Changes in Seroprevalence of \textit{Helicobacter pylori} Infection over 20 Years in Jinju, Korea, from Newborns to the Elderly

\textbf{ABSTRACT}

\textbf{Background:} The objective of this study was to examine changes in the prevalence of cytotoxic-associated gene A (CagA) positive \textit{Helicobacter pylori} infection in Jinju, Korea, over the last 20 years.

\textbf{Methods:} Three cross-sectional analyses were conducted concurrently. A total of 1,305 serum samples were collected from 1994–1995, 2004–2005, and 2014–2015, respectively. The presence of immunoglobulin (Ig) G, IgA, and IgM antibodies against \textit{H. pylori} CagA protein was examined by western blotting.

\textbf{Results:} Overall, seropositivity for anti-CagA IgG antibody was significantly decreased from 63.2\% to 42.5\% over the last 20 years ($P<0.001$). Anti-CagA IgG seropositivities in children and young adults aged 10–29 years decreased from 1994 (60.0\%–85.0\%) to 2015 (12.5\%–28.9\%). The age when plateau of increasing IgG seropositivity was reached in each study period shifted from the 15–19 year-old group in 1994–1995 (85.0\%) to the 40–49 year-old group in 2014–2015 (82.5\%). Overall seropositive rates of anti-CagA IgA and IgM antibodies did not change significantly either over the last 20 years.

\textbf{Conclusion:} \textit{H. pylori} infection rate in children and young adults declined over 20 years in Jinju, probably due to improved sanitation, housing, or economy.

\textbf{Keywords:} Prevalence; \textit{Helicobacter pylori}; CagA protein; Western Blotting

\textbf{INTRODUCTION}

\textit{Helicobacter pylori} is a risk factor of peptic ulcer, atrophic gastritis, and gastric carcinoma.\textsuperscript{1} Environmental, host-related, and virulent factors associated with the bacterium, including cytotoxin-associated gene A (CagA), vacuolating cytotoxin A (VacA), and outer inflammatory protein (OipA) are involved in the development of \textit{H. pylori}-related diseases.\textsuperscript{2} Of these factors,
CagA protein is associated with a severe clinical outcome. Most strains of \( H. pylori \) isolated in East Asia, including Korea, have been confirmed CagA-positive.\(^3,5\)

It is thought that approximately half of the world’s population harbors \( H. pylori \)\(^6,11,15\) and \( H. pylori \)-related diseases represent a significant medical burden.\(^7,8\) Although the prevalence of \( H. pylori \) infection in developed countries has declined over recent decades, infection rate in the rest of the world remains high.\(^6,5,10\) In Korea, \( H. pylori \) infection of late adolescents and adults has decreased due to rapid socio-economic growth and improved housing.\(^11,13\) However, the previous studies had been designed to diagnose \( H. pylori \) infection using enzyme linked immunosorbent assay (ELISA), some of which used crude antigens of \( H. pylori \) isolated outside Korea and had enrolled populations aged 16 years and older due to the limitations of its use in diagnosing \( H. pylori \) infection in children.\(^11,13\) \( H. pylori \) infection can take hold during infancy or early childhood and colonization generally persists for life if left untreated.\(^16,18\)

\( H. pylori \) induces systemic and mucosal immune responses in most infected patients. Serologic tests can detect \( H. pylori \) infection, and serum immunoglobulin (Ig) G, A, and M antibodies may indicate whether infection is acute or chronic.\(^19,20\) \( H. pylori \) CagA antigen might be one of the major antigens that produce the strongest antibody responses and might be related to high levels of anti-\( H. pylori \) antibodies in infected humans.\(^21\) The patients remain positive for anti-CagA antibodies longer than other anti-\( H. pylori \) antibodies even after eradication of \( H. pylori \).\(^3,22\)

Jinju is a city of around 340,000 inhabitants that lies in the southern part of Korea and is surrounded by rural areas. Compared with other cities in Korea, the inflow and outflow of citizens are relatively low (www.index.go.kr). The relatively low migration rate (0.1% during 2006–2016) makes Jinju suitable for evaluating the lifetime trend of \( H. pylori \) infection, together with birth cohort effects. Few reports have examined the prevalence of \( H. pylori \) infection in the general population, including infants and children, along with lifetime trends.\(^23\) Thus the aim of the present study was to investigate changes in the seroprevalence of CagA positive \( H. pylori \) infection over the last 20 years in the general population of Jinju, ranging from neonates to the elderly. In addition, the lifetime trend of \( H. pylori \) infection was estimated by western blot analysis of IgG, IgA, and IgM antibodies against \( H. pylori \) CagA.

