Genomic and vaccine preclinical studies reveal a novel mouse-adapted *Helicobacter pylori* model for the hpEastAsia genotype in Southeast Asia

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Abstract

Introduction. *Helicobacter pylori* infection is a major global health concern, linked to the development of various gastrointestinal diseases, including gastric cancer. To study the pathogenesis of *H. pylori* and develop effective intervention strategies, appropriate animal pathogen models that closely mimic human infection are essential.

Gap statement. This study focuses on the understudied hpEastAsia genotype in Southeast Asia, a region marked by a high *H. pylori* infection rate. No mouse-adapted model strains has been reported previously. Moreover, it recognizes the urgent requirement for vaccines in developing countries, where overuse of antimicrobials is fuelling the emergence of resistance.

Aim. This study aims to establish a novel mouse-adapted *H. pylori* model specific to the hpEastAsia genotype prevalent in Southeast Asia, focusing on comparative genomic and histopathological analysis of pathogens coupled with vaccine preclinical studies.

Methodology. We collected and sequenced the whole genome of clinical strains of *H. pylori* from infected patients in Vietnam and performed comparative genomic analyses of *H. pylori* strains in Southeast Asia. In parallel, we conducted preclinical studies to assess the pathogenicity of the mouse-adapted *H. pylori* strain and the protective effect of a new spore-vectored vaccine candidate on male Mlac:ICR mice and the host immune response in a female C57BL/6 mouse model.

Results. Genome sequencing and comparison revealed unique and common genetic signatures, antimicrobial resistance genes and virulence factors in strains HP22 and HP34; and supported clarithromycin-resistant HP34 as a representation of the hpEastAsia genotype in Vietnam and Southeast Asia. HP34-infected mice exhibited gastric inflammation, epithelial erosion and dysplastic changes that closely resembled the pathology observed in human *H. pylori* infection. Furthermore, comprehensive immunological characterization demonstrated a robust host immune response, including both mucosal and systemic immune responses. Oral vaccination with candidate vaccine formulations elicited a significant reduction in bacterial colonization in the model.

Conclusion. Our findings demonstrate the successful development of a novel mouse-adapted *H. pylori* model for the hpEastAsia genotype in Vietnam and Southeast Asia. Our research highlights the distinctive genotype and pathogenicity of clinical *H. pylori* strains in the region, laying the foundation for targeted interventions to address this global health burden.

DATA AVAILABILITY

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession numbers can be found in the article/Supplementary Material.

INTRODUCTION

Helicobacter pylori is the predominant bacterium in the human stomach microbiota of infected individuals and is the aetiological agent in most cases of gastric cancer, gastric mucosa-associated lymphoid tissue (MALT) lymphoma and gastroduodenal ulcer disease. Carcinogenic infections contribute to approximately 13% of global cancer cases, with *H. pylori*-associated gastric cancer being the most common type [1, 2]. Over 77% of new gastric cancer cases and more than 89% of new non-cardiac gastric cancer

cases are attributed to *H. pylori* infection [3]. Therefore, efforts to address and manage *H. pylori* infections are crucial for reducing the associated disease burden and improving global health outcomes.

H. pylori infection affects approximately half of the global population, with higher rates observed in Asia, Latin America and Africa compared to North America and Oceania. In poorer nations, infection rates range from 85–95%, while in industrialized nations the range is 30–50% [3–6]. These variations may be linked to ancient human migrations and the population structure of *H. pylori* worldwide, which is classified into seven major groups [7]. The hpEastAsia subgroup, prevalent in East and Southeast Asian countries with high gastric cancer incidence [8], exhibits higher virulence and has distinct virulence factors and proteins adapted to local geographical conditions [9, 10].

The global rise in antimicrobial resistance (AMR) adds to the challenge of developing a universal antimicrobial therapy for *H. pylori* due to its diverse genetic makeup [11]. Co-infections with multiple *H. pylori* strains of varying genotypes have demonstrated altered pathogenicity and antibiotic susceptibility [12, 13], emphasizing the need for comprehensive *in vivo* experiments. Consequently, the establishment of an appropriate model of animal and animal-adapted pathogens is crucial.

Mice are commonly chosen as the preferred host animal for studying the *in vivo* effects of *H. pylori* infections due to their scientific relevance and cost-effectiveness. They serve as an important model for assessing the efficacy of vaccines and antimicrobials [14]. However, it is important to note that the gastric physiology of mice does not perfectly replicate the human condition [15]. Although early mouse models provided crucial initial data, they lacked the ability to fully mimic the characteristics of *H. pylori* infection in humans. Therefore, mouse models with persistent colonization by *H. pylori* are crucial from both biological and economic perspectives. Such models enable the analysis of host responses against the pathogen, the influence of microenvironmental and bacterial factors and, most importantly, the development of vaccines targeting clinically relevant strains of *H. pylori* [14].

In mouse colonization studies, it is common for researchers to employ approximately 10 *H. pylori* isolates from various genotypes and geographical sources, as outlined in Table 1. In our recent research, we have introduced an *H. pylori* HP34 strain in Vietnam for preclinical test of vaccine candidates [16] but this clarithromycin-resistant strain has not been analysed in terms of genomics and histopathology to identify the genotype and pathological mechanism, which can be coupled with and compared to wider geographical and disease infection contexts. This study aims to address the existing knowledge gap concerning *H. pylori* infection in Southeast Asian populations by focusing on comparative genomic and histopathological analysis of gastric colonization of HP34 as well as preclinical evaluation of a novel vaccine candidate to establish a novel mouse-adapted model specifically designed for the hpEastAsia genotype in this region. The primary objective is to contribute to the development of interventions that are urgently needed to combat *H. pylori* as a significant global health burden. Moreover, comparative genomics approaches have been shown to improve our understanding of how genetic variation amongst *H. pylori* strains influences disease progression [17, 18]. By introducing this genotype/region-specific model, the study aims to fill the gaps in our understanding of hpEastAsia *H. pylori* infection in Southeast Asia and lay the groundwork for future research and interventions in this specific population. The research not only emphasizes the uniqueness of the HP34 strain but also contributes essential insights into *H. pylori*-associated diseases in the region, paving the way for region-specific interventions to combat this global health issue.

METHODS

Microbial strains

The clinical strains HP22 and HP34 of *H. pylori* were isolated from patients by the Hospital of the University of Medicine and Pharmacy, Hue University, Vietnam. HP22 was obtained (3 February 2020) from a patient with gastritis who had received two previous *H. pylori* treatments. Endoscopy revealed inflammation in the fundus. HP22 displayed resistance to clarithromycin, metronidazole, amoxicillin and levofloxacin but sensitivity to tetracycline. HP34 was isolated (5 May 2020) from a patient with a

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Table 1. An update of common mouse-adapted model strains of *H. pylori*

