Article

Development of serological assays to identify *Helicobacter suis* and *H. pylori* infections

Two gastric *Helicobacter* species in humans

H. suis transmission

H. pylori transmission

Diseases

H. suis

Gastritis
Gastric MALT lymphoma
Peptic ulcer

Diseases

H. pylori

Gastritis
Peptic ulcer
Gastric MALT lymphoma
Gastric cancer

serum

ELISA for simultaneous detection

H. suis infection

Sample	H. suis ELISA	H. pylori ELISA	Decision
14092	3.01	0.74	*H. suis*
04312	2.99	0.30	*H. suis*
04002	2.74	0.41	*H. suis*
94058	2.56	1.67	*H. suis*
94059	2.55	0.50	*H. suis*
14077	2.49	0.70	*H. suis*
04095	2.44	0.46	*H. suis*
04103	2.32	0.26	*H. suis*
14250	2.31	1.29	*H. suis*
04080	2.24	0.19	*H. suis*
04262	2.18	1.10	*H. suis*
14005	1.97	0.20	*H. suis*
04252	1.93	0.19	*H. suis*
04122	1.80	0.09	*H. suis*
14245	1.73	0.38	*H. suis*

H. pylori infection

Sample	H. suis ELISA	H. pylori ELISA	Decision
04172	0.70	2.89	*H. pylori*
04232	2.46	2.68	*H. pylori*
04133	2.68	2.20	*H. pylori*
14328	0.92	2.07	*H. pylori*
14207	0.32	1.80	*H. pylori*
04044	0.24	1.39	*H. pylori*
04318	0.30	1.32	*H. pylori*
04205	0.46	1.19	*H. pylori*
04108	0.00	1.14	*H. pylori*
14218	0.00	1.05	*H. pylori*
04311	0.19	1.01	*H. pylori*
04325	0.00	0.93	*H. pylori*
04220	0.21	0.91	*H. pylori*
04100	0.05	0.89	*H. pylori*
04279	0.19	0.88	*H. pylori*

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Highlights

Gastric non-*Helicobacter pylori* Helicobacter species in humans

ELISA for the simultaneous diagnosis of *Helicobacter suis* and *H. pylori* infection

Evaluation of the anti-*H. suis* antibody titers after eradication

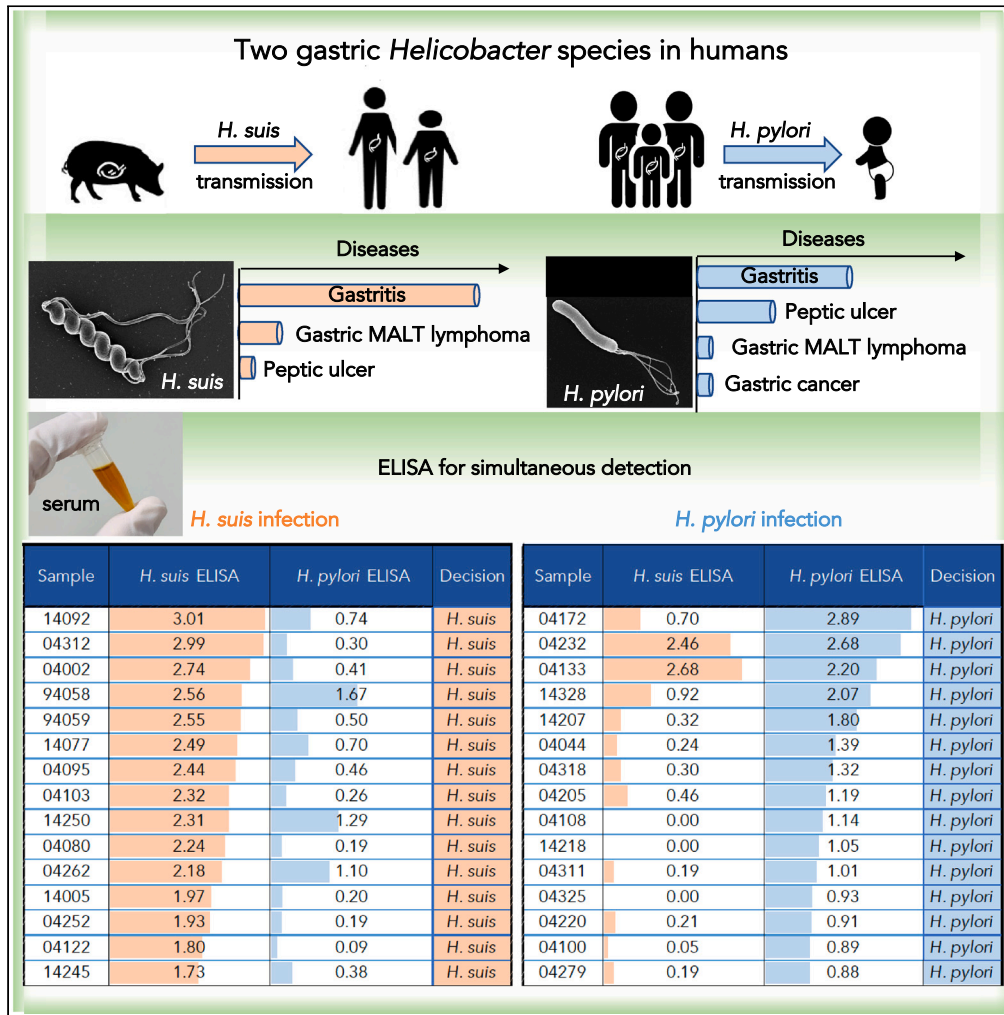Whole-genome sequences of *H. suis* strains isolated from humans

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Article

Development of serological assays to identify *Helicobacter suis* and *H. pylori* infections

