420	100	100
	N-acetyltransferase	HP0935	100	100

[a]Genes for virulence factor of *Helicobacter pylori* .
[b]Based on the *H. pylori* reference strain 26695 as the gene number base.

C. Materials and methods

1. Preparation of *H. pylori* isolates

Clinical strains v225/v226 and v290/291 were isolated from the gastric antrum and corpus of two distinct patients, respectively [8]. *H. pylori* 26695 (ATCC 700392) was the reference strain for whole genome sequence comparisons. All *H. pylori* strains were cultured on Brucella agar plates (Becton Dickinson) supplemented with 10% sheep blood.

2. Library construction

Whole-genome sequencing was conducted by Welgene Biotech Company. Total DNA (10 μg) was fragmented to a size range of 400–500 bp using a Misonix 3000 sonicator, and the fragment sizes were verified using a Bioanalyzer DNA 1000 chip (Agilent Technologies). Subsequently, 1 μg of the sonicated DNA was subjected to end-repair, A-tailing, and adaptor ligation following Illumina's TruSeq DNA preparation protocol.

3. Genome *de novo* assembly

Each sample comprised paired-end reads with a length of approximately ±300 b.p. Quality control, trimming, and filtering procedures were conducted utilizing BBDuk (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/, accessed on 3rd May 2022). Subsequently, the cleaned and filtered nuclear reads were subjected to *de novo* assembly using Spades 3.15.1 with default settings [9].

4. Gene annotation

Open reading frames (ORFs) were annotated employing Rapid Annotation using Subsystem Technology (RAST) [10]. The generated predictions were cross-referenced against the NCBI non-redundant (nr) database using BLASTp.

5. Cell culture

Human gastric adenocarcinoma AGS cells (ATCC CRL 1739) will be cultured in F-12 medium (Sigma-Aldrich) supplemented with 10% decomplemented fetal bovine serum (HyClone) at 37°C in 5% CO_2 for 24-48 h.

6. Bacterial adhesion assay

The adhesion of *H. pylori* to AGS will be analyzed by counting standard colony formation assay. AGS cells (2×10^5) will be seeded in 12-well plates containing F-12 medium supplemented

with 10% FBS at 37°C. Cells will be infected with *H. pylori* at a MOI of 100 for 6h. After infection, the nonadherent *H. pylori* will be removed by washing twice with PBS, and then the infected AGS cells will be lysed with 1 ml distilled water for 10 min. The lysates will be serially diluted in PBS, plated on brucella blood agar plates, and incubated for 4-5 days, after which the CFU will be counted.

7. NF-κB reporter luciferase assay

To perform the transient transfection of NF-κB reporter gene and luciferase activity assay, AGS cells (2×10^5) will be grown in 12-well plates for 24 h and transfected with a NF-κB-luc reporter plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After a 24 h incubation for transfection, cells will be infected with *H. pylori* at a MOI of 100 for 6 h. The cells will be scraped from dishes and an equal volume of luciferase substrate (Promega) will be added to the samples. The luminescence will be measured using a microplate luminometer (Biotek, Winooski, VT, USA). Luciferase activity will be normalized to transfection efficiency, which will then be determined using the β-galactosidase activity of a cotransfected β-galactosidase expression vector (Promega).

8. Statistical analysis

The experimental results will be expressed as means ± SEM. Student's *t*-test will be used to determine the statistical difference between the two groups. The difference will be considered significant when $P < 0.05$. Statistical analysis will be performed by using Prism8.0 (Graph Pad Software, La Jolla, CA, USA).

D. Anticipated results

1. *H. pylori* CagA translocation and phosphorylation diverse between the isolates

The *cag* pathogenicity island (*cag-PAI*) encodes key virulence factors of *H. pylori*, including the toxin CagA and the bacterial type IV secretion system (T4SS). CagA is translocated into gastric epithelial cells via T4SS, where it is subsequently phosphorylated by Src homology phosphotyrosyl phosphatase 2 (SHP2) and influences various cellular processes. The present findings reveal that the two pairs of clinical isolates contain previously uncharacterized genes within the *cag-PAI* cluster, some of which are located near essential genes, such as *cagA* and genes encoding T4SS components. It remains unclear whether these unknown genes interact with CagA or T4SS. This study aims to further investigate the translocation and phosphorylation of CagA in gastric epithelial cells and to explore the functional roles of *cag-PAI*-encoded CagA

and T4SS during infection by clinical isolates.

2. The severity of CagA-induced inflammation in *H. pylori* isolates

The translocation of *H. pylori* CagA activates IL-8 transcription via the NF-κB signaling pathway, contributing to the severity of inflammation in patients with *H. pylori*-induced gastritis. This highlights the pivotal role of CagA in promoting gastric epithelial cell inflammation. Therefore, this study aims to investigate the role of CagA in inducing inflammation following its translocation into gastric epithelial cells by analyzing NF-κB activity and IL-8 production in different *H. pylori* clinical isolates.

F. References

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