No.	Mouse strain/ sex	Bacterial strain	Bacterial source	Bacterial genotype	Bacterial determinants				Disease signatures				Reference
					Cag PAI	CagA	VacA	T4SS	(1)	(2)	(3)	(4)	
	Male or female C57BL/6, female BALB/c	SS1	Australia	hpEurope	Intact	+ve	+ve s2m2	Non-functional	+ve	+ve	+ve	+ve	[15, 63, 64]
2	Female C57BL/6 and BALB/c	SS2000	Australia	ND	–ve	–ve	ND	ND	ND	+ve	+ve	+ve	[36, 65]
3	Female C57BL/6	PMSS1	Australia	hpEurope	Intact	+ve	+ve s2m2	Functional	+ve	+ve	+ve	+ve	[32, 66]
4	Female C57BL/6	NSH57	USA	hpEastAsia	Intact	+ve	ND	Functional	+ve	+ve	ND	ND	[67]
5	Male FVB/N, male C57BL/6, male BALB/c	LSH100 (and derivatives of G27)	USA	hpEastAsia	Intact	+ve	ND	Functional	+ve	+ve	ND	ND	[56–58, 68]
6	Female C57BL/6	P12	Germany	hpEurope	Intact	+ve	+ve s1m1	Functional	+ve	ND	+ve	+ve	[32, 69]
7.	Female C57BL/6 and BALB/c	SPM326	Italy	ND	ND	+ve	+ve s1m1	ND	+ve	+ve	+ve	+ve	[46, 70]
8.	Male and female C57BL/6 and FVB/N	B128	USA	hpEurope	Intact	+ve	+ve s1m2	+ve	ND	+ve	+ve	+v	[37–39]
9.	Male C57BL/6	AM1	India	ND	–ve	–ve	+ve s2m2	–ve	ND	–ve	–ve	+ve	[12, 71]
10	Female or male C57BL/6	X 47, X47-2AL (and derivatives)	USA	ND	–ve	–ve	s1m2	–ve	ND	ND	ND	ND	[56, 72–74]
11	Male Mlac:ICR	HP34	Vietnam	hpEastAsia	Intact	+ve	+ve s1m2	+ve, function: ND	ND	+ve	+ve	+ve	This study

*(1), IL–8 induction; (2), Inflammation; (3), epithelial atrophy; (4), intestinal metaplasia; ND, no data found; +ve, positive; –ve, negative; T4SS, type IV secretion system; hpEastAsia, East Asian-originated *H. pylori*'s genotype [75]; hpEurope, Europe-originated genotype [75]; s1m1, s1m2 and s2m2 genotype combinations suggest the highest, intermediate and no cytotoxic vacuolating activities, respectively [41].

peptic ulcer who had received one previous *H. pylori* treatment. Endoscopy showed superficial duodenal ulceration, inflammation in the fundus and antral erosions. HP34 was resistant to clarithromycin but sensitive to tetracycline, metronidazole, amoxicillin and levofloxacin.

For culturing *H. pylori* strains, selective horse blood agar (HBA) (Oxoid, UK) supplemented with 8% (w/v) defibrinated horse blood (IVAC, Vietnam) or brain heart infusion (BHI) (Oxoid, UK) medium supplemented with 5% (v/v) foetal bovine serum (FBS) (Thermo Fisher Scientific, UK) was used. Incubation took place in a microaerophilic chamber using an Oxoid CampyGen 2.5 l sachet at 37°C with regular passaging. The strains were preserved in BHI supplemented with 15% (v/v) glycerol at −80°C.

Bacillus subtilis strain PY79, derived from the type strain 168, was utilized as a prototrophic laboratory strain [19]. Standard methods described previously were employed for working with *B. subtilis* [20]. Difco sporulation medium (DSM) (Merck, Germany), supplemented with thymine ($50\,\mu g\,ml^{-1}$) and trimethoprim ($3\,\mu g\,ml^{-1}$), was recommended for growth; and the addition of $1\,mM\ MgSO_4$, $1\,mM\ Ca(NO_3)_2$ and $10\,\mu M\ MnCl_2$ was used for sporulation of *B. subtilis* [21].

Genomic DNA extraction and genome sequencing

The revived *H. pylori* strains were subcultured on selective HBA or BHI solid medium at 37°C for 3–5 days. Genomic DNA was extracted from confluent growth using the Qiagen DNA Mini kit (Germany). The quality of the extracted DNA was assessed using NanoDrop One C spectrophotometer (Thermo Fisher) and agarose gel electrophoresis. DNA samples with concentrations over $100\,ng\,\mu l^{-1}$, $A_{260/280}$ ratios between 1.8 and 2.0 and $A_{260/230}$ ratios between 2.0 and 2.2, displaying clear bands on agarose gel, were considered to be good quality and used for sequencing. The DNA samples for sequencing preparation were qualified by 2100 Bioanalyzer (Agilent) and quantified using the Qubit 4 Fluorometer with the Invitrogen Qubit 1× dsDNA HS Assay kit (Invitrogen, USA).

High-quality DNA samples were subjected to sequencing using both Illumina and ONT platforms. The Illumina platform, specifically the NovaSeq 6000 system, generated paired-end reads (2×150 bp) from DNA libraries prepared with NEBNext dsDNA fragmentase and the NEBNext Ultra II DNA Library Prep kit for Illumina (NEB, USA). For ONT sequencing, the NEBNext Companion Module for ONT Ligation Sequencing (NEB #E1780) and the ligation sequencing kit SQK-LSK 109 (ONT, UK) were utilized to prepare the sequencing libraries, which were subsequently loaded onto a flow cell R9.4.1. The sequencing process was processed on the MinION 1B device (ONT, UK) and monitored using the MINKNOW v22.12.7 program (ONT, UK), with a minimum read length of 200 bp. Base-calling was performed using Guppy v6.4 (ONT, UK) with the high accuracy option and a minimum score of 7. The raw data from both Illumina and ONT platforms were stored in the fastq format.

Genomic data analysis

The raw data obtained from the Illumina and ONT platforms underwent quality checks using FastQC and MinIONQC, respectively, as referenced in previous studies [22, 23]. To address any low-quality reads present in the raw data, the Trimmomatic program was employed for Illumina data to remove adapters, low-quality reads (Q score below 20) and reads containing N bases [24]. For ONT data, the Prowler program was utilized to trim and filter out good-quality reads from the raw fastq data [25]. Subsequently, the filtered Illumina and ONT data were assembled using the Unicycler program, which combines short- and long-read data [26], to obtain complete genomes. The complete genome sequences of strains HP22 (average coverage depth: 5406×) and HP34 (average coverage depth: 4966×) were deposited in GenBank with the accession numbers CP122515 and CP122516, respectively.

To annotate the gene composition in the complete chromosomes of HP22 and HP34 strains, the National Center for Biotechnology Information (NCBI) Prokaryotic Genome Annotation Pipeline (version 6.5) with default parameters was employed [27]. Virulence gene composition was identified using the chromosomal data of strain HP26695 (GenBank accession number NC000915). The circular map of the studied strains was illustrated using the GenoVi software [28]. In order to locate changes in amino acid composition associated with AMR, substitution data of AMR-related genes in *H. pylori* strains were referenced according to AMR patterns and genetic elements from Shanghai [29].

Additionally, the complete genomes of HP22 and HP34 strains were uploaded to the *Helicobacter pylori* Typing Tool (HpTT) to identify their geographical distributions [30]. The HpTT employed single-nucleotide polymorphisms and whole-genome sequences from 1211 *H. pylori* strains to construct the geographical relationship at the continent and country levels [30]. To compare the geographical distribution of HP34 and HP22 strains, other *H. pylori* strains belonging to genotypes hpAfrica (including J99 and SouthAfrica7), hpEurope (B38, B128, SS1, PMSS1 and P12), and hpEastAsia (F57, G27, India7 and PeCan5) were included in the analysis.

Preparation of a candidate vaccine strain

The construction of the candidate vaccine strain *B. subtilis* A1.13 involved the design and development of molecular cloning methods, as detailed in our recent studies [31]. The strain was engineered to carry the 5′ segment (including promoter) of the

cotB gene from *B. subtilis*, which encodes a spore coat protein. The *cotB* segment was fused at the 3′-end to the full-length *ureA* gene from *H. pylori*, which encodes urease A (UreA). To evaluate the expression of UreA in the *B. subtilis* strains, Western blotting and whole-spore enzyme-linked immunosorbent assay (ELISA) were performed as described in our previous study [16].