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SUMMARY

***Helicobacter suis*, hosted by hogs, is the most prevalent gastric non-*Helicobacter pylori Helicobacter* species found in humans. Recent studies have suggested that *H. suis* infection has caused many cases of gastric disease, 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. This study presents the world's first use of whole-bacterial cell ELISA to simultaneously assess *H. suis* and *H. pylori* infections. The ELISAs showed high accuracy, with an area under the ROC curve of 0.96, 100% sensitivity, 92.6% specificity, 76.9% positive predictive value, and 100% negative predictive value for the *H. suis* test, and an area under the ROC curve of 0.92, 88.2% sensitivity, 87.5% specificity, 65.2% positive predictive value, and 96.6% negative predictive value for the *H. pylori* test.**

INTRODUCTION

Helicobacter suis, which differs from *Helicobacter pylori* in its bipolar flagella and corkscrew-like spiral morphology, is classified as a member of the gastric non-*H. pylori Helicobacter* species (NHPH), a group of bacteria that have been known to infect the human stomach since the 1980s.[1–4] The natural hosts of *H. suis* are macaques and hogs, whereas other NHPH such as *Helicobacter heilmannii* sensu stricto (s.s.), *Helicobacter ailurogastricus*, *Helicobacter felis*, *Helicobacter salomonis*, *Helicobacter baculiformis*, *Helicobacter cynogastricus,* and *Helicobacter bizzozeronii* infect the stomachs of cats and dogs.[5–7] Comparative genomic assessment of multilocus sequencing typing (MLST) of *H. suis* based on seven housekeeping genes (*atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ureAB*, and *yphC*) revealed high genomic homology between hog- and human-derived isolates, suggesting that *H. suis* infection in humans is a zoonosis originating from hogs.[5,8,9]

Because the conventional *H. pylori* diagnostic tests based on the urease activity, such as the urea breath test (UBT) and rapid urease test (RUT), often yield negative judgments in the case of NHPH infecting the human stomach, PCR or histological examination from gastric biopsy specimens has been performed to diagnose NHPH infection for research purposes. In past reports, the prevalence of NHPH infection was shown to be less than 0.5% by microscopic detection of the NHPH morphology in gastric sections.[10–12] In more recent reports, Tsukadaira et al. estimated the NHPH infection rate in Japan to be approximately 3.35%.[13] When a PCR assay targeting urease genes was performed on the 30 patients with NHPH-associated gastritis, 28 were infected with NHPH as follows (urease genes were not detected in 2 patients): 26 with *H. suis* and 2 with *H. heilmannii* s.s. or *H. ailurogastricus.*[13] They concluded that *H. suis* was the most common NHPH that infected human stomachs.[13] The *H. suis* infection leads to diseases such as gastric mucosa-associated lymphoid tissue (MALT) lymphoma, nodular gastritis, chronic gastritis, and peptic ulcer.[14–20] However, the above reports did not provide proof of *H. suis* or other NHPH infection because they only used PCR assay and did not isolate the bacteria or analyze the isolated bacteria's genome to identify the infecting bacteria.

Because most *H. pylori* infections occur in infancy, usually via transmission from family members, the infection rate of *H. pylori* has dramatically declined mainly because of improvements in the general hygiene environment in childhood in Japan.[21] As a result, the prevalence of *H. suis* infection could become more significant than the *H. pylori* infection rate if no measures are taken to prevent *H. suis* infection.[22] Because

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Continued

eradication therapy of *H. suis* is effective in *H. pylori*-negative gastric MALT lymphoma,[17,23] the *H. suis* infection could be a predictive marker for the efficacy of eradication therapy for *H. pylori*-negative gastric MALT lymphoma.[24] Therefore, the development of comprehensive methods to diagnose *H. suis* infection has become a matter of urgency for clinical practice.

Nonetheless, given the current worldwide infection status—i.e., more than half of the world's population is still infected with *H. pylori*[25]—there is a need for rapid diagnostic products that can detect both *H. suis* and *H. pylori* infections simultaneously. This is the first report of a serological diagnosis that simultaneously assesses *H. suis* and *H. pylori* infection in humans; the study also reports the evaluation of the anti-*H. suis* antibody titers after eradication.

RESULTS

Classification of the specimens

As shown in Table 1, by using routine methods, PCR, and culture for diagnosing *Helicobacter* infections, we classified all 101 subjects into the following four groups in advance: an *H. suis*-infection group (n = 20), an *H. pylori*-infection group (n = 17), a group with eradication of *H. pylori* infection (n = 20), and a group with neither *H. suis* nor *H. pylori* infection (n = 44). The patients in these groups had gastritis (n = 52), gastric mucosa-associated lymphoid tissue (MALT) lymphoma (n = 23), duodenal MALT lymphoma (n = 1), peptic ulcer (n = 20), autoimmune (metaplastic atrophic) gastritis (n = 4), and endoscopic mucosal resection (EMR) for early gastric cancer (EGC; n = 1). The percentage of men in the *H. suis* infection group was 85%, which was significantly different from the percentages in the *H. suis* non-infection groups, i.e., the *H. pylori* infection group (58.8%), the post-eradication of *H. pylori* infection group (45.0%), and the non-infection group (45.0%) (all p< 0.05). Table S1 (Individual data of 101 examinees) shows the individual data of the 101 examinees. The partial 23S rRNA gene sequences of the 13 *H suis* and 16 *H pylori* strains isolated in this study were compared with those of 21 reference *Helicobacter* strains, shown in Table S2 (Reference strains of gastric *Helicobacter* species). The genomic analysis successfully classified every *Helicobacter* strain with species specificity. All *H. suis* and *H. pylori* strains belonged to the same phylogenetic clades of *H. suis* and *H. pylori* reference strains, indicating that the genomic identification in this study was correct. We proved that all *H. suis* strains have more than 99% average nucleotide identity (ANI) values against the *H. suis* type strain HS1, shown in Figure S1 (Phylogenetic analysis of *Helicobacter* strains).