## METHODS

### Study population

Three retrospective cross-sectional analyses using collected serum samples were conducted concurrently. These cross-sectional analyses covered 1994–1995, 2004–2005, and 2014–2015, respectively, spanning a total of 20 years. A total of 1,305 serum samples matched with age, sex and collection period were obtained from the Gyeongsang National University Hospital (GNUH) Biobank, a member of the Korea Biobank Network and analyzed. All of the serum samples were preserved in the deep freezer before analysis. To evaluate the age when acute or initial \( H. pylori \) infection occurs, and to differentiate serologic positivity due to infection from that due to transplacental transmission of anti-\( H. pylori \) CagA IgG antibodies during infancy and early childhood, samples were grouped according to age as follows: neonate, 1–6 months, 7–12 months, 13–24 months, 2–4 years, and 5–9 years (infants and children), and 5 to 10-year intervals thereafter (Table 1).
Antigen preparation and western blot analysis

Whole cell extracts of CagA positive-\textit{H. pylori} strain 51 (obtained from the Korean type Culture Collection; HpKTCC; http://hpktcc.kncrc.or.kr, NCBI Taxonomy ID: 290847) were prepared as described previously.\textsuperscript{24} \textit{H. pylori} strain 51 was isolated from a patient with duodenal ulcer at GNUH in 1988 and has been studied extensively since then.\textsuperscript{14,15,18,22,24-27} Briefly, \textit{H. pylori} strain 51 was cultured for 18 hours under the environment of 37°C, 5%–10% CO\textsubscript{2} and 100% humidity on Mueller-Hinton agar supplemented with 10% bovine serum. Bacterial cells from each plate were harvested and pelleted by centrifugation at 4,000 × g for 15 minutes. These cells were then suspended in sterile phosphate buffer, broken by ultrasonic treatment using an Ultrasonic W380 (Sonics & Materials Inc., Danbury, CT, USA), and stored at −70°C. The sonicated \textit{H. pylori} whole cell lysate was then used as an antigen. The presence of anti-CagA IgG, IgA, and IgM antibodies in the serum was then examined by western blot analysis. Briefly, cell lysates were run on 10%–20% sodium dodecyl sulfate-polyacrylamide gels overlaid with a 3% stacking gel, as described by Laemmli.\textsuperscript{28} These gels were loaded with samples containing 100 µg of antigen along with molecular mass markers (Bio-Rad Laboratories Inc., Philadelphia, PA, USA). Proteins were separated under a constant current of 15 mA for 60 minutes until the bromophenol blue dye migrated out of the gel. Proteins were then transferred onto pre-wetted nitrocellulose (NC) membranes (0.2 micron, Bio-Rad Laboratories Inc.). These membranes were then cut into strips. Strips were incubated with 1:20 diluted serum for 30 minutes at 37°C, rinsed three times with Tris buffered saline containing Tween-20 (TBST; 50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.5), and incubated at 37°C for 30 minutes with alkaline phosphatase-conjugated goat anti-human IgG, IgA, or IgM antibodies (Bethyl Laboratories Inc., Montgomery, TX, USA). Three different NC membrane strips incubated with one serum sample were used for incubation with goat anti-human IgG, IgA and IgM antibodies, respectively. After washing with TBST buffer, these strips were incubated with 5-bromo-4-chloro-3-indolyl phosphate as a substrate and nitroblue tetrazolium as a chromogenic indicator. Reactions were stopped after 15 minutes by rinsing these strips several times with buffer (20 mM Tris-HCl, 50 mM EDTA, pH 8.0). These strips were then dried before mounting. Samples showing a band at 116–120 kDa were considered positive for \textit{H. pylori} CagA IgG, IgA, or IgM antibody (Fig. 1).\textsuperscript{14,15} Results were analyzed by two investigators who were blinded to information about the serum samples including age and sex of subjects, and collection time. The authors interpreted results of western blotting based on Fig. 1 to avoid inconsistency.
Statistical analysis