Histopathological and challenge studies on mice

Male Mlac:ICR mice at 5–6 weeks of age and weighing approximately 20 g were used in this study. The protocols for *H. pylori* infection and subsequent histopathological studies were adapted from the methods developed by Dyer *et al.* [32]. *H. pylori* strains were cultured as described above and resuspended in phosphate-buffered saline (PBS) at a concentration of approximately 2×10^8 c.f.u. $0.2 \, \text{ml}^{-1}$. Two sets of mice were orally infected with 0.2 ml of HP34 strain (*n*=6) on separate days, while the remaining group (*n*=6) received a mock infection (non-infected control) with the same volume of PBS. Subsequently, the mice were euthanized by cervical dislocation, and their stomachs were surgically extracted. Following the removal of the forestomach, the stomach was dissected into longitudinal sections to facilitate subsequent analysis.

To facilitate histopathological studies, gastric tissue sections were immersed in 4% paraformaldehyde for 24 h for fixation. Subsequently, the sections were embedded in paraffin and cut into sections measuring 1–2 µm in thickness. These sections were subsequently dewaxed and stained with haematoxylin and eosin (H and E). Additional staining of neutral mucins with periodic acid–Schiff (PAS) was performed. The gastric tissues were assessed for several features, including the presence of *Helicobacter*, inflammation, epithelial atrophy and intestinal metaplasia (Table 1).

For challenge studies, the mice were divided into three groups: group 1 consisted of six mice and received sterile PBS as a control (naïve group); group 2 consisted of six mice and received PK118 (WT) spores; and group 3 consisted of eight mice and received A1.13 (*cotB-ureA*) spores. Oral immunizations were conducted by administering a volume of 0.2 ml of either PBS (group 1) or spores (1×10^{10} c.f.u.) (group 2 and 3) on days 0, 14, 28 and 53. On the 56th day, the mice were challenged with a daily dose of 0.2 ml of freshly grown *H. pylori* HP34 culture. The optical density at 600 nm (OD_{600}) was monitored to estimate a density of approximately 10^8 *H. pylori* c.f.u. On the 79th day, the stomach samples were taken to enumerate the *H. pylori* c.f.u. by plating them on selective HBA.

Immune response studies on mice

Female C57BL/6 inbred mice at 9 weeks of age were grouped (six mice in each group) and housed together. Group 1, the naïve group, received only PBS as a baseline control. Group 2 was dosed with PK118 (wild-type) spores, whereas group 3 was dosed with A1.13 (*cotB-ureA*) spores, the vaccine candidates. The dosing regimen contained four doses in total with each dose administered over 3 consecutive days. The volume of each dose was 0.2 ml, comprising either PBS or the spore vaccine. For groups 2 and 3, the intra-gastric administration of the spore vaccine consisted of 1×10^{10} spores. Daily administrations were used due to the high viscosity of spore suspensions at high concentrations. The specific dosing schedule was as follows: dose 1 was administered on days 1–3, dose 2 on days 16–18, dose 3 on days 32–34 and dose 4 on days 47–49. Faecal samples were collected from the mice 1 day prior to dosing and on the 15th, 31st, 46th and 61st days for subsequent analysis. On the 62nd day, the mice were sacrificed and serum samples were collected via cardiac bleed. The analysis of immunological responses of these faecal and serum samples was performed as detailed in our recent study [16].

Statistical analysis

Statistical significance was assessed by the Mann–Whitney U test to compare two independent groups or Dunnett's test for comparing multiple treatment groups to a control group using Prism (GraphPad, Dotmatics).

RESULTS

Genomic features of *H. pylori* isolates HP22 and HP34

In this study, we employed both Illumina and Oxford Nanopore sequencing platforms to generate closed genome sequences of HP22 and HP34 isolates (Figs S1, S2 and Table S1, available in the online version of this article). The assembly yielded complete circular genomes for both strains: HP22 with a length of 1582823 base pairs and HP34 with a length of 1600602 base pairs (as summarized in Table S2). Upon analysing the HP22 chromosome, we identified 1304 protein-coding genes (excluding hypothetical coding sequences), along with 36 tRNAs, 6 rRNAs, 2 ncRNAs and 1 tmRNA. Although the HP34 strain shared the same number of non-coding RNAs as the HP22 isolate, it possessed 1312 protein-coding genes. Additionally, both chromosomes exhibited similar GC contents of approximately 38.8 and 38.9%, respectively.

Further investigation revealed that HP34 possessed 111 virulence gene records categorized into 6 functional groups: adherences, effector delivery system, motility, exotoxin, immune modulation and stress survival. These findings are summarized in Table 2. On the other hand, HP22 exhibited the same virulence gene records as HP34, with one exception. HP22's genome did not contain *cag2* record, distinguishing it from HP34 in terms of virulence gene composition (Table 2). The *cag* and *vir* genes were located

Table 2. List of virulence genes in *H. pylori* strains HP22 and HP34

Function	Genes
Adherence	*sabB/hopO, babA/hopS, babB/hopT, hopZ*
Effector delivery system	*cag1, cag2*, cag3, cag5/virD4, cagA, cagD, cagH, cagI, cagM, cagN, cagS, cagU, cagX/virB9, cagY/virB10, cagZ, virB1/cag4, virB11, virB2/ cagC, virB4/cagE,*
Motility	*cds6, cheA, cheV1, cheV2, cheV3, cheW, cheY, flaA, flaB, flgA, flgB, flgC, flgD, flgE, flgE_1, flgG, flgG_2, flgH, flgI, flgK, flgL, flgM, flgR, flgS, flhA, flhB, flhB2, flhF, fliA, fliD, fliE, fliF, fliG, fliH, fliI, fliL, fliM, fliN, fliP, fliQ, fliR, fliS, fliY,HP_RS02435, cheV/HP_RS03030, rpoN/ HP_RS03480, kdtA/HP_RS04690, HP_RS07240, HP0256, hpaA2, motA, motB, pdxA, pdxJ, pflA, pseB, pseC, pseFG, pseH/flmH/flaG1, pseI, tlpA, tlpB, tlpC, ylxH*
Exotoxin	*vacA*
Immune modulation	*oipA/hopH, wbpB, lpxB, futB, futA, gluE, gluP, kdtB, napA, rfaC, rfaJ, futC1, futC2, wbcJ, rfbD, rfbM,*
Stress survival	*ureA, ureB, ureE, ureF, ureG, ureH, ureI*

*Genes absent in HP22.

in *cag*PAI. Some *vir* genes, including *virB2*, *virB4*, *virB8*, *virB9* and *virB10*, were in other positions of HP34 and HP22 genomes and formed *tfs* fragments rather than complete *tfs* regions such as *tfs3* and *tfs4*.

Both strains possessed AMR-related genes, including *gyrA, gyrB, rdxA, frxA, pbp1, pbp2, rpoB* and *rpsL*. However, the specific substitutions associated with AMR differed between the two strains (Tables S3 and S4). Notably, neither strain exhibited the substitutions in *rpsL* and *gyrB*, which are responsible for quinolone and streptomycin resistance, respectively. While various substitutions were found in *rpoB*, these changes did not confer AMR to rifampicin/rifabutin in either of the *H. pylori* strains. Substitutions in the *gyrA* gene enabled quinolone resistance exclusively in the HP22 strain, while those in *frxA* conferred metronidazole resistance solely in the HP34 strain.