Measurement of the anti-*H. suis* and anti-*H. pylori* antibody titers

As shown in Figure 1, the mean with standard deviation (SD) of the absorbance at 450 nm of ELISA for identifying *H. suis* infection in the *H. suis* infection group was 2.1 ± 0.5, which was significantly higher than those of the *H. suis* non-infection groups—i.e., the *H. pylori* infection group (0.5 ± 0.8, p< 0.0001), the post-eradication of *H. pylori* infection group (0.4 ± 0.5, p< 0.0001), and the non-infection group (0.3 ± 0.4, p< 0.0001) (Figure 1A). The cut-off value (1.0) for the absorbance at 450 nm of *H. suis* infection was calculated by receiver operating characteristic (ROC) curve analysis between the *H. suis* infection group (n = 20) and the *H. suis* non-infection groups (n = 81) —i.e., the *H. pylori* infection (n = 17), post-eradication of *H. pylori* infection (n = 20), and non-infection with either *H. suis* or *H. pylori* (n = 44) groups (Figure 1B). Meanwhile, the mean with SD of the absorbance at 450 nm of ELISA for identifying *H. pylori* infection in the *H. pylori* infection group was 1.4 ± 0.7, which was significantly higher than those of two of the *H. pylori* non-infection groups—i.e., the *H. suis* infection group (0.5 ± 0.4, p< 0.0001) and the non-infection group (0.3 ± 0.4, p< 0.0001)—but not the post-eradication of *H. pylori* infection group (0.9 ± 0.8, p> 0.05) (Figure 1C). Then, the cut-off value (0.8) for the absorbance at 450 nm of *H. pylori* infection was calculated by ROC curve analysis between the *H. pylori* infection group (n = 17) and the *H. pylori* non-infection groups (n = 64), which were the *H. suis* infection group (n = 20) and the group with non-infection of either *H. suis* or *H. pylori* (n = 44) (Figure 1D). The sensitivity, specificity, and positive and negative predictive values (PPV and NPV, respectively) of ELISA for identifying the *H. suis* infection group against the *H. suis* non-infection groups were 100% (95% confidence interval [CI]: 83.9–100%), 92.6% (95% CI: 84.8–96.6%), 76.9% (95% CI: 55.9–90.2%), and 100% (95% CI: 93.9–100%), respectively (Table 2). On the other hand, the sensitivity, specificity, PPV, and NPV of ELISA for identifying the *H. pylori* infection group against the *H. pylori* non-infection groups were 88.2% (95% CI: 65.7–97.9%), 87.5% (95% CI: 77.2–93.5%), 65.2% (95% CI: 42.8–82.8%) and 96.6% (95% CI: 87–99.4%), respectively (Table 3).

To validate the test methods, ELISA for identifying *H. suis* infection was performed at another institute using the same protocol and serum from a separate container. The cut-off value obtained was reproducible at

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Table 1. Examinee background characteristics

	H. suis infection (n = 20)	*H. suis* non-infection (n = 81)		
		H. pylori infection (n = 17)	Post-eradication of *H. pylori* infection (n = 20)	Non-infection (n = 44)
Year of age				
Average (interquartile range)	48.5 (44.5–53.8)	49.0 (40.0–55.0)	52.0 (40.0–64.5)	53.5 (46.0–66.5)
Sex (%)				
man	17 (85.0)	10 (58.8)	9 (45.0)	20 (45.0)
woman	3 (15.0)	7 (41.2)	11 (55.0)	24 (55.0)
Disease (%)				
gastritis	15 (75.0)	10 (58.8)	14 (70.0)	13 (29.5)
gastric MALT lymphoma	3 (15.0)	1 (5.9)	1 (5.0)	18 (41.0)
duodenal MALT lymphoma	0 (0.0)	0 (0.0)	1 (5.0)	0 (0.0)
peptic ulcer	1 (5.0)	5 (29.4)	4 (20.0)	10 (22.7)
autoimmune (metaplastic atrophic) gastritis	1 (5.0)	0 (0.0)	0 (0.0)	3 (6.8)
EGC	0 (0.0)	1 (5.9)	0 (0.0)	0 (0.0)

1.0. Correlation analysis of the data between the two institutes yielded a value of Pearson's correlation coefficient (R) of 0.99 (95% CI: 0.97 to 0.99; p< 0.0001). Furthermore, the ELISA was always validated with 1800-fold diluted serum in addition to 3600-fold diluted serum, shown in Figure S2 (ELISA for identification of *H. suis* and *H. pylori* infection using 1800-fold diluted sera). Cohen's kappa for the dichotomous results of the positive and negative assessments was 1.0 (complete agreement) between the two ELISAs.

Decline of anti-*H. suis* antibody titers after eradication

Among 20 individuals belonging to the *H. suis* infection group, six individuals (nos. 94058, 94059, 04080, 04252, 04262, and 14005) suffering from gastritis were administered the eradication therapy as described in the Methods section. After the eradication therapies of *H. suis*, the upper gastrointestinal (GI) endoscopic pathology and PCR for the detection of *H. suis* were performed, and successful eradication of *H. suis* infection was judged in all six of these patients. The anti-*H. suis* antibody titers decreased over time after eradication. The estimated times required for the anti-*H. suis* antibody titers to decline to 1.0 (the cut-off value for the absorbance at 450 nm) in the 6 specimens were as follows: 15.1 months (no. 94058), 12.3 months (no. 94059), 9.1 months (no. 04080), 1.6 months (no. 04252), 3.1 months (no. 04262), and 2.8 months (no. 14005). Then, the six individuals were divided into the following two groups: a group of individuals exhibiting a slow decline of anti-*H. suis* antibody titers after eradication (nos. 9405894059, and 04080) and a group exhibiting a fast decline of anti-*H. suis* antibody titers after eradication (nos. 04252, 04262, and 14005). The time required for the anti-*H. suis* antibody titers to decline until the cut-off value was re-calculated at 13.6 and 2.6 months after eradication in the slow (Figure 2A) and first (Figure 2B) decline groups, respectively. The difference in eradication efficacy between the slow and fast decline groups seems to be attributed to the difference in antibody titers before eradication (2.4 ± 0.2 and 2.0 ± 0.1 in the slow and fast decline groups, respectively, p< 0.05).

