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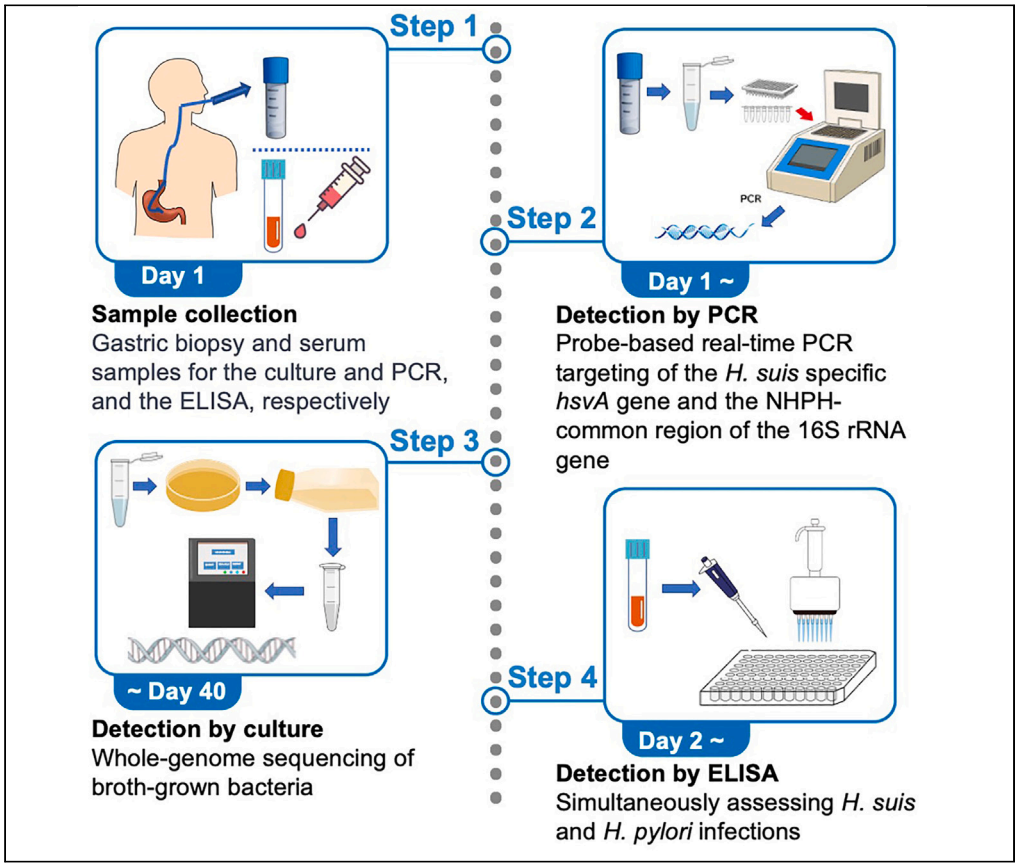
# Protocol for detecting *Helicobacter suis* infection in gastric biopsies and serum by PCR and ELISA




Infection with *Helicobacter suis*, which causes many cases of gastric disease, is not reliably diagnosed. Here, we present a protocol for detecting *H. suis* infection. We describe steps for collecting gastric biopsies and sera from patients, preparing DNA for PCR, and targeting the *H. suis*-specific gene. We then define procedures for inoculating biopsies onto primary agar plates and transferring colonies to secondary agar plates. Finally, we detail whole-genome sequencing of bacteria and assess *H. suis* infection in sera with ELISA.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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## Highlights

Protocol for detecting *H. suis* infection in clinical practice

Steps to collect and transport gastric biopsies and serum specimens

Detecting *H. suis* in gastric biopsies by PCR, culture, and whole-genome sequencing

Establishing serological detection of *H. suis* infection by ELISA

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# Protocol for detecting *Helicobacter suis* infection in gastric biopsies and serum by PCR and ELISA

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## SUMMARY

**Infection with *Helicobacter suis*, which causes many cases of gastric disease, is not reliably diagnosed. Here, we present a protocol for detecting *H. suis* infection. We describe steps for collecting gastric biopsies and sera from patients, preparing DNA for PCR, and targeting the *H. suis*-specific gene. We then define procedures for inoculating biopsies onto primary agar plates and transferring colonies to secondary agar plates. Finally, we detail whole-genome sequencing of bacteria and assess *H. suis* infection in sera with ELISA.**

**For complete details on the use and execution of these protocols, please refer to Matsui et al.[1]**

## BEFORE YOU BEGIN

### Institutional permissions

⚠ CRITICAL: This study has been approved by the Research Ethics Committees (RECs) of NIID and Kitasato University under registration numbers 1284 and 18100, respectively.

⚠ CRITICAL: Ethical reviews have also been approved by medical institutes.

⚠ CRITICAL: Written informed consent has been obtained from all of the patients participating in the study.

*Note:* Individuals with healthy stomachs or gastric disease under 20 years old are excluded from this study.

### Processing of samples

Timing: 1–7 days

1. Collect gastric biopsies and serum samples from individuals undergoing medical check-ups or those with gastric disorders who underwent upper gastrointestinal endoscopy.

   *Note:* The presence of a mottled pattern or marbling findings in the glandular border region from the antrum or vestibule of the stomach to the gastric angle raises the suspicion of an ongoing *H. suis* infection.

   *Note:* Gastric biopsies are collected from each of the following three sites: the greater curvature of the gastric antrum, the greater curvature of the lower gastric body, and the lesser curvature of the gastric angle.[2]

   *Note:* Use separate gastric biopsy transport media for each gastric biopsy.

2. Transport gastric biopsies to the laboratory at 19°C–22°C within five days of collection using the non-*H. pylori Helicobacter* species (NHPH) biopsy transport media.[2]

   ⚠ CRITICAL: Step 2 is crucial for successful *H. suis* cultivation.

3. Remove each gastric biopsy from the biopsy transport media and homogenize it with 300 μL of *H. suis culture* broth using a microtube homogenizer.

   ⚠ CRITICAL: Of the 300 μL homogenate, a 200 μL aliquot should be used immediately for cultivation, while the remaining 100 μL aliquot should be stored at −20°C for PCR until ready for use.