Genomic comparison of *H. pylori* strains in Vietnam and Southeast Asia

In this study, the genome data for two *H. pylori* strains, HP22 and HP34, isolated in central Vietnam, were added to a collection of 85 *H. pylori* isolates recently reported from southern Vietnam [33, 34]. Collectively, the majority of *H. pylori* isolates in Vietnam (80.5%, 70/87) belonged to the hpEastAsia population, while the remaining isolates belonged to the hpEurope population (19.5%, 17/87). Among the isolates, 70 (70/87) were found to be *cagA*-positive. The most frequent genotypes among the *cagA*-positive isolates were *vacA s1m1/cagA+* and *vacA s1m2/cagA+*, accounting for 51.4 (36/70) and 48.6% (34/70) of the isolates, respectively [33, 34].

Based on the analysis of AMR patterns and genetic elements from Shanghai [29], we suggested that HP34 and HP22 shared a similar AMR profile to that of most *H. pylori* genomes reported in Vietnam, Cambodia and Thailand (Tables S3 and S4). Specifically, they were likely to be resistant to metronidazole (associated with substitutions in the *rdxA* and *frxA* genes) and amoxicillin (*pbp1* and *pbp2*), but sensitive to rifampicin/rifabutin (*rpoB*) and streptomycin (*rpsL*) [33]. However, further studies are required to confirm the AMR characteristics of these *H. pylori* strains.

Genomic and geographical distribution analysis using the HpTT webtool confirmed that both HP22 and HP34 strains originated from Asia, specifically the hpEastAsia genotype group (Fig. 1). This genotype group was also predominant in strains identified in Vietnam, Cambodia, Malaysia, Singapore and Indonesia (Fig. 1). Additionally, HP22 exhibited the closest genetic relationship to two strains isolated in Cambodia, while the HP34 strain showed similarities to strains from Australia and Cambodia (Tables S5 and S6), indicating a close relationship to Southeast Asian strains. This finding supports HP34's representation of the hpEastAsia genotype in Vietnam and the Southeast Asian region, where no mouse-adapted model strains had been reported previously (Table 1).

We summarized the common *H. pylori* isolates used in mouse colonization studies, representing different genotypes and geographical sources, and for the first time identified a novel mouse-adapted model strain HP34 representative for the hpEastAsia genotype in Southeast Asia (Table 1). Among these isolates, Sydney strain 1 (SS1) is the most frequently utilized in such studies [35]. SS1 possesses the *cag* pathogenicity island (*cag*PAI) but lacks a functional type IV secretion system (T4SS) [36]. Other widely employed strains include B128 (*cag*PAI+/T4SS+) [37] and X47-2AL (*cag*PAI−/T4SS−) [38, 39]. These strains with the hpEurope genotype were often found in Europe, the USA and Australia. HP34 shared a *cag*PAI+ genotype with SS1 and B128 but possessed a T4SS-positive genotype with several of the most common genes essential for its T4SS function loss [40], including *cagY*, *cag5* and *cagA* (Table 2). It also had the same *vacA* s1m2 subtype as that in B128 and X47-2AL, which may present intermediate cytotoxic vacuolating activities [41], compared to s2m2 strains such as SS1 with little or no cytotoxin production.
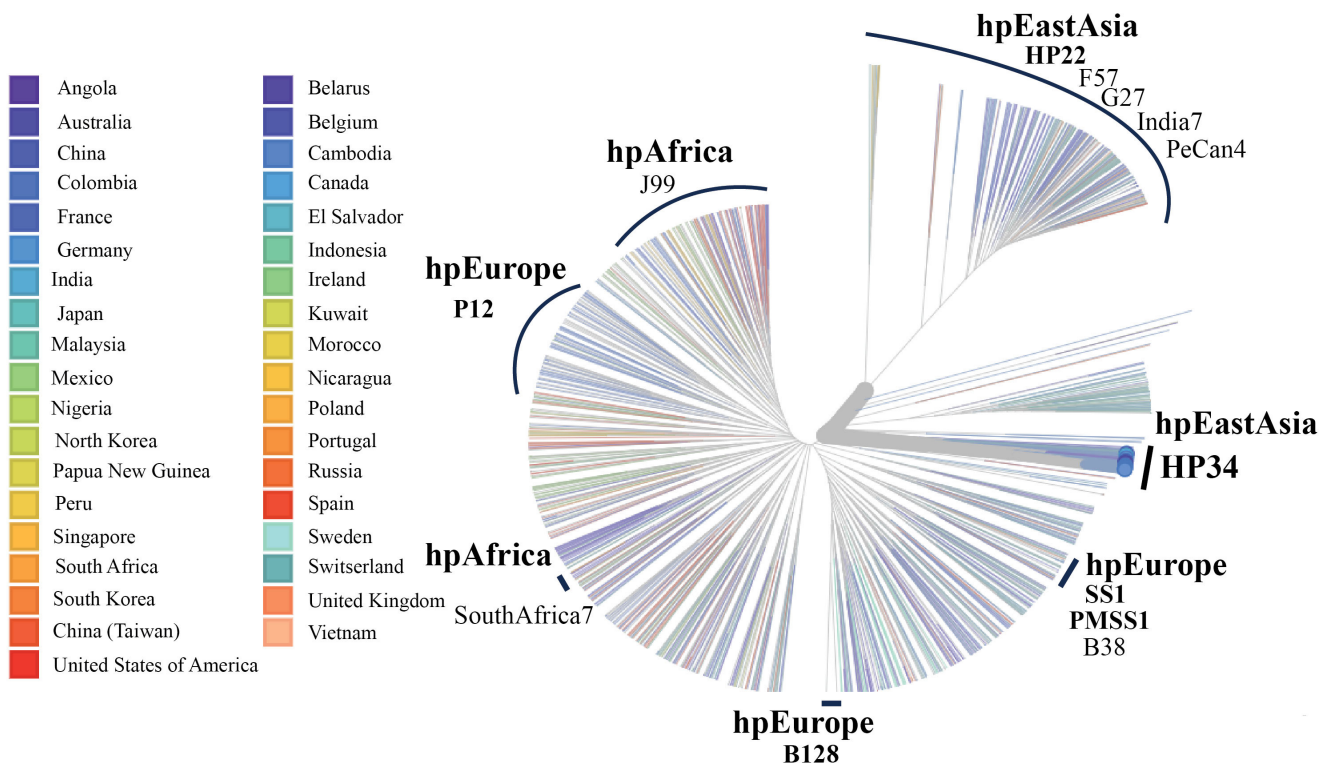
Fig. 1. Genomic and geographical distribution analysis of *H. pylori* strains using the HpTT webtool. The bold names indicate mouse-adapted model strains as the representatives for different genotypes and geographical regions.

Histopathological features of *H. pylori* HP34 infection in gastric tissue of Mlac:ICR mice

Fig. 2 illustrates the overall appearance of gastric mucosal epithelium infected with HP34 cells and sections of stomach embedded and cut as closely as possible to the perpendicular axis. The sections were stained with (H and E for the visualization of cellular details, tissue morphology and general histological features (Fig. 2b–f) and PAS staining for the detection of specific cellular structures (Fig. 2g). Microscopic images of the stained sections were captured and examined under different magnifications. Pathogenic cells were found in the overall appearance under a microscope at 10× (Fig. 2a). Chronic gastritis characterized by the presence of focal areas of inflammatory cells, including neutrophils, plasma cells, lymphocytes and occasionally scattered lymphoid follicles, within the gastric mucosa is shown (Fig. 2a–c). Lymphoid follicles interspersed with gastric glands were also observed (Fig. 2d). A mild acute inflammatory reaction was visible, showing a few polymorphonuclear neutrophils (PMNs) within the stomach body (Fig. 2e). The signs of epithelial erosion of the gastric mucosa could result from an acute inflammatory reaction or acute exacerbation of chronic gastritis (Fig. 2f). Finally, the presence of intestinal metaplastic lesions in chronic gastritis was observed, indicated by the presence of intestinal metaplasia cells (Fig. 2g). These histological findings provided insights into the structural changes and inflammatory responses occurring in the gastric tissues of mice infected with *H. pylori* strain HP34, highlighting the pathological consequences of infection.