DISCUSSION

The infection source of *H. suis*

Twenty out of 101 examinees were infected with *H. suis*, but no other NHPH were identified by the culture and PCR (negative and positive judgments in PCR targeting the *hsvA* and NHPH 16S rRNA genes, respectively). NHPH other than *H. suis*, which colonize the stomachs of cats and dogs, are thought to have a lower risk of infection in the human stomach. On the other hand, no *H. suis* infection has yet been identified in the stomachs of cats and dogs.[5–7,26,27] Although *H. suis* colonizes the hog stomach and is the source of zoonotic infection in a high percentage of human cases of *H. suis* infection,[28–30] the infection rate among hog farmers is not higher than that of individuals with no contact with animals.[31,32] It has been hypothesized that *H. suis* infects humans via the consumption of contaminated pork.[33,34] However, there is no clear evidence regarding the *H. suis* transmission route. In the present study, sampling was not random, and the population was subject to selection bias. Nevertheless, mixed infections with *H. pylori* and *H. suis* were
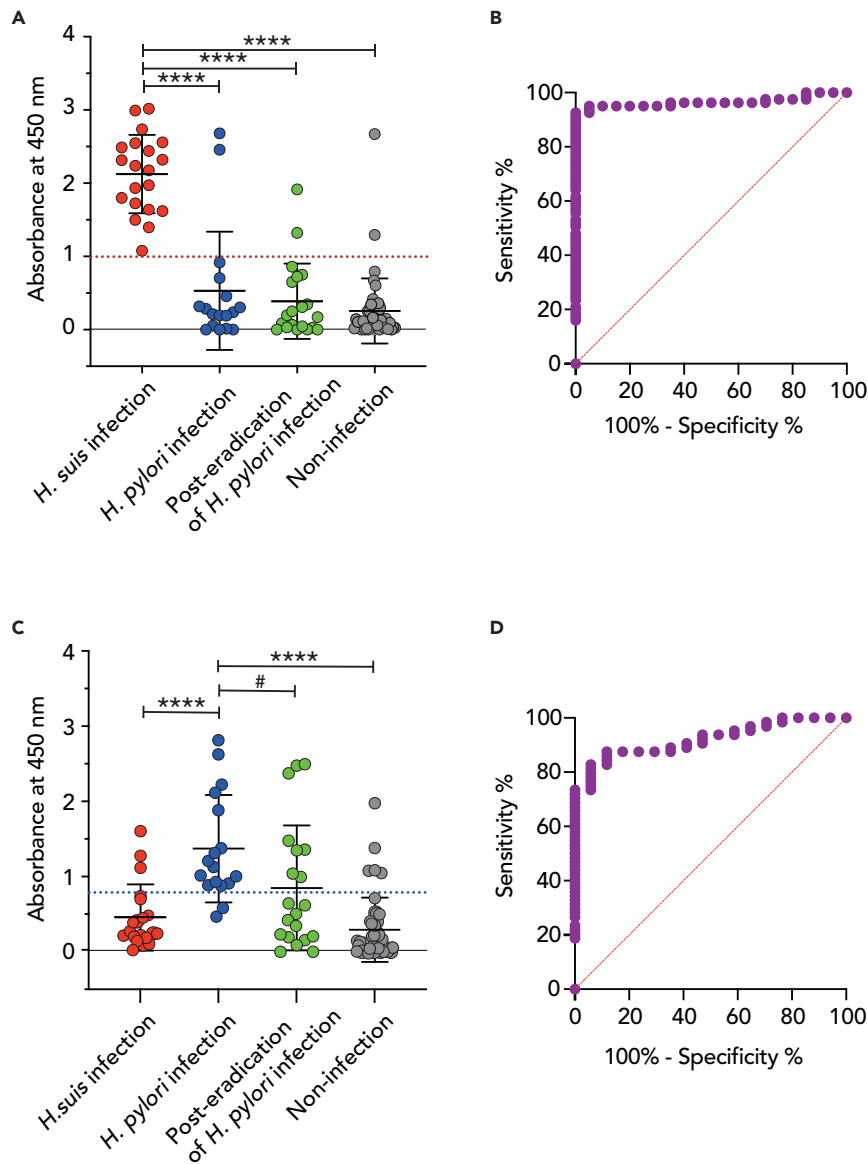
Figure 1. ELISA for identification of *H. suis* and *H. pylori* infection

Serum specimens (n = 101) were divided into four groups: an *H. suis*-infection group (n = 20), an *H. pylori*-infection group (n = 17), a group with eradication of *H. pylori* infection (n = 20), and a group with neither *H. suis* nor *H. pylori* infection (n = 44).

(A) ELISA for detecting *H. suis* infection. ***p< 0.0001, the *H. suis* infection group vs. the *H. pylori* infection, the post-eradication of *H. pylori* infection, or the non-infection group. The red dotted line indicates the cut-off value (1.0). The bar represents the mean with SD.

(B) ROC curve constructed from the *H. suis*-infection group vs. the *H. suis* non-infection groups, which consisted of the *H. pylori* infection, the post-eradication of *H. pylori* infection, and the non-infection groups. The red slanted line is the reference line. The area under the ROC curve (AUC): 0.96 ± 0.02; 95% CI: 0.93 to 1.00; p< 0.0001.

(C) ELISA for detecting *H. pylori* infection. ****p< 0.0001, the *H. pylori* infection group vs. the *H. suis* infection or non-infection group. #p> 0.05, the *H. pylori* infection group vs. the post-eradication of *H. pylori* infection group. The blue dotted line indicates the cut-off value (0.8). The bar represents the mean with SD.