   *Alternatives:* To obtain a gastric specimen from gastric juice, promptly transfer the juice to a sterile tube and transport it to the laboratory at 19°C–22°C. Centrifuge 3.0 mL of the juice at 15,000 × *g* for 3 min at 22°C using a Microfuge 20R. Then, homogenize the resulting precipitate with 300 μL of *H. suis* culture broth using a microtube homogenizer.

4. Transport serum samples to the laboratory at 4°C within one week of collection, aliquot, and store at −80°C until ready for ELISA testing.

## Preparation of ELISA

Timing: 7 days

5. Prepare ELISA antigens from broth cultures of *H. pylori* and *H. suis*.
   a. Inoculate the frozen stock of *H. pylori* strain TN2GF4 onto a *Helicobacter*-selective agar plate for three days in a humidified gas mixture (5% $O_2$, 10% $CO_2$, and 85% $N_2$) at 37°C in a multigas incubator.[3]
   b. Inoculate the grown colonies of *H. pylori* into 25 mL of *H. pylori* culture broth in a 100 mL sterile glass Erlenmeyer flask.[3]
   c. Maintain the culture for three days with orbital shaking at 120 rpm in the humidified gas mixture (5% $O_2$, 10% $CO_2$, and 85% $N_2$) at 37°C.[3]
   d. Inoculate the frozen stock of *H. suis* strain NHP 19-4004 onto NHPH selective agar coated with *H. suis* culture broth.[4] Use five 25 $cm^2$ tissue culture flasks, each containing 5 mL of culture broth.
   e. Maintain the culture for five days under orbital shaking at 50 rpm using an ultra-compact rotary shaker in a humidified gas mixture (5% $O_2$, 12% $CO_2$, and 83% $N_2$) at 37°C.[4]
   f. Collect whole bacterial cells by centrifugation at 10,400 × *g* for 10 min at 4°C by a compact multifunctional centrifuge Allegra X-30R. Use two 15 mL centrifuge tubes for each culture of *H. pylori* and *H. suis*.

g. Wash the collected cells twice with 12.5 mL of PBS and once with 12.5 mL of distilled water by centrifugation at 10,400 × *g* for 10 min at 4°C by the Allegra X-30R using 15 mL centrifuge tubes.
h. Suspend the washed cells in 0.5 mL of distilled water at 4°C in a 1.5 mL microtube.
i. Disrupt using a Bioruptor II through sonication (20 kHz for five cycles of 30 s each, with a 30-s interval between each sonication at 0°C).
j. Remove the insoluble components by centrifugation at 15,000 × *g* for 20 min at 4°C using the Microfuge 20R.
k. Measure the protein concentration of the whole bacterial cell solution using a BioSpectrometer with a μCuvette G1.0, employing BSA solution (1 mg/mL) as the standard.
l. Aliquot and store the prepared whole bacterial cell solutions at −20°C until ready for ELISA testing.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Horseradish peroxidase (HRP)-conjugated goat anti-human IgA + IgG + IgM (H + L) antibody (1:10,000) | Jackson ImmunoResearch | Cat#109-035-064; PRID: AB_2337583 |
| Bacterial and virus strains | | |
| *H. suis* strain NHP19-4004 | Rimbara et al.[2] | N/A |
| *H. pylori* strain TN2GF4 | Matsui et al.[3] | N/A |
| Biological samples | | |
| Gastric biopsies from patients | Matsui et al.[1] | N/A |
| Serum samples from patients (1:3,600) | Matsui et al.[1] | N/A |
| Positive control serum for *Helicobacter suis* infection (no. 04029) (1:3,600) | Matsui et al.[1] | N/A |
| Positive control serum for *Helicobacter pylori* infection (no. 04279) (1:3,600) | Matsui et al.[1] | N/A |
| Negative control serum (no. 04075) (1:3,600) | Matsui et al.[1] | N/A |
| Chemicals, peptides, and recombinant proteins | | |
| Bovine serum albumin (BSA) standard solution (1 mg/mL) | BMS | Cat#BR-220700053 |
| Sodium carbonate | FUJIFILM Wako | Code#199-01605 |
| Sodium bicarbonate | FUJIFILM Wako | Code#199-05985 |
| Phosphate-buffered saline (PBS) 10×, pH 7.4 | Gibco | Cat#70011044 |
| 10% (wt/vol) Tween 20 solution | FUJIFILM Wako | Cat#161-24801 |
| Blocker BSA (10% [wt/vol] BSA in PBS) | Thermo Fisher Scientific | Cat#37525 |
| Glycerol | FUJIFILM Wako | Cat#070-04941 |
| Critical commercial assays | | |
| DNeasy Blood & Tissue Kit | QIAGEN | Cat#69504/69556 |
| Nextera XT DNA Library Prep Kit | Illumina | Cat#TG-131-1096 |
| ELISA enzyme substrate KPL SureBlue TMB Microwell Peroxidase Substrate (1 component) | SeraCare Life Sciences | Cat##5120-0075 |
| Nunc-Immuno 96-well microtiter plate | Thermo Fisher Scientific | Cat#439454 |
| Plate Seal T (PET sticking seal) | Watson | Cat#547-SBS-PET |
| 5 mL V-bottom freestanding tube (sterile product) | Sarstedt | Cat#62.558.201 |
| Petri dish (ø90 × 15 mm; sterile product) | AS ONE | Cat#GD90-15 |
| Tissue culture flask (vent cap, 25 $cm^2$; sterile product) | Violamo | Cat#VTC-F25P |
| *Helicobacter*-selective agar plate | Nissui Pharmaceutical | Cat#51035 |
| Brucella broth | BD BBL | Cat#211088 |
| Brucella agar | BD BBL | Cat#211086 |
| Bacto agar | BD BBL | Cat#63-6531-35 |
| Heat-inactivated fetal bovine serum (FBS) | Biowest | Cat#S181H |
| Skirrow (*Campylobacter* selective supplement) | Oxoid | Cat#OXSR0069E |

(*Continued on next page*)