Preparation of candidate vaccine strain with spore surface expression of *H. pylori* antigen UreA for preclinical studies

To express the UreA protein of *H. pylori* on the surface of *B. subtilis* spores, we genetically fused the relevant *ureA* coding ORFs to the *B. subtilis cotB* gene sequences. Precisely, we fused the complete *ureA* gene to the CotB coding sequences from amino acids 1 to 281 in *B. subtilis* strain A1.13. The design and development of this strain have been described in our latest studies, which were submitted concurrently with the present study [31]. In this study, we confirmed the successful expression of UreA on the surface of the spore of A1.13 (depicted in Fig. 3) and then conducted preclinical studies of this strain as a promising vaccine candidate in a novel mouse-adapted model challenged with *H. pylori* HP34 (presented in next sections of the results).

To assess the surface expression of UreA in *B. subtilis* vaccine strains, we examined spore coat proteins using Western blotting (Fig. 3a) and performed whole-spore ELISA (Fig. 3b). Upon blotting size-fractionated spore coat extracts for A1.13 (CotB-UreA), three distinct bands were observed (Fig. 3a). These bands had approximate molecular weights of 40 kDa, 58–60 kDa and 65–70 kDa. Notably, these bands were absent in PK118 spores, which had an insertional disruption in both thyA and thyB but
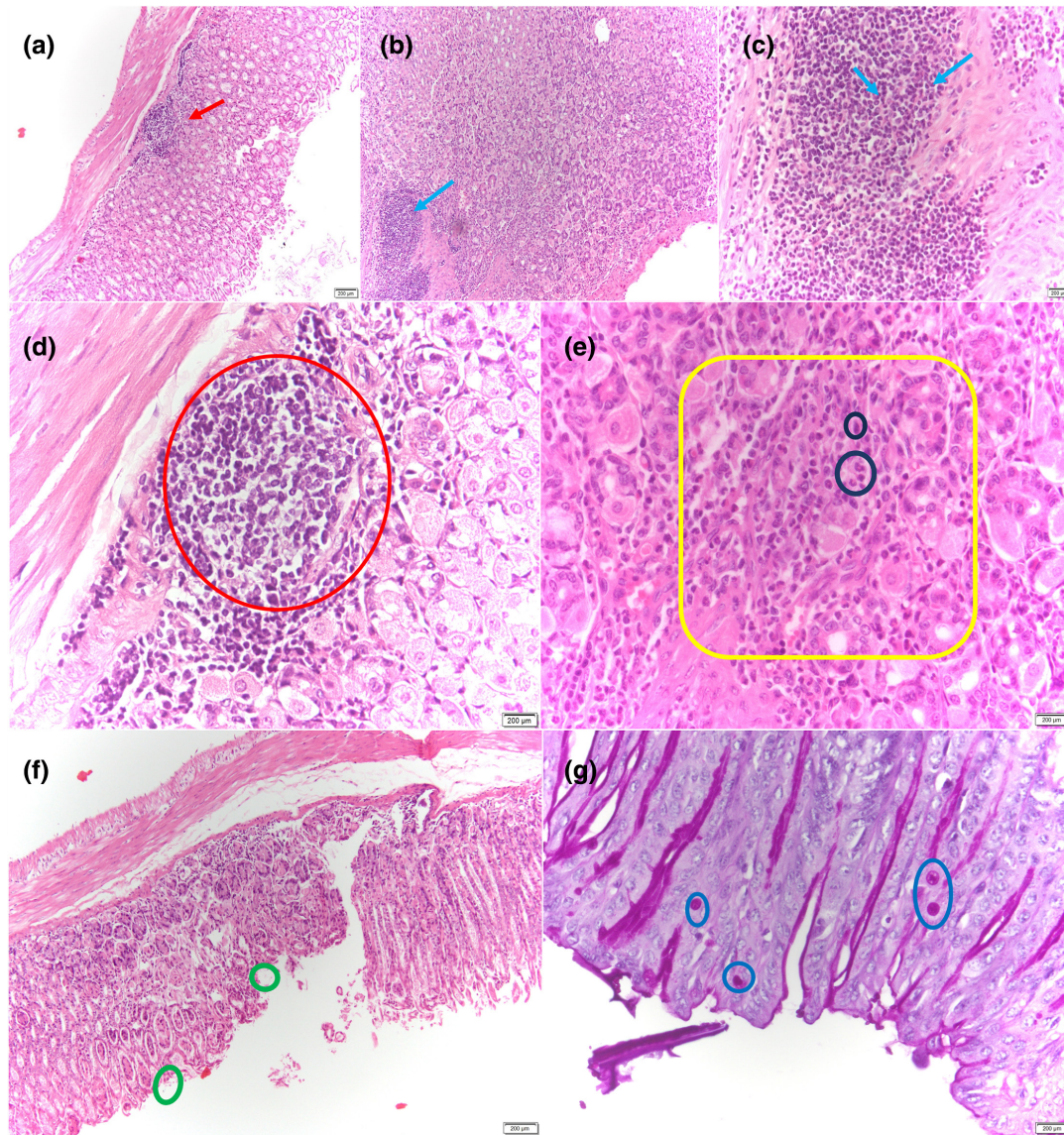
Fig. 2. Histology of *H. pylori* HP34-infected male Mlac:ICR gastric tissues. Histological sections were photographed through a microscope and stained with haematoxylin and eosin (H and E) (a–f) and periodic acid–Schiff (PAS) (g). Mice were infected with the strain HP34 for 4 weeks. (a–c) Chronic gastritis with focal areas of inflammatory cells (red arrow) such as neutrophils, plasma cells, lymphocytes and sometimes scattered lymphoid follicles (blue arrow) in the gastric mucosa are shown. (d) Image of lymphoid follicles (circled in red) interspersed with gastric gland (glands surrounding the lymphoid follicle). (e) Mild acute inflammatory reaction (squared area) with a few polymorphonuclear neutrophils (PMNs) seen in the stomach body (dark blue circle). (f) Epithelial erosion (green circles) of gastric mucosa due to acute inflammatory reaction or acute exacerbation of chronic gastritis. (g) Intestinal metaplastic lesions in chronic gastritis (intestinal metaplasia cells in blue circle). Original magnifications at 10× (a, b, f) and 40× (c–e, g).

lacked any chimeric genes. One of these bands corresponded to the expected size of CotB-UreA at approximately 58.2 kDa. The other bands are likely multimeric or breakdown species.