(D) ROC curve constructed from the *H. pylori* infection group vs. the *H. pylori* non-infection groups, including the *H. suis* infection and the non-infection groups. The red slanted line is the reference line. AUC: 0.92 ± 0.03; 95% CI: 0.86 to 0.98; p< 0.0001.

not observed. Most patients infected with *H. suis* were men (Table 1). Tsukadaira et al. also noted that 25 out of 26 patients infected with *H. suis* were men.[13] However, this study was limited to cases with endoscopic gastritis, so it cannot provide information on infection rates, gender, or other characteristics. To

Table 2. ELISA to identify *H. suis* infection

		Positive	Negative	Total
Absorbance	≥1.0	20	6	26
at 450 nm	<1.0	0	75	75
	Total	20	81	101

Positive includes the *H. suis* infection group (n = 20). Negative includes the *H. suis* non-infection groups (n = 81). All specimens (n = 101) were classified into two groups: a group with an *H. suis*-infection value greater than or equal to the cut-off value (n = 26) and a group with an *H. suis*-infection value smaller than the cut-off value (n = 75). Cut-off value, 1.0; sensitivity, $\frac{20}{20+0}$ = 100%; specificity, $\frac{75}{6+75} \approx 92.6\%$; PPV, $\frac{20}{20+6} \approx 76.9\%$; NPV, $\frac{75}{0+75} \approx 100\%$.

establish a more accurate correlation between infection rates, gender, age, symptoms, endoscopic findings, and disease, large-scale epidemiological studies that cover the general population, including those who undergo serological diagnosis, are necessary.

Comparison of the serological assays for the detection of *H. suis* and *H. pylori* infection

Marini et al. have tried detecting anti-*Helicobacter* antibodies of macaques by ELISA using the *H. pylori* and *H. suis* outer membrane protein preparations as antigens.[35] In the identification of *H. suis* infection, the sensitivity and specificity were 70.0% (95% CI: 34.8%–93.3%) and 75.0% (95% CI: 19.4%–99.4%), respectively.[35] Meanwhile, in the identification of *H. pylori* infection, the sensitivity and specificity were 66.7% (95% CI: 9.2%–99.2%) and 63.6% (95% CI: 30.8%–89.1%), respectively.[35] Therefore, the ELISA data using the whole bacterial cell lysates as antigens and the 3600-fold diluted sera in this study (Figure 1 and Tables 2 and 3) showed more accuracy than the ELISA data using the outer membrane protein preparations as antigens. When synthetic peptides constructed from each *H. suis* or *H. pylori*-specific autotransporter protein sequence were used as antigens, ELISA did not show higher accuracy compared to whole-cell lysates as antigens.
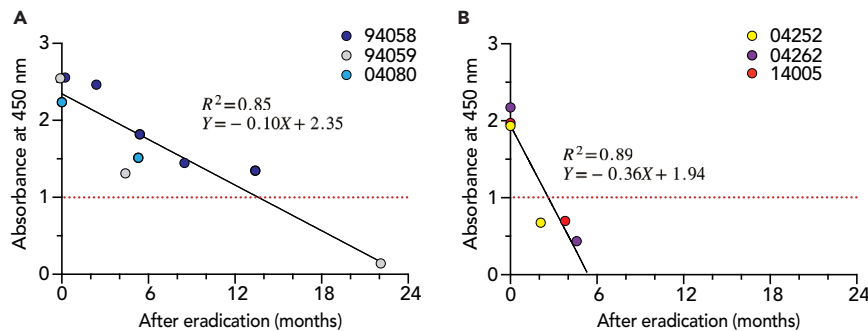
The discrepancy in the judgments made between the serological assay and the PCR or culture

We conducted conventional Giemsa staining and immunohistochemistry (IHC) to identify *H. suis* and *H. pylori* in gastric biopsy specimens, shown in Table S1 (Individual data of 101 examinees). To compare the discriminatory ability between the serological assay and PCR or culture using gastric biopsy specimens, we attempted to detect *H. suis* and *H. pylori* by Giemsa staining and IHC analysis of the biopsy specimens. Among 17 (*H. pylori* infection group) and 20 (post-eradication of *H. pylori* infection group) individuals, four serum specimens (nos. 04232 and 04133 of *H. pylori* infection group, and nos. 04150 and 04162 of post-eradication of *H. pylori* infection group) were *H. suis*-positive by ELISA (Table S1 [Individual data of 101 examinees]). Because the microscopy did not detect *H. suis* in these specimens, we suggest that the whole-cell lysate of *H. suis* SNTW101c reacted with anti-*H. pylori* serum antibodies. For this reason, we advocate simultaneous serological testing for *H. suis* and *H. pylori* infections. Among 44 individuals in the non-infection group (PCR and culture negatives), two serum specimens (nos. 04293 and 04018 from patients with gastric MALT lymphoma and peptic ulcer, respectively) were *H. suis*-positive by ELISA, shown in Table S1 (Individual data of 101 examinees). Histology could not explain the discrepancies between serology and PCR or culture. However, we presume that the individuals had prior infections with *H. suis* (no. 04293) and *H. pylori* (no. 04018), because the serum specimens tested strongly positive for *H. suis* infection (no. 04293) and *H. pylori* infection (no. 04018) by ELISA, shown in Table S1 (Individual data of 101

Table 3. ELISA to identify *H. pylori* infection

		Positive	Negative	Total
Absorbance	≥0.8	15	8	23
at 450 nm	<0.8	2	56	58
	Total	17	64	81

Positive includes the *H. pylori* infection group (n = 17). Negative includes the *H. suis* infection group and the non-infection group (n = 64). All specimens (n = 81) were classified into two groups: a group with an *H. pylori*-infection value greater than or equal to the cut-off value (n = 23) and a group with an *H. pylori*-infection value smaller than the cut-off value (n = 58). Cut-off value, 0.8; sensitivity, $\frac{15}{15+2} \approx 88.2\%$; specificity, $\frac{56}{8+56} = 87.5\%$; PPV, $\frac{15}{15+8} \approx 65.2\%$; NPV, $\frac{56}{2+56} \approx 96.6\%$.

A

Absorbance at 450 nm

- 94058
- 94059
- 04080

$R^2 = 0.85$
$Y = -0.10X + 2.35$

After eradication (months)

B

Absorbance at 450 nm

- 04252
- 04262
- 14005

$R^2 = 0.89$
$Y = -0.36X + 1.94$

After eradication (months)

Figure 2. Measurement of anti-*H. suis* antibody titers after the eradication of *H. suis* infection

Six individuals underwent *H. suis*-eradication treatment, and then were monitored for serum anti-*H. suis* antibody titers by ELISA. Results for three individuals (nos. 94058, 94059, and 04080) (A) were plotted separately from the other three (nos. 04252, 04262, and 14005)

(B). The red dotted line indicates the cut-off value.

examinees). In addition, in the non-infection group, it is assumed that the other four individuals had previously been infected with *H. pylori* and that the *H. pylori* were eradicated spontaneously or accidentally because the serum specimens (nos. 04167, 14327, 04157, and 14202) tested positive for *H. pylori* by ELISA, shown in Table S1 (Individual data of 101 examinees).