*Continued*

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Vitox supplement | Oxoid | Cat#R663090 |
| Dent (*H. pylori* selective supplement) | Oxoid | Cat#OXSR0147E |
| Amphotericin B solution (250 μg/mL) | Sigma-Aldrich | Cat#A2942 |
| Linezolid | Abcam | Cat#ab141991 |
| THUNDERBIRD Probe qPCR Mix | Toyobo | Cat#QPS-101T |
| GoTaq Green Master Mix | Promega | Cat#M7122 |
| QIAquick PCR Purification Kit | QIAGEN | Cat#28104 |
| **Deposited data** | | |
| Whole-genome sequences of *H. suis* 13 strains (GenBank/ENA/DDBJ accession number) | Matsui et al.[1] | N/A |
| The 23S-rRNA gene sequences of *H. pylori* 16 strains (GenBank/ENA/DDBJ accession number) | Matsui et al.[1] | N/A |
| The 23S-rRNA gene sequences of *Helicobacter* 21 type strains (GenBank/ENA/DDBJ accession number) | Matsui et al.[1] | N/A |
| Sequence typing (ST) assignment | Matsui et al.[1] | PubMLST https://pubmlst.org/organisms/helicobacter-suis |
| **Oligonucleotides** | | |
| *H. suis hsvA* targeting probe-based real-time PCR primer sequence: NHP194003_11930_forward: 5′-CTGGTAATGCATCATTAGAAGCAAA-3′ | Rimbara et al.[2] | N/A |
| *H. suis hsvA* targeting probe-based real-time PCR primer sequence: NHP194003_11930_reverse: GATGGGCGCTTCTGGTTTA | Rimbara et al.[2] | N/A |
| *H. suis hsvA* targeting probe-based real-time PCR probe sequence: NHP194003_11930_probe: 56-FAM/TGTACACAC/ZEN/CAAACAGATGAGCCGT/3IABkFQ | Rimbara et al.[2] | N/A |
| NHPH 16S rRNA gene targeting probe-based real-time PCR primer sequence: NHPH_16S_F: CAAGTCGAACGATGAAGCCTA | Matsui et al.[1] | N/A |
| NHPH 16S rRNA gene targeting probe-based real-time PCR primer sequence: NHPH_16S_R: ATTTGGTATTAATCACCATTTCTAGT | Matsui et al.[1] | N/A |
| NHPH 16S rRNA gene targeting probe-based real-time PCR probe sequence: NHPH_16S_probe: 56-FAM/TTACTCACC/ZEN/CGTGCGCCACTAATC/3IABkFQ | Matsui et al.[1] | N/A |
| *H. pylori* 651 bp DNA fragment of the 23S rRNA gene targeting colony PCR primer sequence: F3: CCGTAGCGAAAGCGAGTCT | Matsui et al.[1] | N/A |
| *H. pylori* 651 bp DNA fragment of the 23S rRNA gene targeting colony PCR primer sequence: R3: CCCGACTAACCCTACGATGA | Matsui et al.[1] | N/A |
| **Software and algorithms** | | |
| PrimerQuest Tool | IDT | https://www.idtdna.com/pages/tools/primerquest?returnurl=%2FPrimerQuest%2FHome%2FIndex |
| Shovill v1.1.0 | Open source | https://github.com/tseemann/shovill |
| pyani 0.2.12 | Open source | https://github.com/widdowquinn/pyani |
| GraphPad Prism 9.4.1 | GraphPad Software | https://www.graphpad.com/scientific-software/prism/ |
| **Other** | | |
| *H. suis hsvA* targeting probe-based real-time PCR protocol | Rimbara et al.[2] | N/A |
| NHPH 16S rRNA gene targeting probe-based real-time PCR protocol | Matsui et al.[1] | N/A |
| *H. pylori* 651 bp DNA fragment of the 23S rRNA gene targeting colony PCR protocol | Matsui et al.[1] | N/A |
| Multigas incubator (water jacket) | Astec | Cat#APM-30D |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Ultra-compact rotary shaker | Nissin | Cat#NA-M301 |
| Compact multifunctional centrifuge Allegra X-30R | Beckman Coulter | Cat#A99473 |
| Falcon 15 mL conical centrifuge tube (sterile product) | Thermo Fisher Scientific | Cat#352096 |
| Microfuge 20R | Beckman Coulter | Cat#B30-144 |
| 1.5 mL Microtube (sterile product) | BMBio | Cat#WNB615601 |
| Microtube homogenizer (sterile product) | AS ONE | Cat#2-3869-2 |
| 2.0 mL Screw cap microtube (self-standing type) | AS ONE | Cat#1-2960-02 |
| Bioruptor-II | BMBio | Cat#BR2006A |
| BioSpectrometer | Eppendorf | Cat#P1EPD1000194-1 |
| μCuvette G1.0 | Eppendorf | Cat#6138000018 |
| Applied Biosystems 7500 Fast Real-Time PCR | Thermo Fisher Scientific | Cat#45509 |
| MicroAmp Fast 96-well reaction plate | Thermo Fisher Scientific | Cat#4346907 |
| MicroAmp Optical Adhesive Film | Thermo Fisher Scientific | Cat#4311971 |
| CronoSTAR Portable 8 Real-Time System (4 channels) | Clontech | Cat#640249 |
| CronoSTAR Portable Control PC | Clontech | Cat#WN820 |
| 0.2 mL 4-strip tube, individual for CronoSTAR | Clontech | Cat#WN100A |
| Mastercycler X50s | Eppendorf | Cat#6311000010 |
| Fast PCR tube strips, 0.1 mL | Eppendorf | Cat#0030124928 |
| Illumina MiSeq | Illumina | Cat#SY-410-1003 |
| Microplate shaking incubator MyBLP25 | AS ONE | Cat#1-2934-01 |
| Microplate washer Wellwash | Thermo Fisher Scientific | Cat#5165000 |
| Microplate Spectrophotometer Multiskan SkyHigh | Thermo Fisher Scientific | Cat#A51119700C |
| Electric pipette Xplorer single channel (0.1–10 μL) | Eppendorf | Cat#4861000.015 |
| Electric pipette Xplorer single channel (1–20 μL) | Eppendorf | Cat#4861000.017 |
| Electric pipette Xplorer single channel (10–200 μL) | Eppendorf | Cat#4861000.027 |
| Electric pipette Xplorer single channel (50–1000 μL) | Eppendorf | Cat#4861000.163 |
| Electric pipette Xplorer 8-channel (15–300 μL) | Eppendorf | Cat#4861000.147 |
| Electric pipette Xplorer 8-channel (50–1200 μL) | Eppendorf | Cat#4861000.163 |
| Electric pipette single channel (0.1–10 mL) | A&D | Code#MPA10000 |
| Autoclave | Tomy | Code#LSX-500 |
| Vortex-Genie 2 mixer | M&S | Code#S1-0286 |
| Class II biological safety cabinet | Airtech | Code#BHC-1010IIA2 |
| Medical refrigerated showcase (4°C) | Nihon Freezer | Code#NC-ME31HC |
| Deep freezer (−80°C) | Nihon Freezer | Code#CLN-32U |
| Biomedical freezer (−20°C) | PHC | Code#MDF-MU549DH-PJ |