## Immunity effect in mouse model dosed with the vaccine candidate

To evaluate the immune responses induced by the spore vaccines expressing *H. pylori* antigens, female C57BL/6 mice at 9 weeks of age were orally dosed with the spores of strains A1.13 (CotB-UreA), along with the isogenic control PK118 strain that lacked *H. pylori* antigens. The measurement of antigen-specific secretory immunoglobulin A (sIgA) in faecal samples demonstrated seroconversion to UreA, as depicted in Fig. 4a, b. Day 61 exhibited the highest antibody responses. Notably, the A1.13-dosed mice displayed significantly ($P<0.05$) higher antibody responses than those administered with PK118 spores or the naïve group. Although PK118-dosed mice exhibited minimal levels of UreA-specific sIgA, the difference was not statistically significant.
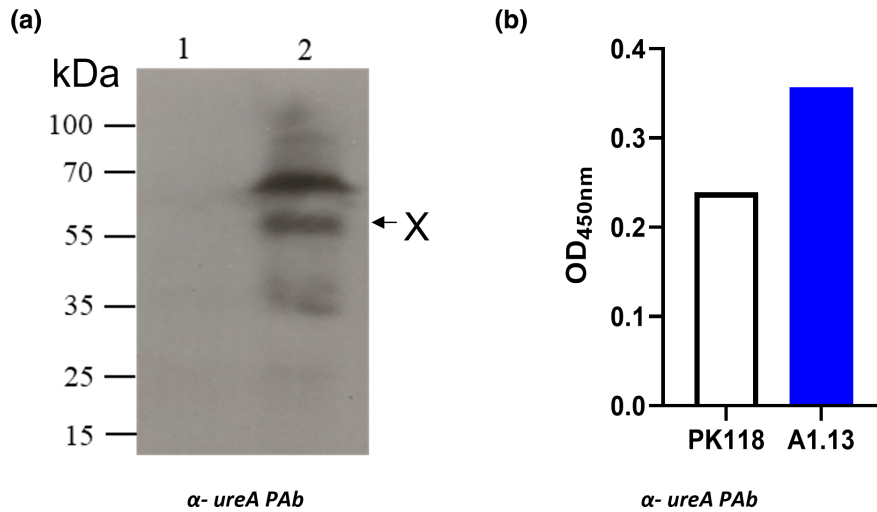
Fig. 3. Spore coat expression of urease A (UreA) proteins. (a) Western blot analysis was conducted using SDS-PAGE (12 % w/v) to fractionate spore coat protein B extracted from preparations of pure spores (approximately 2×10^9 spores/extraction). The blotting membrane was probed with specific polyclonal antibodies (PAbs) as indicated. Blots showed spore coat protein B extracted from the strain A1.13 (CotB$^{(1-281)}$–UreA$^{(1-238)}$) and the isogenic parent strain PK118 (wild-type, WT). Lane 1 represents spores of strain PK118 and lane 2 represents A1.13. The bands observed corresponded to CotB-UreA chimaeras, with molecular weights of approximately 58.2 kDa (x). (b) Whole-spore ELISA was performed using microtitre plates coated with spores at a concentration of 1×10^8 spores per well. The spores used in the ELISA were derived from strains PK118 (WT) and A1.13 (CotB-UreA). They were labelled with anti-UreA PAbs (1:2000) followed by incubation with anti-rabbit IgG secondary antibody, horseradish peroxidase (HRP) conjugate (1:3000).

Additionally, measurement of serum immunoglobulin G (IgG) responses at day 61 indicated that A1.13 induced systemic immunity, as depicted in Fig. 4c. Collectively, these findings demonstrated the mucosal and systemic immune responses elicited by the oral administration of spore vaccines expressing *H. pylori* antigens. The levels of UreA-specific sIgA in faecal samples and UreA-specific IgG in serum samples indicated the generation of antigen-specific immune responses following the vaccination regimen. These results provided valuable insights into the immunogenicity of the spore vaccines and their potential for developing effective strategies against *H. pylori* infection.
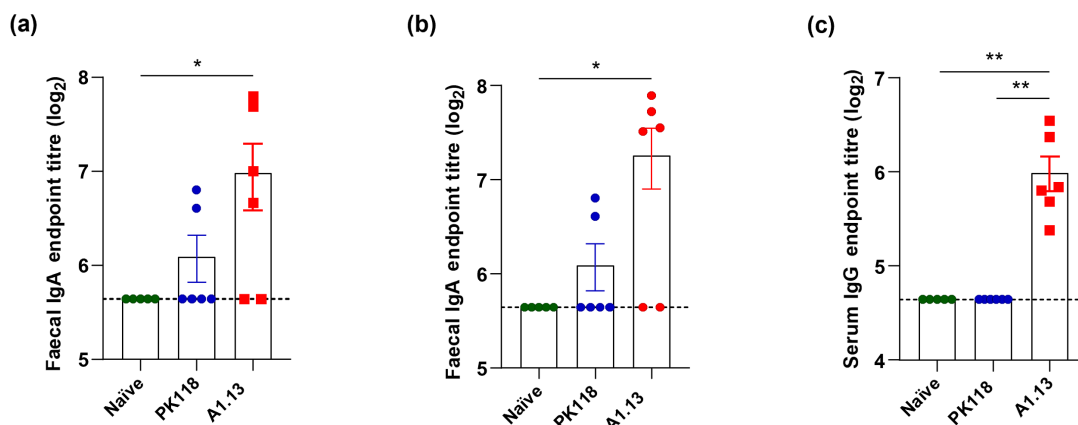
Fig. 4. Mucosal and systemic responses following oral administration of *H. pylori* antigen-expressing spore vaccines. Female mice (C57BL/6) were dosed with PBS (naive), spores of PK118 (WT), or A1.13 (CotB-UreA) four times. Each dose comprised three separate administrations (1×10^{10} spores/administration); dose 1 (days 1–3), dose 2 (days 16–18), dose 3 (days 32–34) and dose 4 (days 47–49). (a, b) UreA-specific responses were determined by enzyme-linked immunosorbent assay (ELISA), which showed faecal samples collected at days 46 (a) and 61 (b), and the levels of anti-UreA-specific sIgA. The optical density at 450 nm (OD$_{450nm}$) was measured as an indicator of the immune response. (c) Anti-UreA-specific immunoglobulin G (IgG) responses in the serum samples collected at day 61 were measured. Statistical analysis was performed using the Mann–Whitney U test, and significance was denoted as * $P=0.0498$ (a), $P=0.0215$ (b); ** naïve vs A1.13, $P=0.0020$, PK118 vs A1.13, $P=0.0022$ (c).
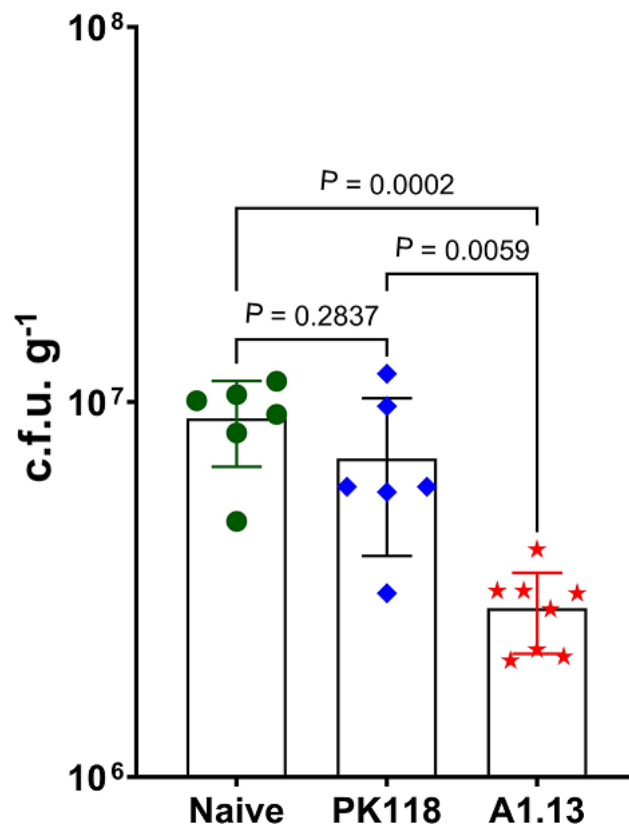
Fig. 5. Protective effect in an immunized male Mlac:ICR mouse model. The bacterial loads of *H. pylori* strain HP34 in stomach samples were assessed 21 days after challenge to evaluate the impact of immunization. The mice were orally dosed four times with spores (1×10^{10} spores per dose) of either PK118 (wild-type, WT) or A1.13 (CotB-UreA) and were challenged with *H. pylori* 7–8–9 days after the last immunization. The naïve mice received phosphate-buffered saline (PBS) as control. The data presented in this figure combine samples from two independent repeat studies, with the corresponding *P* values indicated for statistical significance.