The χ² or Fisher’s exact test was used to compare differences in seropositivities for anti-CagA IgG, IgA, and IgM antibodies according to age, sex, and study period. All statistical analyses were performed using SPSS ver 25.0 (IBM, Armonk, NY, USA). The level of significance was set at 0.05. GraphPad Prism 8 (Graph-Pad Software, San Diego, CA, USA) was used for graphics.

Ethics statement

The study protocol was approved by the Institutional Review Board at Gyeongsang National University Hospital (GNUH 2015-06-010). Informed consent was waived by the board.

RESULTS

A total of 1,305 serum samples were tested, of which 656 were from males and 713 were from subjects younger than 20 years old. Of these samples, 439, 427, and 439 were obtained from 1994 to 1995, 2004 to 2005, and 2014 to 2015 (Table 1).

Anti- H. pylori CagA IgG antibody seropositivity according to age and study period

The overall seropositive rate, except for the anti-CagA IgG positive rate in infants aged 0–12 months, was 63.2% from 1994 to 1995, 54.9% from 2004 to 2005, and 42.5% from 2014 to 2015 (P < 0.001), respectively. There was no difference in overall anti-CagA IgG positive rate between males and females (male, 54.9%; female, 50.4%; P = 0.108).

Age-specific seropositive rates of anti-CagA IgG antibodies in each study period are plotted in Fig. 2. Anti-CagA IgG antibody seropositivities according to age in the three cross-sectional periods were decreased from newborn to 7–12 month-old group or 2–4 year-old group and then increased with age (Fig. 2). Seropositive rate of neonates was 73.7% in 1994–1995, 75.0% in 2004–2005, and 63.2% in 2014–2015 (P = 0.765). The lowest seropositive rate in each study period was 25.0% in those age 2–4 years in 1994–1995, 15.0% in those age 13–24 months in 2004–2005, and 0.0% in those age 7–12 months in 2014–2015. Significant reductions in age-specific anti-CagA IgG seropositivities, particularly in those aged between 7 and 12 months, 10 and 14 years, 15 and 19 years, and 20 and 29 years were presented over
The age when the seropositivity for anti-CagA IgG reached a plateau was delayed over the 20 years and occurred in the 15–19 year-old group in 1994–1995 (85.0%), the 20–29 year-old group in 2004–2005 (72.5%), and the 40–49 year-old group in 2014–2015 (82.5%), respectively. And then the highest rate in each study period occurred in the 15–19 year-old group in 1994–1995 (85.0%), and the 50–59 year-old group in 2004–2005 (92.5%) and 2014–2015 (89.5%), respectively. Seropositivities in early childhood (2–4 years old) during the three study periods decreased numerically from 1994 to 2015 (25% in 1994–1995, 17.5% in 2004–2005 and 13.9% in 2014–2015). However, the decrease was not statistically significant ($P = 0.484$) (Table 2). According to the birth cohort of the 2–4 year-old group in 1994–1995, positive rates were not significantly changed until individuals were in their 20s in 2014–2015 (25.0% in 1994–1995, 35.9% in 2004–2005 and 28.9% in 2014–2015, $P = 0.587$) (Table 2).