## MATERIALS AND EQUIPMENT

**Gastric biopsy transport media**[1,2]

| Reagent | Final concentration | Amount |
|---|---|---|
| Brucella broth | Casein peptone (15.0 g/L), Meat peptone (5.0 g/L), Sodium chloride (5.0 g/L), Dextrose (1.0 g/L), Sodium bisulfite (0.1 g/L), Yeast extract (2.0 g/L) | 28.1 g |
| Bacto agar | 0.4% (wt/vol) | 4 g |
| Heat-inactivated FBS, | 20% (vol/vol) | 200 mL |
| Skirrow | Vancomycin (10.0 μg/mL), Trimethoprim (5.0 μg/mL), Polymyxin B (2.5 IU/mL) | 4 mL (2 vials) |
| Vitox | Glucose (2.0 mg/mL), Vitamin B12 (0.2 μg/mL), Adenine (20.0 μg/mL), L-Glutamine (200.0 μg/mL), Guanine (0.6 μg/mL), p-Aminobenzoic acid (0.26 μg/mL), L-Cystine (22.0 μg/mL), NAD (Coenzyme 1; 5.0 μg/mL), Cocarboxylase (2.0 μg/mL), Iron (III) nitrate (0.4 μg/mL), Thiamine hydrochloride (0.06 μg/mL), Cysteine hydrochloride (518 μg/mL) | 20 mL (2 vials) |
| Amphotericin B (250 μg/mL), | 5 μg/mL | 20 mL |

(*Continued on next page*)

| *Continued* | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Hydrochloric acid (37%) | N/A | Adjusted to pH 5.0 (approx. 1. 35 mL) |
| $ddH_2O$ | N/A | 754.65 mL |
| Total | N/A | 1 L |

*Note:* Suspend the Brucella broth and Bacto agar in $ddH_2O$ and autoclave for 15 min at 121°C. Then cool to 50°C in a hot water bath before adding the remaining reagents. Store 5 mL of the gastric biopsy transport medium in a V-bottom freestanding tube at 4°C for up to 3 months. See Figure 1A.

| **NHPH selective agar plate**[1,2] | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Brucella agar | Casein peptone (15.0 g/L), Meat peptone (5.0 g/L), Sodium chloride (5.0 g/L), Dextrose (1.0 g/L), Sodium bisulfite (0.1 g/L), Yeast extract (2.0 g/L), Agar (15.0 g/L) | 43.1 g |
| Heat-inactivated FBS, | 20% (vol/vol) | 200 mL |
| Skirrow | Vancomycin (10.0 μg/mL), Trimethoprim (5.0 μg/mL), Polymyxin B (2.5 IU/mL) | 4 mL (2 vials) |
| Vitox | Glucose (2.0 mg/mL), Vitamin B12 (0.2 μg/mL), Adenine (20.0 μg/mL), L-Glutamine (200.0 μg/mL), Guanine (0.6 μg/mL), p-Aminobenzoic acid (0.26 μg/mL), L-Cystine (22.0 μg/mL), NAD (Coenzyme 1; 5.0 μg/mL), Cocarboxylase (2.0 μg/mL), Iron (III) nitrate (0.4 μg/mL), Thiamine hydrochloride (0.06 μg/mL), Cysteine hydrochloride (518 μg/mL) | 20 mL (2 vials) |
| Amphotericin B (250 μg/mL), | 5 μg/mL | 20 mL |
| Hydrochloric acid (37%) | N/A | Adjusted to pH 5.0 (approx. 1. 35 mL) |
| $ddH_2O$ | N/A | 754.65 mL |
| Total | N/A | 1 L |

*Note:* Suspend the Brucella agar in $ddH_2O$ and autoclave for 15 min at 121°C. Then cool to 50°C in a hot water bath before adding the remaining reagents. Store 18 mL of the NHPH selective agar medium in a Petri dish at 4°C for up to 3 months.

*Alternatives:* When anticipating *Lactobacillus* contamination in the gastric specimen, supplement the NHPH selective agar medium with linezolid (final concentration of 10 mg/L).

| ***H. suis culture* broth**[1,2] | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Brucella broth | Casein peptone (15.0 g/L), Meat peptone (5.0 g/L), Sodium chloride (5.0 g/L), Dextrose (1.0 g/L), Sodium bisulfite (0.1 g/L), Yeast extract (2.0 g/L) | 28.1 g |
| Heat-inactivated FBS | 20% (vol/vol) | 200 mL |
| Skirrow | Vancomycin (10.0 μg/mL), Trimethoprim (5.0 μg/mL), Polymyxin B (2.5 IU/mL) | 4 mL (2 vials) |
| Vitox | Glucose (2.0 mg/mL), Vitamin B12 (0.2 μg/mL), Adenine (20.0 μg/mL), L-Glutamine (200.0 μg/mL), Guanine (0.6 μg/mL), p-Aminobenzoic acid (0.26 μg/mL), L-Cystine (22.0 μg/mL), NAD (Coenzyme 1; 5.0 μg/mL), Cocarboxylase (2.0 μg/mL), Iron (III) nitrate (0.4 μg/mL), Thiamine hydrochloride (0.06 μg/mL), Cysteine hydrochloride (518 μg/mL) | 20 mL (2 vials) |
| Amphotericin B (250 μg/mL), | 5 μg/mL | 20 mL |
| Hydrochloric acid (37%) | N/A | Adjusted to pH 5.0 (approx. 1. 35 mL) |
| $ddH_2O$ | N/A | 754.65 mL |
| Total | N/A | 1 L |

*Note:* Dissolve the Brucella broth in $ddH_2O$ and autoclave for 15 min at 121°C. Then cool to 50°C in a hot water bath before adding the remaining reagents. Store the *H. suis culture* broth in a sterilized bottle at 4°C for up to 3 months.

| ***H. suis* modified biphasic culture**[1] | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| NHPH selective agar | N/A | 50 mL |
| *H. suis culture* broth | N/A | 50 mL |
| **Total** | **N/A** | **100 mL** |