Protective effect of the vaccine candidate in an immunized mouse model

To assess the impact of immunization on *H. pylori* colonization, male Mlac:ICR mice were orally administered four doses of spores (1×10^{10} c.f.u. per dose) from either PK118 or A1.13. A naïve group was included as a control. Subsequently, the mice were challenged with *H. pylori* HP34 (via oral administration) using a challenge dose of approximately 10^8 c.f.u. Stomach samples were collected 21 days post-challenge to quantify the c.f.u. of *H. pylori*.

The study was conducted with repeat experiments, and the combined c.f.u. data are presented in Fig. 5. Mice immunized with the A1.13 CotB-UreA-expressing spores exhibited a significant reduction in *H. pylori* c.f.u., with a median reduction of approximately 72% (*P*=0.0002) compared to the naïve group. Furthermore, A1.13-immunized mice showed a median reduction of approximately 50% (*P*=0.0059) compared to mice dosed with PK118 spores. Although PK118-dosed animals also demonstrated a reduction in *H. pylori* c.f.u. (approximately 39% reduction in median values) compared to the naïve group, the difference was not statistically significant (*P*>0.1).

These results demonstrated that the spore vaccine strain A1.13, expressing UreA, conferred protective immunity sufficient to significantly reduce *H. pylori* colonization in mice. The findings highlight the potential of this vaccine candidate in mitigating *H. pylori* infection.

DISCUSSION

Among known mouse-adapted *H. pylori* models, SS1 infection was first introduced as being associated with chronic gastritis and gastric atrophy [35]. However, wild-type mouse models, such as C57BL/6 [14], BALB/c [42] and C3H [43], infected with *H. pylori* typically exhibit mild gastritis or slow-progressing diseases, limiting insights into *H. pylori* pathogenicity [44–46]. Infection of mice with *H. pylori* or *Helicobacter felis* results in lymphocytic gastritis without progressing to severe conditions like peptic ulcers or gastric cancer [47–49]. Structural differences between murine and human stomachs, along with the distinct bacterial profiles

in the murine stomach, limit the development of severe gastric pathologies [47–49]. Consequently, wild-type mouse models are suboptimal for experimental *H. pylori* infections. Instead, knockout or transgenic mouse models, such as INS-GAS, IFN-γ/TNF-α knockout, IL-1β transgenic, IL-10 knockout, Fas antigen transgenic, p27-deficient and CagA-transgenic mice, have been used to induce gastric cancer in the context of *H. pylori* infection through high-salt diets or chemical carcinogens [38, 50–55]. These rodent models have contributed significantly to the investigation of *H. pylori* virulence characteristics [36, 39].

In our study, for the first time, we introduced a novel mouse-adapted *H. pylori* HP34 model for the hpEastAsia genotype in Vietnam and Southeast Asia, focusing on the genomic insights, histopathological features and vaccine preclinical trials. Additionally, we evaluated the potential of a candidate spore vaccine, A1.13 (CotB-UreA), as a promising prophylactic vaccine against *H. pylori* infection. The utilization of a mouse and mouse-adapted pathogen model specifically tailored to a particular genotype and geographical region offers an additional advantage in studying *H. pylori* infection and evaluating potential interventions.

We also utilized both female C57BL/6 and male Mlac:ICR mice for the immunization and challenge studies, respectively. They are widely available and extensively used in preclinical research. Mlac:ICR mice are versatile outbred strains suitable for various research applications, including toxicology and immunization studies. On the other hand, C57BL/6 mice offer a well-defined and standardized genetic background, making them preferred for biomedical research on various diseases, genetics and immunology. Mlac:ICR mice, a mouse strain originating from Switzerland, were selected and developed by Dr Hauschka at the American Institute of Cancer Research (1960), and then transferred to the National Laboratory Animal Center of Mahidol University (NLAC – MU) in Thailand in 1980 (https://nlac.mahidol.ac.th/en/index.php/nlac-icr-detail/). In Vietnam, Mlac:ICR mice were imported from Thailand in 2016, and then used for research and quality testing of vaccines and medical biological products at the National Institute for Control of Vaccines and Biologicals (NICVB) and Institute of Vaccines and Medical Biologicals (IVAC).

The selection of C57BL/6 mice for measuring immunogenicity was driven by the question of whether spore vaccines induce an immune response. Importantly, there is no strict mandate that the same animal species be used for both aspects. In fact, employing different species can offer more informative results. It is also essential to note that for human clinical studies it is typically required to demonstrate the proof of concept in at least two animal species, not merely one. The outcomes of our challenge clearly indicated that, in comparison to the placebo group, animals administered with spore vaccines enjoyed a degree of protection. However, it is crucial to underline that immune responses and protection are distinct phenomena. While they are likely interrelated, it is prudent to treat them as separate entities. Our study is oriented towards addressing two discrete questions: immunogenicity and protection, and there is no inherent reason why the same animal species should be employed for both. In fact, we contend that using different animal species can provide a more comprehensive perspective.

Regarding the selection of mouse sex in the models, Table 1 shows that female and/or male mice were used. Female C57BL/6 and BALB/c mice are often used in research on pathological models for *H. pylori* or to develop vaccines against this pathogen. In many other cases, there were still studies using male C57BL/6 or BALB/c or FVB/N mice [12, 56–58] or both sexes [38, 39]. This proves that male or female mice can be used in immune models or challenges for vaccine research to *H. pylori*. There were at least three reasons why male mice were selected in this study. Firstly, in some studies, male mice may tend to show a higher level of gastric colonization by HP compared to females. The reasons for this difference may involve variations in gastric physiology, hormones, or immune responses. For example, in the FVB/N mouse model, male gastric tissue responded more rapidly and aggressively to *H. pylori* infection when compared with females [38]. Secondly, hormonal fluctuations in female mice, particularly during the estrous cycle, can impact on the course of infection. Thirdly, only one sex was selected for the sake of standardization and reproducibility, thus minimizing variability in experimental conditions and facilitating comparisons across studies. For example, there were sex differences in the gastric inflammatory and epithelial response to *H. felis* in the C57BL/6J mouse model [59].

However, female mice may exhibit stronger immune responses, which could be beneficial for studies evaluating the host immune response to *H. pylori* or assessing the efficacy of potential vaccines. Therefore, for immune response studies in this study, female C57BL/6 mice were still used, as discussed above. In terms of relevance to human infection, researchers may choose to study both male and female mice to better understand potential sex-specific differences in disease susceptibility, progression, or immune response. This can enhance the translatability of findings to human populations.

To obtain the complete genomes of two clinical *H. pylori* strains isolated in Vietnam, we employed a combination of Illumina and Nanopore sequencing methods. As a result, we successfully obtained the high-quality closed-form complete genomes of strains HP22 and HP34. The genome size of HP22 and HP34 was approximately 1.6 Mb, with GC contents ranging from 38.8–38.9%, and they contained 1304–1312 protein-coding genes, consistent with previous *H. pylori* sequencing results. The inclusion of genome data from HP22 and HP34 expands our understanding of the genetic diversity, virulence genes and antimicrobial resistance genes present in *H. pylori* strains. Furthermore, the genomic comparison revealed that HP34 well represents the hpEastAsia genotype in Vietnam and the Southeast Asian region, where no previously reported mouse-adapted model strains existed, as listed in Table 1. Global comparative genomic analyses looked at how the virulence genes in *H. pylori* vary around the world and found that in different regions the pathogen evolved in unique ways associated with the human genetic backgrounds [17, 18]. This means that the risk of acquiring gastric cancer from *H. pylori* can be different in each region. Therefore, the complete genome of HP34 as a representative of the Southeast Asian region have provided additional information

that can be useful for future research because it can represent how the pathogen in this region evolved and help us understand the risk of stomach cancer better.