Evaluation of the *H. suis* eradication therapies by serological assay

When *H. suis* infection becomes apparent, eradication of *H. suis* is recommended even if the examinees are not suffering from severe illnesses such as gastric MALT lymphoma, nodular gastritis, or intractable peptic ulcer. The regimen for *H. pylori* eradication has been used for *H. suis* eradication. In most cases, patients are treated with triple-agent eradication therapy consisting of an inhibitor of gastric acid secretion such as a PPI or a potassium-competitive acid blocker (P-CAB) along with two antibiotics such as amoxicillin and clarithromycin or amoxicillin and metronidazole for 7 to 14 days.[14,15,18,36–38] In the current study, the time-dependent decline of antibody titers after eradication confirmed the success of the eradication therapies. The serological assay of *H. suis* infection would be beneficial as a follow-up examination to confirm eradication (Figure 2).

Urgent requirements

Infection with *H. pylori* in infancy might confer years of protection against *H. suis* infection. In the present study, none of the *H. suis* infected examinees had *H. pylori* infection or prior *H. pylori* infection that had been eradicated. The *H. suis* infection rate might have risen with the decline of the *H. pylori* infection rate. Because there is currently no established method for the clinical diagnosis of *H. suis* infection, accurate diagnosis of *H. suis* infection is crucial to avoid wasting precious time on the wrong course of treatment.

Limitations of the study

The PPV of ELISA for discriminating the *H. suis* infection group from the *H. suis* non-infection groups was 76.9% (95% CI: 55.9–90.2%) (Table 2). The PPV of ELISA for discriminating the *H. pylori* infection group from the *H. pylori* non-infection groups was 65.2% (95% CI: 42.8–82.8%) (Table 3). Concerning diagnostic accuracy, the low values of PPV are the limitation of serological assays. The main reason is that previously eradicated infections may test positive by ELISA, even in cases that tested negative by PCR. 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 efficacy. This is the current limitation of the study.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY

SUPPLEMENTAL INFORMATION

ACKNOWLEDGMENTS

AUTHOR CONTRIBUTIONS

Conceptualization, H.M., E.R., and K.S.; Methodology – Cultures and PCR, E.R. and S.A.; Methodology – Genomic Analysis, M.S.; Methodology – ELISA, H.M.; Methodology – Microscopy, H.O.; Medical Practice – Eradication, K.M. and K.T.; Medical Practice – Specimen Collection, K.M., K.T., H.S., M.S., T.U., H.T., S.N., A.T., M.S., S.T., and T.S.; Writing, H.M., E.R., and K.M. All authors verified the underlying data and approved the final version of the manuscript for publication.

DECLARATION OF INTERESTS

M.H., R.E., and S.M. are inventors of the technology described in this manuscript and are listed as applicants in the international patent application under the PCT (PCT/JP2023/002328, PCT/JP2023/002329, and PCT/JP2023/002330). The authors declare that they have no other competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
HRP-conjugated goat anti-human IgA+IgG+IgM (H+L) antibody	Jackson	Code#109-035-064; RRID: AB_2337583
Rabbit monoclonal anti-*H. pylori* antibody (clone EP279)	Cell Marque	Cat#AC-0247; RRID: AB_2936230
Rabbit polyclonal anti-*H. suis* antibody	This paper	N/A
HRP-conjugated goat anti-rabbit antibody	Agilent Technologies	Code#P0448; RRID: AB_2617138
Bacterial and virus strains		
H. suis SNTW101c	Rimbara et al.[9]	N/A
H. pylori TN2GF4	Matsui et al.[39]	N/A
H. suis 13 strains isolated from gastric biopsy specimens of subjects	This paper	See Table S1
H. pylori 17 strains isolated from gastric biopsy specimens of subjects	This paper	See Table S1
Biological samples		
Gastric biopsy specimens from subjects	This paper	N/A
Serum specimens from subjects	This paper	N/A
Chemicals, peptides, and recombinant proteins		
H. pylori eradication triple-combination drug VONOSAP Pack 400 (vonoprazan 40 mg, amoxicillin 1500 mg, and clarithromycin 400 mg)	Takeda Pharmaceutical	YJ Code# 6199104X1023
H. pylori eradication triple-combination drug VONOSAP Pack 800 (vonoprazan 40 mg, amoxicillin 1500 mg, and clarithromycin 800 mg)	Takeda Pharmaceutical	YJ Code# 6199104X2020
Critical commercial assays		
DNeasy Blood & Tissue Kits	Qiagen	Cat#69504/69556
Nextera XT DNA Library Prep Kit	Illumina	Illumina advantage product Cat#TG-131-1096
ELISA enzyme substrate KPL SureBlue TMB Microwell Peroxidase Substrate (1-Component)	Sera Care Life Sciences	Material#5120-0075
Nunc-Immuno 96-well microtiter plate	Thermo Fisher Scientific	Cat#439454
NHPH agar plate	This paper	N/A
Brucella broth	BD	BD BBL™ -211088
Skirrow	Oxoid	Cat#OXSR0069E
Vitox	Oxoid	Cat#R663090
Dent	Oxoid	Cat#OXSR0147E
Helicobacter-selective agar plate	Nissui Pharmaceutical	Cat#51035
Histofine antigen-retrieval solution	Nichirei Biosciences	Code#414251F/414252F
Master mix	IDT	Code#1055770