⚠ CRITICAL: Prepare 5 mL of NHPH selective agar in a 25 $cm^2$ tissue culture flask which can be stored at 4°C for up to 3 months. After inoculation of *H. suis*, add 5 mL of fresh *H. suis culture* broth and begin cultivation. See Figure 1B.

| ***H. pylori* culture broth**[3] | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Brucella broth | Casein peptone (15.0 g/L), Meat peptone (5.0 g/L), Sodium chloride (5.0 g/L), Dextrose (1.0 g/L), Sodium bisulfite (0.1 g/L), Yeast extract (2.0 g/L) | 28.1 g |
| Heat-inactivated FBS | 10% (vol/vol) | 100 mL |
| Dent | Vancomycin (10.0 μg/mL), Trimethoprim (5.0 μg/mL), Cefsulodin (5.0 μg/mL), Amphotericin B (5.0 μg/mL) | 4 mL (2 vials) |
| Vitox | Glucose (2.0 mg/mL), Vitamin B12 (0.2 μg/mL), Adenine (20.0 μg/mL), L-Glutamine (200.0 μg/mL), Guanine (0.6 μg/mL), p-Aminobenzoic acid (0.26 μg/mL), L-Cystine (22.0 μg/mL), NAD (Coenzyme 1; 5.0 μg/mL), Cocarboxylase (2.0 μg/mL), Iron (III) nitrate (0.4 μg/mL), Thiamine hydrochloride (0.06 μg/mL), Cysteine hydrochloride (518 μg/mL) | 4 mL (2 vials) |
| $ddH_2O$ | N/A | 892 mL |
| **Total** | **N/A** | **1 L** |

*Note:* Dissolve the Brucella broth in $ddH_2O$ and autoclave for 15 min at 121°C. Then cool to 50°C in a hot water bath before adding the remaining reagents. Store the *H. pylori culture* broth in a sterilized bottle at 4°C for up to 3 months.

| ***Helicobacter* stock media** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Brucella broth | Casein peptone (15.0 g/L), Meat peptone (5.0 g/L), Sodium chloride (5.0 g/L), Dextrose (1.0 g/L), Sodium bisulfite (0.1 g/L), Yeast extract (2.0 g/L) | 2.81 g |
| Glycerol | 50% (vol/vol) | 50 mL |
| $ddH_2O$ | N/A | 45 mL |
| **Total** | **N/A** | **100 mL** |

*Note:* Dissolve Brucella broth in a mixture containing 45 mL of $ddH_2O$ and 50 mL of glycerol, then add more $ddH_2O$ to make 100 mL.

*Note:* Dispense 0.5 mL of *Helicobacter* stock media into each 2.0 mL screw cap microtube. Store the *Helicobacter* stock media at 4°C for up to one year after autoclaving for 20 min at 120°C.

| **0.1 M Carbonate/bicarbonate buffer, pH 9.4**[1] | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Sodium carbonate | 0.019 M | 2.014 g |

(*Continued on next page*)

| *Continued* | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Sodium bicarbonate | 0.081 M | 6.805 g |
| $ddH_2O$ | N/A | 900 mL |
| Total | N/A | 1 L |

*Note:* Dissolve sodium carbonate and sodium bicarbonate in 900 mL of $ddH_2O$, then add more $ddH_2O$ to make 1 L.

*Note:* Store the 0.1 M carbonate/bicarbonate buffer at 4°C for up to one year after autoclaving for 15 min at 121°C.

| **PBS-T**[1] | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| PBS 10×, pH 7.4 | Potassium phosphate monobasic (144.0 mg/L), Sodium chloride (9.0 g/L), Sodium phosphate dibasic (795 mg/L) | 100 mL |
| Tween 20 solution (10% [wt/vol]) | 0.05% (wt/vol) | 5 mL |
| $ddH_2O$ | N/A | 895 mL |
| Total | N/A | 1 L |

*Note:* Store the PBS-T at 4°C for up to one year after autoclaving for 15 min at 121°C.

| **Blocking buffer**[1] | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| PBS 10×, pH 7.4 | Potassium phosphate monobasic (144.0 mg/L), Sodium chloride (9.0 g/L), Sodium phosphate dibasic (795 mg/L) | 5 mL |
| Blocker BSA (10% [wt/vol]) | 1% (wt/vol) | 5 mL |
| $ddH_2O$ | N/A | 40 mL |
| Total | N/A | 50 mL |

*Note:* Store the blocking buffer in a sterilized bottle at 4°C for up to one year.

## STEP-BY-STEP METHOD DETAILS

### Perform PCR testing

Timing: 1 day

1. Extract DNA from the 100 μL aliquot of the gastric biopsy homogenates using the DNeasy Blood & Tissue Kit.[1,2]
2. Measure the DNA concentration using the BioSpectrometer with the μCuvette G1.0.
3. Use the prepared DNA as the template for probe-based real-time PCR, targeting the *H. suis*-specific *vacA*-type autotransporter protein gene (*hsvA*) and the NHPH-common region of the 16S rRNA gene.
4. The sequences of the two sets of primers and probes, designed using the PrimerQuest Tool, are as follows:

NHP194003_11930_forward (5′-CTGGTAATGCATCATTAGAAGCAAA-3′), NHP194003_11930_reverse (5′-GATGGGCGCTTCTGGTTTA-3′), and NHP194003_11930_probe (5′-/56-FAM/TGTACACAC/ZEN/CAAACAGATGAGCCGT/3IABkFQ-3′) for targeting the *hsvA* gene; NHPH_16S_F (5′-CAAGTCGAACGATGAAGCCTA-3′), NHPH_16S_R (5′- ATTTGGTATTAATCACCATTTCTAGT-

3′), and NHPH_16S_probe (5′-/56-FAM/TTACTCACC/ZEN/CGTGCGCCACTAATC/3IABkFQ/-3′) for targeting the NHPH 16S rRNA gene.

**PCR master mix targeting the *H. suis*-specific *hsvA* gene or the NHPH-common region of the 16S rRNA gene utilizing the THUNDERBIRD Probe qPCR Mix**

| Reagent | Amount |
|---|---|
| DNA Template | 3 pg–100 ng |
| qPCR Mix (2×) | 10 μL |
| Primer 1 | 6 pmol |
| Primer 2 | 6 pmol |
| Probe | 4 pmol |
| ROX reference dye | 0.04 μL |
| $ddH_2O$ | X μL |
| Total Volume | 20 μL |

*Note:* Perform real-time PCR primarily on the Applied Biosystems 7500 Fast using the MicroAmp Fast 96-well reaction plates and MicroAmp Optical Adhesive Film.