**Table 2.** Positive rate of anti-CagA IgG, IgA, and IgM according to age and year

| Age          | IgG, % | $P$ value | IgA, % | $P$ value | IgM, % | $P$ value |
|--------------|--------|-----------|--------|-----------|--------|-----------|
| 1994–1995    | 1994–2005 | 2004–2005 | 2014–2015 |
| Neonate      | 73.7  | 75.0 | 63.2  | 0.765 | 10.5 | 30.0 | 0.0 | 0.021 | 0.0 | 15.0 | 0.0 | 0.100 |
| 1–6 mon      | 55.0  | 55.0 | 25.0  | 0.112 | 0.0 | 5.0 | 0.0 | 1.000 | 0.0 | 5.0 | 0.0 | 1.000 |
| 7–12 mon     | 50.0  | 31.6 | 0.0 | $< 0.001$ | 0.0 | 5.3 | 0.0 | 0.317 | 0.0 | 0.0 | 0.0 | - |
| 13–24 mon    | 45.0  | 15.0 | 15.0  | 0.063 | 15.0 | 5.0 | 0.0 | 0.310 | 0.0 | 0.0 | 0.0 | - |
| 2–4 yr       | 25.0  | 17.5 | 13.9  | 0.484 | 2.5 | 2.5 | 5.6 | 0.686 | 0.0 | 0.0 | 0.0 | - |
| 5–9 yr       | 35.0  | 22.5 | 15.0  | 0.132 | 12.5 | 12.5 | 12.5 | 1.000 | 0.0 | 7.5 | 2.5 | 0.322 |
| 10–14 yr     | 60.0  | 35.9 | 12.5  | $< 0.001$ | 27.5 | 30.8 | 10.0 | 0.047 | 2.5 | 0.0 | 5.0 | 0.772 |
| 15–19 yr     | 85.0  | 52.5 | 17.5  | $< 0.001$ | 40.0 | 35.0 | 17.5 | 0.081 | 2.5 | 0.0 | 10.0 | 0.125 |
| 20–29 yr     | 80.0  | 72.5 | 28.9  | $< 0.001$ | 30.0 | 52.5 | 24.4 | 0.022 | 2.5 | 12.5 | 15.6 | 0.109 |
| 30–39 yr     | 85.0  | 77.5 | 62.5  | 0.070 | 67.5 | 50.0 | 55.0 | 0.259 | 7.5 | 32.5 | 12.5 | 0.010 |
| 40–49 yr     | 62.5  | 77.5 | 82.5  | 0.107 | 27.5 | 50.0 | 52.5 | 0.049 | 5.0 | 20.0 | 20.0 | 0.083 |
| 50–59 yr     | 72.5  | 92.5 | 89.5  | 0.034 | 67.5 | 72.5 | 84.2 | 0.216 | 27.5 | 22.5 | 21.1 | 0.859 |
| ≥ 60 yr      | 72.5  | 69.0 | 75.0  | 0.829 | 57.5 | 48.3 | 62.5 | 0.492 | 22.5 | 13.8 | 20.0 | 0.749 |

Statistically significant differences according to age and study period are shown in bold.
CagA = cytotoxic-associated gene A, Ig = immunoglobulin.

**Fig. 2.** Changes in anti-CagA IgG antibody seropositivity according to age during a 20-year study in Jinju. Each line connects values for each study period: 1994–1995 (line with circle), 2004–2005 (line with square), and 2014–2015 (line with triangle). There were significant differences in age specific seropositivity with time (from 1994 to 2015) between those aged 7–12 months and 10–29 years ($P < 0.05$). Anti-CagA IgG seropositivity in early childhood (age, 2–4 years) remained low and stable over 20 years.

**Anti- *H. pylori* CagA IgA seropositivity according to age and study period**

The overall rate of anti-CagA IgA seropositivity in each study period was 31.4% in 1994–1995, 34.0% in 2004–2005, and 29.4% in 2014–2015 ($P = 0.354$). The anti-CagA IgA seropositive...
rate was relatively low in infancy and early childhood (2–4 years old group) in each study period. After early childhood, anti-CagA IgA positive rate appeared to increase with age (Fig. 3).