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Whole-genome sequences of *H. suis* 13 strains (GenBank/ENA/DDBJ accession number)	This paper	See Table S1
The 23S-rRNA gene sequences of *H. pylori* 16 strains (GenBank/ENA/DDBJ accession number)	This paper	See Table S1
The 23S-rRNA gene sequences of *Helicobacter* 21 type strains (GenBank/ENA/DDBJ accession number)	This paper	See Table S2
Sequence typing (ST) assignment	This paper	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.[9]	N/A
H. suis hsvA targeting probe-based real-time PCR primer sequence: NHP194003_11930_reverse: GATGGGCGCTTCTGGTTTA	Rimbara et al.[9]	N/A
H. suis hsvA targeting probe-based real-time PCR probe sequence: NHP194003_11930_probe: 56-FAM/TGTACACAC/ZEN/CAAACAGATGAGCCGT/3IABkFQ	IDT Rimbara et al.[9]	N/A
NHPH 16S rRNA gene targeting probe-based real-time PCR primer sequence: NHPH_16S_F: CAAGTCGAACGATGAAGCCTA	This paper	N/A
NHPH 16S rRNA gene targeting probe-based real-time PCR primer sequence: NHPH_16S_R: ATTTGGTATTAATCACCATTTCTAGT	This paper	N/A
NHPH 16S rRNA gene targeting probe-based real-time PCR probe sequence: NHPH_16S_probe: 56-FAM/TTACTCACC/ZEN/CGTGCGCCACTAATC/3IABkFQ	IDT This paper	N/A
H. pylori 651 bp DNA fragment of the 23S rRNA gene targeting colony PCR primer sequence: F3: CCGTAGCGAAAGCGAGTCT	This paper	N/A
H. pylori 651 bp DNA fragment of the 23S rRNA gene targeting colony PCR primer sequence: R3: CCCGACTAACCCTACGATGA	This paper	N/A
Software and algorithms		
ClustalW ver. 2.1	Open source	http://www.clustal.org/clustal2/
Shovill v1.1.0.	Open source	https://github.com/tseemann/shovill
Roary version 3.13.0	Open source	https://github.com/sanger-pathogens/Roary
RAxML-NG v. 1.1	Open source	https://github.com/amkozlov/raxml-ng
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/

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
H. suis hsvA targeting probe-based real-time PCR protocol	Rimbara et al.[9]	N/A
NHPH 16S rRNA gene targeting probe-based real-time PCR protocol	This paper	N/A
H. pylori 651 bp DNA fragment of the 23S rRNA gene targeting colony PCR protocol	This paper	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hidenori Matsui (hmatsui@niid.go.jp).

Materials availability

The polyclonal anti-*H. suis* antibody and ELISA prototype generated by this study are available from the lead contact upon reasonable request. Bacterial strains isolated in this study are also available from the lead contact upon reasonable request.

Data and code availability

- The whole-genome data of *H. suis* strains have been deposited at GenBank/ENA/DDBJ and will be available from the date of publication.

- The 23S rRNA genome data of *H. pylori* strains have been deposited at GenBank/ENA/DDBJ and will be available from the date of publication.

- Accession numbers can be found in Table S1 (Individual data of 101 examinees) and Table S2 (Reference strains of gastric *Helicobacter* species).

- This paper does not report any original code.

- Any additional information about the analysis in this paper is available from the lead contact upon reasonable request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Ethics statement

The Research Ethics Committees (REC) of the National Institute of Infectious Diseases (NIID) and Kitasato University approved this study under registration numbers 1284 and 18100, respectively. The ethical review was also approved at the following eight medical institutions: Junpukai Health Maintenance Center, Kyorin University Hospital, Tokai University Hospital, Toyama University Hospital, Takeda Hospital, Aichi Medical University Hospital, Kakogawa Central Municipal Hospital, and Kanazawa Municipal Hospital. We also obtained written informed consent from all examinees, each of whom received an explanation of the study at one of the participating medical institutions.

Collection and transportation of clinical specimens

Serum and gastric biopsy specimens from examinees undergoing medical check-ups or suffering from any gastric disorder who underwent upper GI endoscopy at one of the eight above-named medical institutions were enrolled. However, the examinees with normal stomachs and those with a gastric disease under 20 years of age were excluded from this study. The specimens from participants having healthy stomachs were excluded from this study. All examinees were found to have *H. pylori* infection by means of two of the following five tests: the UBT, RUT, serum *H. pylori* antibody test, stool *H. pylori* antigen test, and histological test. *H. pylori*, *H. suis*, and other NHPH infections were finally confirmed by culture of gastric biopsy specimens. Gastric biopsy specimens were collected from the greater curvature of the gastric antrum, the greater curvature of the lower gastric body, and the lesser curvature of the gastric angle, and they were transported to the NIID using the NHPH biopsy transport media for cultivation, PCR, and microscopy.

Serum specimens were divided into three containers, transported to the NIID, and stored at -80°C until use. The specimen number assigned by the NIID and the clinical data cannot be used to identify medical institutions or examinees.

Agar plate culture for the detection of *Helicobacter* species in clinical specimens

The gastric biopsy specimen was homogenized with 300 μL of Brucella broth (BD, Franklin Lakes, NJ, USA) adjusted to pH 5.0 by hydrochloride. A 200 μL aliquot of the tissue homogenates was inoculated onto NHPH agar plates containing 1.5% (wt/vol) agar, Brucella broth, 20% (v) heat-inactivated bovine serum (FBS), *Campylobacter* selective supplement (Skirrow; Oxoid, Basingstoke, UK), Vitox supplement (Oxoid), and hydrochloric acid to adjust the pH to 5.0, and incubated for more than seven days in a humidified gas mixture (5% O_2, 12% CO_2, and 83% N_2) at 37°C. The grown colonies of primary culture were inoculated onto NHPH agar plates and enriched by modified biphasic culture for 120 h with shaking in a humidified gas mixture at 37°C. The whole-genome sequencing of broth-grown bacteria was carried out as described below. Although both *H. pylori* and NHPH were grown on the NHPH agar plate as primary cultures, the *H. pylori* colonies tended to grow faster and to be larger than the small, slow-growing colonies formed by NHPH.[9] Therefore, the colonies formed by *H. pylori* were inoculated onto a Brucella agar plate containing 5% (vol/vol) horse blood for 72 h in a humidified gas mixture at 37°C. NHPH could not grow on this plate.