*Alternatives:* When dealing with a small number of samples, perform real-time PCR on the CronoSTAR Portable 8 Real-Time System (4 channels) using 0.2 mL 4-strip tubes.

**PCR cycling conditions**

| Steps | Temperature | Time | Cycles |
|---|---|---|---|
| Initial Denaturation | 95°C | 20 s | 1 |
| Denaturation | 95°C | 3 s | 40 |
| Extension | 60°C | 30 s | |

5. The diagnosis of *H. suis* infection is confirmed when positive results are obtained from both types of PCR targeting the *H. suis*-specific *hsvA* and NHPH-common 16S rRNA genes.[4]

*Note:* NHPH, other than *H. suis*, is diagnosed when negative and positive results are obtained by PCR targeting the *H. suis*-specific *hsvA* and NHPH-common 16S rRNA genes, respectively. See Problem 4.

## Perform culture testing

Timing: 19–26 days

6. Inoculate the 200 μL aliquot of the gastric biopsy homogenates onto NHPH selective agar plates.[1,2]
7. Incubate the plates for 2–3 weeks at 37°C in a humidified gas mixture (5% $O_2$, 12% $CO_2$, and 83% $N_2$).

*Note:* Although both *H. pylori* and *H. suis* are grown as primary cultures on NHPH selective agar plates, the *H. pylori* colonies tend to grow faster and larger than the small, slow-growing colonies formed by *H. suis*. See Figure 2.

*Note:* We include the culture as a diagnostic criterion to confirm *H. pylori* infection, whereas we exclude the culture as a diagnostic criterion to confirm *H. suis* infection.

8. To identify *H. pylori* infection, amplify the 651 bp DNA fragment of the 23S rRNA gene by the colony PCR using the following set of primers: F3 (5′- CCGTAGCGAAAGCGAGTCT-3′) and R3 (5′-CCCGACTAACCCTACGATGA-3′).

**PCR master mix targeting the 651 bp DNA fragment of the 23S rRNA gene of *H. pylori* using the GoTaq Green Master Mix.**

| Reagent | Amount |
|---|---|
| Master Mix (2×) | 12.5 μL |
| Primer 1 | 2.5 μL (10 μM) |
| Primer 2 | 2.5 μL (10 μM) |
| $ddH_2O$ | 7.5 μL |
| Total Volume | 25 μL |

*Note:* Suspend the colony in PCR master mix by pipetting it up and down using the tip of a micropipette.

*Note:* Perform the PCR on the Mastercycler X50s using the Fast PCR Tube Strips.

**PCR cycling conditions**

| Steps | Temperature | Time | Cycles |
|---|---|---|---|
| Initial Denaturation | 95°C | 2 min | 1 |
| Denaturation | 95°C | 30 s | 30 |
| Annealing | 55°C | 1 min | |
| Extension | 72°C | 2 min | |
| Final extension | 72°C | 7 min | 1 |
| Hold | 4°C | forever | |

9. After PCR, confirm the presence of amplified bands by performing agarose gel electrophoresis.
10. Purify the amplified DNA fragments using the QIAquick PCR Purification Kit and stored at −20°C for subsequent DNA sequencing.

*Note:* *H. pylori* strains will be identified if the homology of the 23S rRNA gene to *H. pylori* strain ATCC43504 (GenBank #: AP017632.1) is greater than 99.0%.[1]

11. Inoculate the grown colonies of primary culture onto NHPH selective agar coated with *H. suis* culture broth and enriched by modified biphasic culture for five days with orbital shaking at 50 rpm in the humidified gas mixture at 37°C.

*Note:* Use four 25 $cm^2$ tissue culture flasks, each containing 5 mL of culture broth.

12. Collect whole bacterial cells from 20 mL of culture broth by centrifugation at 10,400 × *g* for 10 min at 22°C by the Allegra X-30R using two 15 mL centrifuge tubes. Dissolve the resulting pellet from 10 mL of culture broth with 0.5 mL of *H. suis* culture broth and mix it with 0.5 mL of the *Helicobacter* stock media in the 2.0 mL screw cap microtube. Then, stock the *Helicobacter* strains at −80°C for up to 10 years.
13. Store the resulting pellet from 10 mL of culture broth at −20°C until ready for whole-genome sequencing.

## Perform whole-genome sequencing

**Timing: 14 days**

14. Extract DNA from the culture broth pellet for whole-genome sequencing using the DNeasy Blood & Tissue Kit.[1]
15. Measure the DNA concentration using the BioSpectrometer with the μCuvette G1.0.

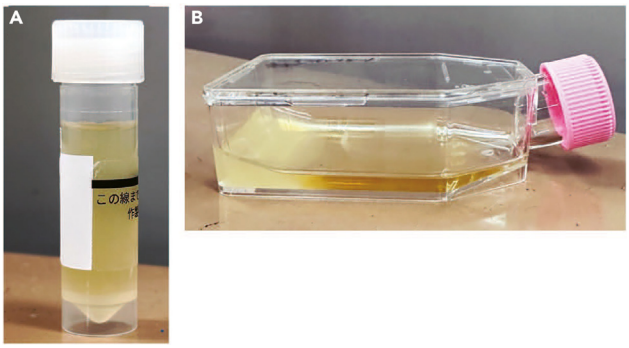


**Figure 1. *H. suis* transport and culture media**

(A and B) The gastric biopsy transport medium (A) and the modified biphasic culture of *H. suis* (B) are provided. The gastric biopsy transport medium contains a line indicating the appropriate location of gastric biopsy insertion (A).

16. Prepare the library for Illumina sequencing (150-bp paired-end, insert size of 500–900 bp) using the Nextera XT DNA Library Prep Kit.
17. Perform whole-genome sequencing of all culture-derived *H. suis* strains using the Illumina MiSeq platform.
18. Use the Illumina reads for *de novo* assembly by employing Shovill v1.1.0. and default parameters, aiming to acquire the draft genome sequences.
19. Determine bacterial species by calculating the average nucleotide identity (ANI) using pyani 0.2.12.