Anti-\textit{H. pylori} CagA IgM seropositivity according to age and study period

Overall seropositivities for anti-CagA IgM antibodies in 1994–1995, 2004–2005, and 2014–2015 were 6.4%, 10.8%, and 9.8%, respectively ($P = 0.060$). Overall seropositive rates of anti-CagA IgM over the last 20 years were generally low compared with those of anti-CagA IgG and anti-CagA IgA (Fig. 4). According to age, anti-CagA IgM seropositive rates were generally lower in individuals younger than 20 compared with those in adults $\geq$ 20 years old.

DISCUSSION

The present study showed that the overall seroprevalence of anti-\textit{H. pylori} CagA IgG antibodies in Jinju, Korea, decreased from 63.2% to 42.5% between 1994 and 2015. Recent decline in the incidence of \textit{H. pylori} infection with time was similar to a previous study. However, the rate of \textit{H. pylori} in adolescents and adults aged 15 years and older was higher in Jinju (73.8% in 2004–2005) than other regions of Korea (59.6% in 2005).
Anti-CagA IgG seropositivity increased with age from early childhood. Subjects older than 10 years of age in 1994–1995, 20 years of age in 2004–2005 and 30 years of age in 2014–2015 showed seropositive rates of over 60% (Table 2 and Fig. 2). Age-related increase of seropositivity observed in each study period in the present study may represent a cohort phenomenon\textsuperscript{23,29} rather than newly acquired \textit{H. pylori} infections as individuals get older. Previous studies have also noted that birth cohort effects can lead to an increasing seroprevalence of \textit{H. pylori} infection with age and decreasing seroprevalence observed in subsequent generations.\textsuperscript{11,30} In this study, the age to a plateau of anti-CagA IgG positive rate tended to be delayed by 10–20 years of age for each study period (Fig. 2). The shifts of age to a plateau over 20 years and the marked reduction of positive rates in the 10–20s age groups in the present study suggests that the prevalence of CagA positive \textit{H. pylori} infection in Jinju will decline further in the coming decades. However, the relatively stable positive rate in the cohort of children aged 2–4 years in 1994–1995 (25%) over 20 years (35.9% of 10–14 year-old group in 2004–2005 and 28.9% of 20–29 year-old group in 2014–2015, \( P = 0.587 \)) (Table 2 and Fig. 2) may contribute to persistently stable colonization of \textit{H. pylori} and the incidence of \textit{H. pylori}-associated disease in Jinju in the future, albeit low. Further studies on incidences of CagA positive \textit{H. pylori}-associated diseases are warranted.

During the three study periods, neonates and infants showed high rates of anti-CagA IgG seropositivity (Table 2 and Fig. 2). However, such high rates could not be evidence of \textit{H. pylori} infection in newborns and infants. Rather, they could represent positive rates in females of childbearing age. This was supported by the finding that rates of anti-CagA IgG seropositivity in those younger than 6 months of age was similar to those in females aged 15–49 years in each study period. The rate of anti-CagA IgG seropositivity in those younger than 6 months of age was 64.1% in 1994–1995, 65.0% in 2004–2005, and 43.6% in 2014–2015. Corresponding rates in females aged 15–49 years were 78.8%, 66.3%, and 46.9%, respectively. Transplacental transmission of anti-CagA IgG might cause high positivities in neonates and infants because IgG can transfer passively from a mother to a fetus. Therefore, the overall seropositive rates of anti-CagA IgG in this study were calculated excluding neonates and infants. Serum samples from babies and females aged 15–49 years were randomly assigned and the mother-infant relationship was unknown in the present study. Further studies with paired serum samples from babies and their mothers would be necessary.

Overall rates of anti-CagA IgA seropositivity were similar among the three study periods (\( P = 0.354 \)). Unlike those measured in a previous study,\textsuperscript{30} the authors found that the anti-CagA IgA seropositivity during each study period gradually increased with age, particularly after 2–4 years of age (Fig. 3). Anti-CagA IgG and IgA seropositivities around 1–4 years of age showed lower rates than others. And IgG and IgA seropositivities in those older than 5 years increased along with age for each period. Thus, the authors tentatively estimated the time of \textit{H. pylori} infection for each period might have occurred in early childhood, especially of those younger than 4 years old in Jinju, Korea (Figs. 2 and 3).