PCR for the detection of *Helicobacter* species in clinical specimens

To identify NHPH infections, the DNA was prepared from the remaining 100 μL of homogenates of gastric biopsy specimens using DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany). Then, the DNA was used as the template for probe-based real-time PCR targeting of the *H. suis*-specific *vacA*-type autotransporter protein gene (*hsvA*) and the NHPH-common region of the 16S rRNA gene. The sequences of the two sets of primers and probes were 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. The diagnosis of *H. suis* infection was made when both positive results occurred in two types of PCR targeting the *hsvA* and NHPH 16S rRNA genes.[9] The diagnosis of NHPH other than *H. suis* was made when negative and positive results occurred in PCR targeting the *H. suis*-specific *hsvA* and NHPH-common 16S rRNA genes, respectively. We included the culture as a diagnostic criterion to confirm *H. pylori* infection, whereas we excluded the culture as a diagnostic criterion to confirm *H. suis* infection. To identify *H. pylori* infection, the 651 bp DNA fragment of the 23S rRNA gene was amplified by the colony PCR using the following set of primers: F3 (5′- CCGTAGCGAAAGCGAGTCT-3′) and R3 (5′-CCCGACTAACCCTACGATGA-3′). The 23S rRNA gene sequences of *Helicobacter pylori* strains were deposited at GenBank/EMBL/DDBJ as shown in Table S1. The nucleotide sequences of PCR products were aligned by ClustalW ver. 2.1 (http://www.clustal.org/clustal2/). The 23S rRNA gene sequences of the reference *Helicobacter* strains were taken from GenBank/EMBL/DDBJ.

METHOD DETAILS

Broth culture of *H. pylori* and *H. suis* for the preparation of ELISA antigens

The colonies of *H. pylori* TN2GF4 grown on a *Helicobacter*-selective agar plate (Nissui Pharmaceutical, Tokyo) were inoculated into a Brucella broth containing 10% (vol/vol) FBS supplemented with 1% (vol/vol) Vitox supplement and *Helicobacter pylori*-selective supplement (Dent; Oxoid) for 72 h by shaking in a humidified gas mixture (5% O_2, 10% CO_2, and 85% N_2) at 37°C.[39] The frozen stock of *H. suis* SNTW101c was inoculated onto NHPH agar plates and enriched by modified biphasic culture for 120 h with shaking in a humidified gas mixture at 37°C as described above.

ELISA

Bacterial cells collected by centrifugation were washed twice with phosphate-buffered saline, pH 7.4 (PBS), suspended in distilled water and disrupted by sonication with a Bioruptor II (BMBio, Tokyo) to prepare whole-cell lysates. For the measurement of serum anti-*H. suis* or anti-*H. pylori* antibody titer, Nunc-Immuno 96-well microtiter plates (No. 439454; Thermo Fisher Scientific, Waltham, MA, USA) containing 100 μL of whole-cell lysates of *H. suis* SNTW101c or *H. pylori* TN2GF4 (4 μg/mL in 0.1 M carbonate/bicarbonate buffer, pH 9.4) were incubated overnight at 4°C. After washing three times with PBS containing

0.05% (vol/vol) Tween 20 (PBS-T), the wells were saturated with 200 μL of blocking buffer (PBS containing 1% BSA). The plates were incubated for 1 h at 37°C while shaking at 500 rpm. After washing three times with PBS-T, the wells were filled with 50 μL of serum samples diluted at 1:1,800 (Figure S2 [ELISA for identification of *H. suis* and *H. pylori* infection using 1800-fold diluted sera]) and 1:3,600 (Figure 1) with blocking buffer. The plates were incubated for 1 h at 37°C while shaking at 500 rpm. After washing three times with PBS-T, the wells were filled with 50 μL of horseradish peroxidase (HRP)-conjugated goat anti-human IgA+IgG+IgM (H+L) secondary antibody (Jackson, Bar Harbor, ME, USA) diluted at 1:10,000 with blocking buffer. The plates were incubated for 1 h at 37°C while shaking at 500 rpm. After washing three times with PBS-T, the wells were filled with 50 μL of KPL SureBlue TMB Microwell Peroxidase Substrate (1-Component) (Sera Care Life Sciences, Milford, MA, USA). After 5 min, the color reaction was stopped by adding 50 μL of 1 N hydrochloric acid into the wells. The absorbance at 450 nm (reference wavelength, 630 nm) was measured on a ChroMate-6 microplate reader (Microtec, Chiba, Japan).

Eradication of *H. suis*

For the treatment of gastric diseases with *H. suis* infection, each of the following three agents was administered twice daily for a week for eradication of *H. suis*: vonoprazan (P-CAB: 20 mg), amoxicillin (750 mg), and clarithromycin (200 mg for nos. 04080, 04252, 04262, and 14005 or 400 mg for nos. 94058 and 94059). At least two months after the eradication treatment, an upper GI endoscopy was performed to confirm gastric disease remission and the disinfection of *H. suis* by PCR.

QUANTIFICATION AND STATISTICAL ANALYSIS

Differences in the gender ratio among four groups were compared by Fisher's exact test. The anti-*H. suis* and anti-*H. pylori* antibody values were compared among four groups using one-way ANOVA, followed by Tukey's post hoc test. Cut-off values, AUCs, sensitivities, and specificities based on the ROC curves were calculated using GraphPad Prism software version 9.5.0 for Mac (GraphPad Software, San Diego, CA, USA). As the diagnostic accuracy by ELIS, the PPV and NPV with 95% CI were calculated using Clinical Calculator 1 (http://vassarstats.net/clin1.html). Pearson's correlation coefficient (R) was determined using GraphPad Prism software. Cohen's kappa was calculated using the following formula. $K = \frac{P_0 - P_e}{1 - P_e}$. Po: observed agreement between the rates. Pe: expected agreement based on chance. Regression lines and R-squares were calculated using GraphPad Prism software. Differences were considered significant when the *P*-value (two-tailed) was less than 0.05. **** and # indicate $P<0.0001$ and $P>0.05$, respectively.