The genome data also showed distinct nucleotide changes in the *rdxA* gene of the HP22 and HP34 genomes, some of which were associated with metronidazole and amoxicillin resistance. However, our *in vitro* investigations indicated that HP22 displayed resistance to metronidazole and amoxicillin, as predicted by its genome profile, whereas HP34 was found to be sensitive to these antibiotics (data not shown). This discrepancy suggests the involvement of other factors or mechanisms in the antibiotic resistance phenotype of HP34, warranting further studies focusing on functional genomics to better understand the underlying mechanisms and genes associated with antibiotic resistance in these strains.

Both HP22 and HP34 strains have been identified as being resistant to clarithromycin. It is important to note that the prevalence of clarithromycin-resistant *H. pylori* strains has been increasing rapidly worldwide. For instance, in PR China the rate of clarithromycin resistance rose from approximately 15% in 2000 to around 53% in 2014 [60]. This surge in resistance is attributed to the widespread use of clarithromycin in standard triple-therapy regimens for *H. pylori* infection globally. Consequently, clarithromycin-resistant *H. pylori* strains have been included in the World Health Organization's (WHO's) list of 12 antibiotic-resistant priority pathogens that pose the greatest threat to human health [61]. Therefore, the HP34 strain serves as a valuable model for AMR research, particularly in the context of Asia.

Histopathological analysis of gastric tissue infected with *H. pylori* strain HP34 provided valuable insights into the structural changes and inflammatory responses induced by the infection. Microscopic examination of H and E-stained sections confirmed the successful colonization of HP34 cells and revealed the presence of pathogenic cells in the gastric mucosal epithelium. The infected mice exhibited chronic gastritis, characterized by focal areas of inflammatory cells, mild acute inflammatory reactions, epithelial erosion of the gastric mucosa and the presence of intestinal metaplastic lesions. These findings align with previous reports on mouse models of *H. pylori* infection [11, 14, 35, 49]. Notably, the observed histopathological alterations in the mouse model closely resembled the pathological manifestations typically observed in human *H. pylori* infection. Specifically, HP34, isolated from a patient with peptic ulcer, exhibited similarities with endoscopic findings of superficial duodenal ulceration, fundal inflammation and antral erosions.

HP34 also showed histopathological similarities with common mouse-adapted model strains SS1 and PMSS1, specifically in terms of inflammation, epithelial atrophy and intestinal metaplasia (Table 1). This may suggest that HP34 is likely to induce more severe pathology in mice. The primary pathogenicity determinant in *H. pylori* infection is the Cag pathogenicity island (*cag*PAI), an approximately 40 kb DNA segment encoding a type IV secretion system (T4SS) responsible for translocating the CagA oncoprotein into host gastric epithelial cells. However, this has some impact on an argument that chronic *H. pylori* colonization in animal models with a *cag*+ strain, such as PMSS1, often results in *cag*PAI gene loss and T4SS function impairment (Hansen et al., 2020). This was attributed to *cag*Y recombination as the predominant mechanism and associated with indels or nonsynonymous changes in 13 other essential *cag*PAI genes, with *cag5*, *cag10* and *cagA* being the most affected (Hansen et al., 2020). Importantly, the genome of HP34 contains these crucial genes, including *cagY*, *cag5* and *cagA*; therefore, further studies on the functional genomics of mouse-adapted model strains are required in terms of T4SS function.

Evaluation of immune responses induced by oral administration of the candidate spore vaccine (A1.13) expressing *H. pylori* antigens revealed promising results. The increase of antigen-specific secretory immunoglobulin A (sIgA) in faecal samples and immunoglobulin G (IgG) in serum samples confirmed the effectiveness of the oral spore vaccine in eliciting both mucosal and systemic immune responses. In addition, there is a correlation between the faecal sIgA and serum IgG responses with regard to induction and seroconversion, as shown by the fact that oral dosing with spore vaccines induced both IgA and IgG. These findings highlight the immunogenicity of the spore vaccines and their potential for developing effective strategies against *H. pylori* infection.

To assess the protective effect of the vaccine candidate, we evaluated its impact on *H. pylori* colonization in male Mlac:ICR mice. The results demonstrated a significant reduction in *H. pylori* c.f.u. in mice immunized with A1.13 spores compared to the naïve group and PK118 wild-type spores, with reductions of approximately 72 and 50%, respectively. These findings underscore the effectiveness of the A1.13 spore vaccine in conferring protective immunity and suggest its potential for mitigating *H. pylori* infection and reducing bacterial colonization in mice. These results are consistent with previous studies conducted by Zhou *et al.* [62] using spores expressing the full-length UreB protein (CotC-UreB) and our recent studies employing spores expressing the full-length UreA protein (CotB-UreA) (strain PK82) or truncated UreB domain (CotB-UreB) (strain PK78) [16]. Both strains A1.13 and PK82 expressing CotB fused with full-length UreA protein, but CotB sequences were designed differently from amino acids 1 to 281 in A1.13 [31] and from amino acids 1 to 338 in PK82 [16].

It is also important to note that the results obtained from the spores alone (PK118 spores) showed a low level of protection (39% reduction in gastric c.f.u.), which was not found by Zhou *et al.* [62]. Nevertheless, albeit without statistical significance, this observation is consistent with our previous study [16], which also demonstrated a 40% reduction.

In conclusion, this study successfully obtained complete genomes of two clinical *H. pylori* strains in Vietnam, with HP34 representing the hpEastAsia genotype in Southeast Asia. The high-quality consensus sequences of the circular genomes of the HP34 and HP22 strains could serve as novel references for *H. pylori*. Histopathological analysis of HP34-infected mice showed similarities to human *H. pylori* infection, and immunization with the A1.13 spore vaccine elicited immune responses and reduced bacterial colonization, highlighting its potential as a prophylactic vaccine against *H. pylori* infection. Our study successfully developed a novel mouse-adapted *H. pylori* model tailored for the hpEastAsia genotype in Vietnam and Southeast Asia. This model holds significant promise for advancing our understanding of *H. pylori*-associated diseases in humans and developing region-specific interventions to combat this global health problem.

Author contributions
T.K.C.N., T.C.N. and T.L.P. carried out the microbiological and molecular studies. H.D.K.D., B.N.M. and M.T.V. performed the whole-genome sequencing and analysis. T.L.P.N. led the preclinical studies. T.T.P. and H.A.H. contributed to methodology, resources, validation and review of the manuscript. P.M.K. carried out the microbiological and immunological studies. H.T.D. contributed to resources and project administration. A.N.P. contributed to resource administration and reviewed the manuscript. V.D.N. and S.M.C. contributed to conceptualization, methodology, supervision, project administration and funding acquisition. V.D.N. wrote the manuscript. M.T.V. is co-corresponding author with V.D.N. All authors read and approved the final manuscript for publication.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The challenge studies were conducted with approval from the Research and Ethics Committee of the Institute of Vaccines and Biological Medicals (IVAC) (decision no. 241/QD-VXSPYT 29/07/2022). Murine studies were conducted with approval from Royal Holloway University of London Ethics Committee and the approved UK Home Office animal project licence PB9FA6ABB. The clinical *H. pylori* strains were isolated from two patients by the Hospital of the University of Medicine and Pharmacy, Hue University, Vietnam through routine clinical practice and informed consent to participate in the study was obtained from these individuals.

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