Generally, IgM and IgA antibodies could be produced as a primary response. Greater quantity of IgG antibodies could be produced as a secondary response after antigen exposure. Specific IgG could persist several months or years. Positive anti-CagA IgA could represent mucosal immune response in \textit{H. pylori} infection. In addition, mucosal immune response in a person infected with \textit{H. pylori} might occur repeatedly.\textsuperscript{21} Based on humoral immune response sequences and our results of higher anti-CagA IgG positivities in the older birth cohort and anti-CagA IgA seropositivity patterns along with age, especially in those older than 5 years,
anti-CagA IgA antibodies might be repetitively produced by the active mucosal immune response of already existing bacteria rather than acute infection. Our findings could support that *H. pylori* infection is acquired during early childhood while new infections during adulthood are rare (Figs. 2 and 3). Development of atrophic gastritis, intestinal metaplasia, or gastric cancer in the elderly may have contributed to the lower anti-CagA IgA seropositivity because *H. pylori* disappears from the gastric mucosa after these diseases develop. However, further studies are warranted to determine the relationship between anti-*H. pylori* CagA IgA seropositivity and mucosal inflammation or histologic status of the gastric mucosa.

Humoral IgA response in newborns and infants is usually weak. The low rate of anti-CagA IgA seropositivity in neonates and infants younger than 2 years of age in the present study might represent sporadic acute *H. pylori* infection. Rates of anti-CagA IgA seropositivity in those aged 60 years and older were lower than those in subsequent generations during the three study periods (Fig. 3). Development of atrophic gastritis, intestinal metaplasia, or gastric cancer in the elderly may have contributed to the lower anti-CagA IgA seropositivity because *H. pylori* disappears from the gastric mucosa after these diseases develop.

The present study showed that age-specific rates of anti-CagA IgM seropositivity during the three study periods were very low compared with those of anti-CagA IgG and IgA seropositivity (Fig. 4). Anti-CagA IgM antibody has not been well established as a meaningful marker of *H. pylori* infection yet. However, a small number of acute *H. pylori* infections could be acquired sporadically at any age including the newborn period. In addition, *H. pylori* might invade the gastric mucosa or re-infect an already infected person during their lifetime, although this is likely to be uncommon.

This study has several limitations. First, any information regarding housing or economic circumstance was not provided. Neither medical information such as *H. pylori* eradication nor clinical outcome of subjects associated with CagA-positive *H. pylori* was provided. Second, this was a retrospective cross sectional cohort study based on limited geography. Regional limitation may account for our finding of a higher seroprevalence of *H. pylori* infection than that of a previous nationwide survey. Third, the authors used western blotting for qualitative analyses of IgG, IgA and IgM antibodies. However, western blotting has been extensively validated as a method for the detection of several antibodies at the same time. And it is, in fact, more sensitive and specific for anti-*H. pylori* antibodies in younger children including infants than an ELISA. Finally, presence of CagA negative *H. pylori* among the study population and change in antigenicity of CagA over 20 years might be possible. However, since CagA positive *H. pylori* comprises a large proportion of strains identified in Korea so far, CagA negative *H. pylori* might account for a small portion in the present study. Nevertheless, the present study has a strength; it examined temporal changes in CagA positive *H. pylori* infection in a relatively constant population from birth to old age, over a period of 20 years.

Results of this study suggest that Jinju may experience a lower prevalence of CagA positive *H. pylori* infection and associated diseases in the future. However, the low and stable seroprevalence of CagA positive *H. pylori* infection in early childhood over 20 years suggests that low, albeit constant, colonization by the bacterium may continue. Transmission of *H. pylori* could be highly affected by intrafamilial and environmental exposure.
and infection could occur in early childhood and persist without treatment. Therefore, education regarding personal hygiene such as hand washing and eating habits in family, and aggressive diagnosis and eradication could help reduce colonization. Establishment of a diagnostic and therapeutic strategy in children with H. pylori infection in Korea is necessary.

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