*Note:* We have shown that all *H. suis* strains have more than 99% ANI values against the *H. suis* type strain HS1.[1]

## Perform ELISA testing

Timing: 2 days

20. Prepare the Immuno microtiter plates by adding 100 μL of whole bacterial cell solution of *H. suis* strain NHP19-4004 or *H. pylori* strain TN2GF4 (at a concentration of 4 μg/mL) in 0.1 M carbonate/bicarbonate buffer, pH 9.4 to each well.[1]
21. Seal the plates with the Plate Seal T and incubate for 8–16 h at 4°C.
22. Remove the whole bacterial cell solution from the wells.
23. Wash the wells three times with 200 μL of PBS-T using a microplate washer (Wellwash), then saturate the wells with 200 μL of blocking buffer.
24. Seal the plates with the Plate Seal T and incubate for 1 h at 37°C while shaking at 500 rpm on a microplate shaking incubator (MyBLP25).
25. Discard the blocking buffer from the wells.
26. After three washes with 200 μL of PBS-T on the Wellwash, fill the wells with 50 μL of serum samples that have been diluted at a ratio of 1:3,600 with blocking buffer.
27. Seal the plates with the Plate Seal T and incubate for 1 h at 37°C while shaking at 500 rpm on the MyBLP25.
28. Discard the serum samples from the wells.
29. After three washes with 200 μL of PBS-T on the Wellwash, fill the wells with 50 μL of HRP-conjugated goat anti-human IgA + IgG + IgM (H + L) secondary antibody that has been diluted at a ratio of 1:10,000 with blocking buffer.
30. Seal the plates with the Plate Seal T and incubate for 1 h at 37°C while shaking at 500 rpm on the MyBLP25.
31. Discard the secondary antibody from the wells.
32. After three washes with 200 μL of PBS-T on the Wellwash, fill the wells with 50 μL of KPL SureBlue TMB Microwell Peroxidase Substrate (1-Component).

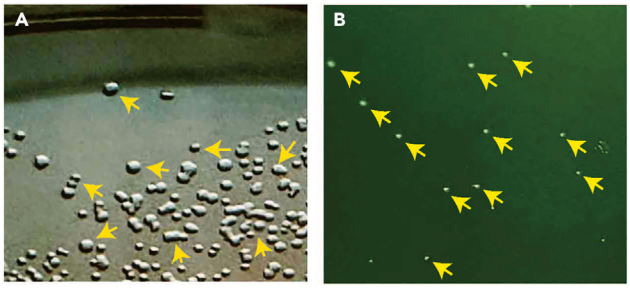


**Figure 2. Primary cultures of *Helicobacter***

(A and B) The image shows colonies (indicated by arrows) of *H. pylori* (A) and *H. suis* (B) grown on NHPH selective agar plates after three days and 14 days of cultivation, respectively.

33. Allow the substrate to react for 5 min at 22°C.
34. Stop the color reaction by adding 50 μL of 1 N hydrochloric acid into the wells.
35. Measure the absorbance at 450 nm (with a reference wavelength 620 nm) using a microplate reader (Multiskan SkyHigh).
36. Calculate the cut-off index as follows:

$$\text{Cut} - \text{off index} = \frac{(\text{Abs of test serum}) - (\text{Abs of negative control})}{(\text{Abs of positive control}) - (\text{Abs of negative control})}$$

37. Determine the positive and negative results based on the cut-off index values obtained.

***Note:*** The positive control for *H. suis* infection, the positive control for *H. pylori* infection, and the negative control are serum sample numbers 04029, 04279, and 04075 from the previous study, respectively obtained from a previous study.[1]

***Note:*** Positive cut-off index $\geq$1.0. Negative cut-off index<1.0. See Table 1.

## EXPECTED OUTCOMES

*Helicobacter suis*, which is hosted by wild boars, hogs, and macaques,[5,6] is the most prevalent NHPH found in the human stomach.[1,7] The infection rate of *H. suis* may have increased due to the decrease in *H. pylori* infection rate. Recent studies have suggested that *H. suis* infection has caused many cases of gastric disease,[7–9] but the transmission route from hogs remains unclear. Diagnostic methods based on *H. suis* urease activity often yield negative results, and there is no reliable method for diagnosing *H. suis* infection in clinical practice without gastric biopsy specimens. In this study, we include the culture as a diagnostic criterion to confirm *H. pylori* infection, whereas we exclude the culture as a diagnostic criterion to confirm *H. suis* infection. The ELISA showed high accuracy with an area under the receiver operating characteristic (ROC) curve (AUC) of 0.96 $\pm$ 0.02 (95% CI: 0.93 to 1.00), 100% (95% CI: 83.9%–100%) sensitivity, 92.6% (95% CI: 84.8%–96.6%) specificity, 76.9% (95% CI: 55.9%–90.2%) positive predictive value, and 100% (95% CI: 93.9%–100%) negative predictive value for the *H. suis* test.[1] The *H. suis* infection could be a predictive marker for the efficacy of eradication therapy for *H. pylori*-negative gastric mucosa-associated lymphoid tissue (MALT) lymphoma.[10] Therefore, the development of comprehensive methods to diagnose *H. suis* infection has become a matter of urgency for clinical practice. From now on, accurate diagnosis of H. suis infection will help avoid wasting valuable time with incorrect treatment.

## LIMITATIONS

Validation of serological tests for diagnosing infectious diseases requires agreement between several independent analytical and measurement results under defined conditions. However, this study represents the first to develop a serological diagnostic method for *H. suis* infection in humans. Therefore, this assay has only one independent validation group to confirm its validity. This is the current limitation of the study.

## TROUBLESHOOTING

### Problem 1

- The interpretations may show discrepancies between the ELISA and the PCR/culture (handling of infection diagnosis results, steps 5, 7, and 37).

### Potential solution

- In case the PCR/culture results are negative, there is a possibility of a positive ELISA due to a previous infection in which *H. suis* or *H. pylori* was eradicated spontaneously or by chance.[1]

### Problem 2

- The individual tested shows a strong positive result for *H. pylori* infection, but there is a possibility of a positive result for *H. suis* infection based on ELISA testing (handling of infection diagnosis results, step 37).

### Potential solution

- Because whole bacterial cell solution of *H. suis* may react with high titer anti-*H. pylori* serum antibodies, as shown in Table 1, sample 12, we advocate simultaneous ELISA testing for *H. suis* and *H. pylori* infection.

### Problem 3

- Even if *H. suis* and *H. pylori* infection test negative, positive results can still be obtained in PCR targeting the NHPH-common 16S rRNA gene (handling of infection diagnosis results, steps 5 and 37).

### Potential solution

- This case indicates the presence of gastric disease caused by NHPH other than *H. suis*, such as *H. ailurogastricus* infection.[11] Therefore, it is crucial to isolate infectious bacteria from gastric biopsies in order to accurately diagnose the condition.

### Problem 4

- If an *H. suis* infection is detected in a healthy individual, can it be identified to be the cause of future gastric disease, such as gastric MALT lymphoma? (handling of infection diagnosis results, steps 5, 7, 37)

### Potential solution

- Because *H. suis* infection has been detected in individuals with gastric diseases, including gastritis, peptic ulcers, and gastric MALT lymphoma,[1] monitoring the individual's gastric health and being aware of any symptoms or changes are essential.

**Table 1. Example of raw ELISA data for the detection of *H. suis* (Hs) and *H. pylori* (Hp) infections measured on a plate reader**

| Sample | Hs | Hs | Hs | Hs | Hs | Hs | Hp | Hp | Hp | Hp | Hp | Hp | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Abs. 1 (450 nm) | Blank substraction (450 nm) | Abs. 2 (620 nm) | Blank substraction (620 nm) | Basic calculation | Cut-off index | Abs. 1 (450 nm) | Blank substraction (450 nm) | Abs. 2 (620 nm) | Blank substraction (620 nm) | Basic calculation | Cut-off index | Decision |
| Blank | 0.153 | | 0.039 | | | | 0.158 | | 0.039 | | | | |
| Positive control | 1.106 | 0.953 | 0.053 | 0.014 | 0.939 | | 0.962 | 0.804 | 0.034 | -0.005 | 0.809 | | |
| Negative control | 0.287 | 0.134 | 0.042 | 0.003 | 0.131 | | 0.220 | 0.062 | 0.042 | 0.003 | 0.059 | | |
| Sample 1 | 0.316 | 0.163 | 0.038 | -0.001 | 0.164 | 0.041 | 0.239 | 0.081 | 0.047 | 0.008 | 0.073 | 0.019 | Negative |
| Sample 2 | 0461 | 0.308 | 0.040 | 0.001 | 0.307 | 0.218 | 0.372 | 0.214 | 0.051 | 0.012 | 0.202 | 0.191 | Negative |
| Sample 3 | 2.617 | 2.464 | 0.037 | -0.002 | 2.466 | **2.890** | 0.687 | 0.529 | 0.039 | 0 | 0.529 | 0.626 | *H. suis* |
| Sample 4 | 1.599 | 1.446 | 0.041 | 0.002 | 1.444 | **1.625** | 0.335 | 0.177 | 0.041 | 0.002 | 0.175 | 0.155 | *H. suis* |
| Sample 5 | 1.500 | 1.347 | 0.038 | -0.001 | 1.348 | **1.506** | 0.326 | 0.168 | 0.037 | −0.002 | 0.170 | 0.148 | *H. suis* |
| Sample 6 | 2.306 | 2.153 | 0.035 | -0.004 | 2.157 | **2.507** | 0.211 | 0.053 | 0.043 | 0.004 | 0.049 | −0.013 | *H. suis* |
| Sample 7 | 1.836 | 1.683 | 0.036 | -0.003 | 1.686 | **1.925** | 0.427 | 0.269 | 0.052 | 0.013 | 0.256 | 0.263 | *H. suis* |
| Sample 8 | 0.364 | 0.211 | 0.037 | -0,002 | 0.213 | 0.101 | 2.565 | 2.407 | 0.038 | −0.001 | 2.408 | **3.132** | *H. pylori* |
| Sample 9 | 0.580 | 0.427 | 0.036 | -0.003 | 0.430 | 0.370 | 1.506 | 1.348 | 0.038 | −0.001 | 1.349 | **1.720** | *H. pylori* |
| Sample 10 | 0.261 | 0.108 | 0.039 | 0 | 0.108 | −0.028 | 1.377 | 1.219 | 0.044 | 0.005 | 1.214 | **1.540** | *H. pylori* |
| Sample 11 | 0.873 | 0.720 | 0.036 | -0.003 | 0.723 | 0.733 | 1.903 | 1.745 | 0.036 | −0.003 | 1.748 | **2.252** | *H. pylori* |
| Sample 12 | 2.071 | 1.918 | 0.039 | 0 | 1.918 | **2.212** | 4.063 | 3.905 | 0.058 | 0.019 | 3.886 | **5.103** | *H. pylori* |

Bold indicates a Cut-off index of 1.0 or greater.

### Problem 5

- Which method is effective in eradicating *H. suis* infection? (handling of infection diagnosis results, steps 5, 7, 37)

### Potential solution

- Based on our experience,[1] the treatment for gastric diseases with *H. suis* infection involved the administration of the following three agents twice daily for one week to eradicate *H. suis*: vonoprazan (20 mg), amoxicillin (750 mg), and clarithromycin (200 mg or 400 mg). Following the eradication treatment, an upper GI endoscopy was performed at least two months later to confirm the remission of gastric disease and the eradication of *H. suis* through PCR testing. The anti-*H. suis* antibody titers also decreased over time after eradication.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to the lead contact, Emiko Rimbara (rimbara@niid.go.jp).

### Materials availability

ELISA prototypes, bacterial strains, and control antibodies for ELISA are available from the lead contact upon reasonable request.

### Data and code availability

- We have deposited the 23S rRNA gene data of *H. pylori* strains at GenBank/ENA/DDBJ.
- We have deposited the complete whole-genome sequences of *H. suis* strains at GenBank/EMBL/DDBJ .
- No original code is reported in this paper.
- Additional information about the analysis in this paper is available from the lead contact upon reasonable request.

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## AUTHOR CONTRIBUTIONS

Conceptualization, H.M., E.R., K.S., and T.K.; methodology – cultures and PCR, E.R. and S.A.; methodology – genomic analysis, M.S.; methodology – ELISA, H.M.; medical practice, K.T., H.S., and K.M.; writing, H.M. and E.R. All authors verified the underlying data and approved the final version of the manuscript for publication.

## DECLARATION OF INTERESTS

H.M., E.R., and M.S. are inventors of the technology described in this manuscript and are listed as applicants in the international patent applications under the PCT (PCT/JP2023/002328, PCT/JP2023/002329, and PCT/JP2023/002330).

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